Coloraciones cosméticas y bacterias en contextos de selección sexual de abubillas y calaos

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COLORACIONES COSMÉTICAS Y BACTERIAS EN CONTEXTOS DE SELECCIÓN SEXUAL DE ABUBILLAS Y CALAOS

COSMETIC COLORATIONS AND BACTERIA IN CONTEXTS OF SEXUAL SELECTION IN HOOPOES AND HORNBILLS

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RESUMEN

Las bacterias son simbiontes habituales de macroorganismos con los que, en muchas ocasiones, forman asociaciones mutualistas, en las que tanto el simbionte como el hospedador obtienen beneficios. Uno de los beneficios que los hospedadores pueden obtener de las bacterias simbiontes es la protección frente a un amplio abanico de patógenos, mediante el aporte de sustancias defensivas que las bacterias sintetizan para su competencia con otros microorganismos. La comunidad microbiana asociada al hospedador puede ser diferente entre individuos de la misma especie y, en consecuencia, ser una propiedad que cause diferencias en las capacidades de esos individuos. Estas diferencias podrían ser de interés para las potenciales parejas y, en tal caso, seleccionarse la producción de señales asociadas a ese tipo de calidad. De esta manera, la calidad del microbioma de un individuo podría ser la información transmitida por algunas señales sexuales existentes en los hospedadores de bacterias beneficiosas. Dado que la evolución y mantenimiento de las señales depende de la fiabilidad de la información transmitida, sería de esperar que en este escenario surgieran señales dependientes de las propias bacterias simbiontes que se poseen, a través de los colores u olores causados por los metabolitos de las bacterias. Un ejemplo de ello podrían ser las aves del orden *Upupiformes*, como la abubilla europea (*Upupa epops*) y la abubilla arbórea africana (*Phoeniculus purpureus*). Estas especies presentan propiedades especiales en las secreciones de su glándula uropigial. Se sabe que albergan bacterias productoras de sustancias antimicrobianas en la glándula uropigial y se ha comprobado experimentalmente que esos simbiontes son responsables de algunas propiedades de la secreción como el color y la presencia de compuestos químicos volátiles. La existencia de simbiontes en la secreción uropigial de las aves podría ser un fenómeno más extendido. Por ejemplo, en el orden *Bucerotiformes*, clado hermano de las abubillas formado por 61 especies de calaos, algunas especies presentan secreciones uropigiales coloreadas (amarillentas), que usan para teñir distintas partes de su cuerpo, posiblemente con una función ornamental. Sin embargo, hasta el momento se desconoce si las secreciones uropigiales pigmentadas de los calaos están vinculadas a la presencia de comunidades microbianas de la glándula uropigial, tal y como se ha descrito en las abubillas. En esta tesis se estudia la hipótesis de que las secreciones coloreadas, tanto de la abubilla europea como de los calaos, funcionan como señales de

calidad usadas por los portadores para transmitir información sobre la comunidad de simbiontes que albergan en sus glándulas uropigiales.

Las señales sexuales pueden actuar antes (para conseguir pareja) o después del apareamiento (para conseguir una mayor inversión parental de la pareja). Tanto las abubillas como los calaos tienen un estricto reparto de tareas reproductivas entre los dos sexos, encargándose exclusivamente las hembras de la incubación y cuidados de los pollos mientras son pequeños, y aportando los machos todo el alimento que las hembras y los pollos consumen en ese periodo. Por ello, el éxito reproductivo de ambos miembros de la pareja está muy vinculado y es esperable que la inversión reproductora de ambos dependa de la calidad de su pareja. En los calaos, las secreciones teñidas son usadas por los dos sexos desde antes del emparejamiento y por tanto podrían actuar como señales pre-emparejamiento. Sin embargo, en las abubillas, las secreciones coloreadas y cargadas de bacterias solo están presentes en las hembras, y solo las desarrollan después del emparejamiento, durante el periodo que pasan dentro del nido. Por tanto, en este caso podrían funcionar como señales post-emparejamiento de las hembras dirigidas a conseguir una mayor inversión parental de los machos. Además, el hecho de que la asociación con bacterias en esta especie sea temporal y esté vinculada al hábitat del nido, plantea la duda de cómo obtienen su bacterioma en cada temporada. Una posibilidad sería la adquisición de simbiontes que puedan quedar en reservorios en los nidos usados por otras abubillas los años anteriores. En tal caso sería beneficioso para las abubillas ser capaces de detectar esos nidos y seleccionarlos para la nidificación. Los tres objetivos generales planteados en esta tesis están destinados a comprobar tres predicciones de la hipótesis de que las glándulas de abubillas y calaos albergan comunidades de bacterias simbiontes diferentes entre individuos y que se han seleccionado señales para transmitir su calidad: (I) Las abubillas pueden incorporar a su glándula bacterias beneficiosas del material de nidos reutilizados, y por ello seleccionarían nidos previamente usados por otras abubillas. (II) Las propiedades de la secreción de las hembras de abubilla son usadas por los machos para inferir la calidad de las hembras y por ello realizarán un esfuerzo reproductor mayor cuando están emparejados con hembras que poseen secreciones de mayor calidad. (III) Las secreciones uropigiales pigmentadas de los calaos mostrarán evidencias de estar pobladas por bacterias más frecuentemente que las no pigmentadas. A continuación se exponen los antecedentes, los métodos de estudio utilizados y los resultados principales obtenidos, para cada predicción por separado.

I. Capítulo I. Selección de sitio de nidificación en la abubilla. Efectos sobre el bacterioma uropigial.

Uno de los factores clave para el establecimiento y la evolución de las simbiosis, es el modo de transmisión de los simbiontes. Varios mecanismos han sido propuestos para explicar la compleja comunidad bacteriana de la secreción uropigial de la abubilla. Se sabe que algunas de las bacterias simbiontes presentes en las glándulas son transmitidas verticalmente y otras parecen hacerlo horizontalmente. Los *enterococos* presentes en la secreción parecen provenir del tracto gastro-intestinal de las hembras (cloaca) por lo que también podrían transmitirse a través las heces. Dado que las heces se acumulan en el material de los nidos usados, una posibilidad sería que el material del nido de las abubillas actúe como reservorio de sus bacterias simbiontes. Siendo así, las hembras podrían aumentar la diversidad bacteriana de su glándula u obtener cepas más competitivas a través de su selección de nidos previamente utilizados frente a otros sin esas bacterias. Para comprobarlo se realizó un experimento manipulando la presencia de material procedente de nidos usados de abubilla. Con ello se testó: (1) si había preferencia por aquellas cajas nido que contenían material viejo de nido de abubilla, (2) las consecuencias de su elección en la carga bacteriana de la secreción y de la cáscara de los huevos, en contacto con ese material y (3) su posible efecto en el éxito reproductivo. Las abubillas mostraron una preferencia por las cajas nido que contenían material blando añadido a las cajas frente a las cajas vacías, independientemente de si el material agregado provenía o no de nidos de abubilla previamente utilizados. Las características del material experimental no afectaron a su éxito reproductivo, pero sí afectaron a las cargas bacterianas de la cáscara del huevo y a la composición de la comunidad bacteriana de la glándula uropigial. Esta es la primera vez que se muestra un efecto del material del nido en la secreción uropigial de la abubilla. Además, varios OTUs (Unidad Taxonómica Operacional) de la secreción uropigial de las hembras se asociaron positivamente con el éxito de eclosión. Los resultados se suman a otros previos que muestran que una función principal de la secreción uropigial de las hembras de abubilla es proteger a los huevos de infecciones microbianas. También apoyan la predicción de que la re-utilización de nidos afecta a la comunidad de bacterias de la glándula. Sin embargo no se cumplió la

predicción que implicaba una preferencia por los nidos reutilizados basada en la presencia de material viejo.

II. Capítulos II y III. Los machos de abubilla realizan una inversión reproductiva diferencial basándose en las propiedades de la secreción uropigial de la hembra.

Se sabe que el color oscuro de la secreción uropigial de la abubilla está asociado a la presencia de bacterias simbiontes, siendo rojiza cuando éstas son eliminadas con antibióticos. Además, la saturación del color de la secreción se relaciona negativamente con su capacidad antimicrobiana. Por otra parte, un comportamiento único en las abubillas es que las hembras manchan activamente la cáscara de los huevos con su secreción uropigial, cambiando su color de un tono azul inicial a un verde-marrón final. La secreción que recubre la cáscara reduce la entrada de bacterias patógenas en los huevos, y esa capacidad depende de los *enterococos* que alberga. De esta manera, el color cosmético de los huevos de abubilla podría haber evolucionado como una señal sexual post-apareamiento de la capacidad antimicrobiana de la hembra. Numerosos estudios en otras especies de aves han demostrado cómo el color de los huevos refleja la calidad de la hembra y cómo esta coloración influye en la inversión del macho. Es la denominada hipótesis de la coloración de los huevos seleccionada sexualmente (Hipótesis SSEC). Se ha testado con coloraciones intrínsecas producidas por pigmentos, como la biliverdina y la protoporfirina, pero nunca con coloraciones cosméticas causadas por la secreción uropigial. En esta tesis se investiga, por primera vez, si la coloración cosmética de los huevos de abubilla influye en el aporte de alimento al nido por parte del macho (Capítulos II y III). En el capítulo II se realizó un estudio descriptivo de la relación entre el color de los huevos de las hembras y el esfuerzo reproductor de sus machos. En el capítulo III se realizó un experimento de intercambio de puestas entre hembras para comprobar si los cambios en el color de los huevos causaron cambios en la misma estima de esfuerzo reproductor de los machos. En ambos estudios se obtuvo que los machos de abubilla invirtieron menos en nidos que contenían huevos con una coloración más saturada. Además, la saturación del color de la cáscara de los huevos estuvo negativamente relacionada con la abundancia de bacterias simbióticas en la secreción de la hembra (Capítulo II). Estos resultados son consistentes con la hipótesis de que la coloración

cosmética de la cáscara del huevo debida a la secreción uropigial estaría actuando como una señal sexual post-apareamiento de las hembras. Esta señal mostraría el potencial antimicrobiano de su secreción, que los machos usarían para ajustar su esfuerzo parental. Esta es la primera demostración experimental (Capítulo III) de los beneficios asociados a la señal cosmética de las hembras.

III. Capítulos IV y V. Las secreciones coloreadas de los calaos contienen bacterias simbiontes y los ornamentos de estas aves han evolucionado para señalizar su calidad en contextos de defensa frente a patógenos.

Hasta el momento no se ha estudiado si las secreciones uropigiales de los calaos están habitadas por bacterias al igual que sucede con las de la abubilla. En el Capítulo IV de esta tesis se aborda por primera vez la comprobación de esta hipótesis con 13 especies de calaos alojados en diferentes zoológicos de España, Portugal y Francia. Para ello se recogieron muestras de distintas superficies de su cuerpo, incluyendo el penacho de la glándula y su secreción uropigial. Se realizaron siembras en medios de cultivo, así como extracciones de ADN y secuenciación del gen 16S bacteriano para así estudiar la composición de las comunidades bacterianas presentes. Los resultados mostraron crecimiento bacteriano a partir de las muestras de secreción en todas las especies de calaos africanas y en una especie asiática. Además, varias de estas especies de calaos tenían secreciones densamente pobladas de bacterias cuando se observaban en un microscopio. También se observó una clara asociación de taxones bacterianos particulares con las glándulas uropigiales o las áreas del cuerpo cubiertas con secreción en varias especies. Varios de esos taxones bacterianos son productores de sustancias antibióticas y están comúnmente asociados a relaciones mutualistas en varios grupos de animales. Además, en una especie de calao asiático, un grupo de bacterias pigmentadas productoras de carotenoides se asoció a la glándula uropigial, y a las áreas manchadas del cuerpo. Esta evidencia sugiere que los calaos son un grupo de aves involucradas en interacciones coevolutivas con bacterias que viven en o alrededor de sus glándulas uropigiales y que algunos de esos simbiontes podrían ser responsables de las propiedades especiales de las secreciones.

La coloración de las secreciones de algunas especies de calaos podría estar también relacionada con la incorporación de carotenoides obtenidos en la dieta. Los carotenoides

son responsables de los colores rojo, amarillo y naranja. Además, son importantes para varias funciones biológicas y son un recurso limitado y escaso en la naturaleza. Así, la coloración basada en carotenoides puede indicar el acceso y la capacidad de obtener recursos nutritivos, pero también otros rasgos de calidad, tales como la condición corporal, la capacidad antioxidante, la carga parasitaria y su capacidad para luchar frente a las infecciones microbianas. De esta manera, las diferencias en la coloración basada en carotenoides entre especies de calaos podrían indicar diferencias en su eficiencia en el uso de carotenoides y en la importancia de señales que reflejen la capacidad de respuesta inmune. Muchas especies de calaos presentan, además, un plumaje conspicuo con amplias áreas blancas que contrastan con un color negro general. Estos parches blancos podrían desempeñar un papel importante en la señalización sexual, ya que, en aves, habitualmente son dependientes de la condición física y predictores de la calidad fenotípica. Los calaos poseen, además, una estructura queratinizada única situada sobre el pico denominada casco, que podría actuar como un indicador de madurez sexual, habiendo evolucionado mediante selección sexual. La coloración del casco y del pico es muy variable entre las distintas especies de calaos. Dado que el pico y las cubiertas del casco están cubiertos de queratina, su coloración podría indicar la calidad individual relacionada con su capacidad para hacer frente a los microorganismos queratinolíticos. Existen numerosas diferencias entre las especies de calaos en todos estos caracteres ornamentales. Por lo tanto, son un grupo en el que un enfoque comparativo entre especies podría permitir conocer los rasgos de historia vital o las presiones selectivas que han estado asociados a la evolución de dichas señales. Dado que todos estos caracteres ornamentales pueden estar relacionados de una manera u otra con las presiones selectivas ejercidas por las bacterias, en el Capítulo V de esta tesis se analiza si su presencia y/o tamaño están vinculados con la carga de distintos grupos bacterianos.

Los principales hallazgos de este análisis comparativo son, en primer lugar, que el grado de exageración de dos adornos se correlaciona positivamente a través de la filogenia de los calaos. Estos son: la superficie de plumas blancas en el cuerpo y la superficie coloreada del pico (amarillo, naranja, o/y rojo). Además, la variación en la ornamentación entre especies se relaciona con las cargas bacterianas de diferentes partes del cuerpo. Por último, la prevalencia en la glándula uropigial de un grupo particular de bacterias conocidas por su producción de bacteriocinas (*enterococos*) se relacionó con la abundancia de posibles bacterias patógenas (queratinolíticas) en el casco. Estos resultados

sugieren que las bacterias han jugado un papel importante en la evolución de los complejos diseños de color encontrados en los calaos.

Todos los resultados de la tesis apoyan la hipótesis general de que en abubillas y calaos, las coloraciones cosméticas de la secreción uropigial pueden estar siendo seleccionadas como señales de calidad asociada a la posesión de comunidades bacterianas simbiontes. En el caso de las abubillas, los machos respondieron a los cambios de la coloración de los huevos ajustando su esfuerzo parental. En el caso de los calaos se confirma que algunas especies también mantienen simbiosis con bacterias en sus secreciones, algo que no se conocía hasta el momento. Al contrario de lo esperado en nuestra hipótesis inicial, las bacterias en este grupo no estuvieron presentes solo en las especies con secreciones coloreadas. Sin embargo, algunos simbiontes en esas especies podrían ser responsables de las propiedades especiales de la secreción, por sus capacidades biosintéticas particulares. Además, el estudio comparativo de los ornamentos de estas especies muestra asociaciones entre los ornamentos y distintos grupos de bacterias, tanto patógenas como productoras de sustancias defensivas, e interacciones entre estos grupos bacterianos entre sí. En conjunto, estos hallazgos sugieren que el grupo de los calaos es un nuevo modelo de estudio de gran interés para comprender la evolución de simbiosis mutualistas con bacterias en aves, así como de señales asociadas a esa relación.

Resumen

ABSTRACT

Bacteria are common symbionts of macro-organisms frequently establishing mutual associations, in which both the symbiont and the host get benefits. One of the benefits that the hosts can obtain from the symbiotic bacteria is the protection against a wide range of pathogens, by means of the contribution of defensive substances that the bacteria synthesize for their competition with other microorganisms. The microbial community associated with the host may be different between individuals of the same species and, consequently, be a property that causes differences in the abilities of those individuals. These differences could be of interest to potential partners and, in this case, the production of signals associated with that type of quality can be selected. In this way, the quality of an individual's microbiome could be the information transmitted by some existing sexual signals in hosts of beneficial bacteria. The evolution and maintenance of the signals depends on the reliability of the information transmitted . In this scenario, it would be expected the evolution of signals dependent on the symbiotic bacteria hosted, through the colours or odours caused by the metabolites of the bacteria An example could be the birds of the order *Upupiformes*, such as the European hoopoe (*Upupa epops*) and the African woodhoopoe (*Phoeniculus purpureus*). These species have special properties in the secretions of their uropygial glands. It is known that they harbor bacteria producing antimicrobial substances in the uropygial gland and it has been shown experimentally that these symbionts are responsible for some of the properties of the secretion, such as its colour and the presence of volatile chemical compounds in their composition. The existence of symbionts in the uropygial secretion of birds could be a more widespread phenomenon. For example, in the order *Bucerotiformes*, a sister clade of the hoopoes, formed by 61 species of hornbills, some species have coloured (yellowish) uropygial secretions, which they use to stain different parts of their body, possibly with an ornamental function. However, it is unknown if the pigmented uropygial secretions of the hornbills are linked to the presence of microbial communities in the uropygial gland, as described in hoopoes. In this thesis, we study the hypothesis that coloured secretions, both of the European hoopoe and of the hornbills, function as quality signals that they use to

transmit information about the community of symbionts associated to their uropygial glands.

Sexual signals can act before (to get a partner) or after mating (to get a greater parental investment of the partner). Both, hoopoes and hornbills, have a strict distribution of roles between the two sexes. Only females incubate and take care of the nestlings while they are small, and the males provide all the food that the females and the nestlings need in that period. Therefore, the reproductive success of both members of the couple is closely linked and it is expected that the reproductive investment of both depend on the quality of their partner. In hornbills, stained secretions are used by both sexes throughout the year, and therefore they could act as pre-mating signals. However, in the hoopoes, the coloured and secretions loaded with bacteria are only present in the females, and they only develop them after pairing, during the period they are inside the nest. Therefore, in this case they could function as post-mating signals of females to obtain a greater parental investment of males. In addition, the fact that the association with bacteria in hoopoes is temporary and is linked to the nest habitat, raises the question of how they obtain their bacterioma in each season. One possibility would be the acquisition of symbionts that may remain in reservoirs in the nests used by other hoopoes the previous years. In such a case, it would be beneficial for the hoopoes to be able to detect those nests and select them for nesting. The three general objectives of this thesis intend to test three predictions of the hypothesis that the uropygial glands of hoopoes and hornbills harbour communities of different symbiotic bacteria between individuals and that signals have been selected to show their quality: (I) Hoopoes can incorporate into their gland beneficial bacteria from the material of reused nests, and thus they would select nests previously used by other hoopoes. (II) The properties of the secretion the female hoopoes are used by the males to infer the quality of the females. Therefore males will make a greater reproductive effort when they are paired with females that have higher quality secretions. (III) The pigmented uropygial secretions of hornbills will show evidence of presence of bacteria more frequently than the non-pigmented ones. Background, methods used, and main results obtained, are indicated below for each prediction separately.

I. Chapter I. Selection of nesting site in the hoopoe. Effects on uropygial bacterioma.

One of the key factors for the establishment and evolution of symbioses, is the mode of transmission of symbionts. Several mechanisms have been proposed to explain the complex bacterial community of the uropygial secretion of the hoopoe. It is known that some of the symbiotic bacteria present in their glands are transmitted vertically and others seem to be horizontally transmitted. *Enterococci* present in the secretion seem to come from the female gastro-intestinal tract, so they could also be transmitted through faeces. Since faeces are accumulated in the material remaining in used nests, one possibility would be that such material serves as a reservoir of symbiotic bacteria. Thus, females could increase the bacterial diversity of their gland or obtain more competitive strains by selecting previously used nests compared to nests without those bacteria. To test this prediction, an experiment was carried out manipulating the presence of material from nests previously used by hoopoes. It was tested: (1) if there was a preference for nest boxes containing old hoopoe nest material, (2) the consequences of nest choice on the bacterial load of the secretion and the eggshells, in contact with that material, and (3) possible effect on reproductive success. The hoopoes showed a preference for nest boxes containing soft material added to the boxes versus empty ones, regardless if the added material came from previously used hoopoe nests. The characteristics of the experimental material did not affect reproductive success, but they affected bacterial loads of the eggshells and the composition of the bacterial community of the uropygial gland. This is the first time that is shown an effect of the nest material on the bacteriome found in the uropygial secretion in hoopoes. In addition, several OTUs (Operational Taxonomic Units) of female uropygial secretions were positively associated with hatching success. These results agree with previous ones that showed that a main function of the uropygial secretion of females of hoopoes is to protect the eggs from microbial infections. They also support the prediction that the reuse of nests affects the gland's bacterial community. However, the prediction that reused nests should be preferred based on the presence of old material was not fulfilled.

II. Chapters II and III. Hoopoe males perform a differential reproductive investment based on the properties of females' uropygial secretions.

It is known that the dark colour of the uropygial secretion of hoopoes is associated with the presence of symbiotic bacteria, being reddish when they are eliminated with antibiotics. In addition, secretion colour saturation is negatively related to its antimicrobial capacity. A unique behavior in the hoopoe is that the females actively stain the eggshells with their uropygial secretion, changing their colour from an initial blue tone to a final green-brown. The secretion that covers the eggshells reduces the entry of pathogenic bacteria into the eggs, and that capacity depends on the *enterococci* it harbors. In this way, the cosmetic colour of the eggshells of hoopoes could have evolved as a post-mating sexual signal of the female's antimicrobial capacity. Numerous studies in other bird species have shown how the colour of the eggs reflects the quality of the female and how this coloration influences male investment. It is the sexually selected eggshell colouration hypothesis (SSEC hypothesis). It has been tested with intrinsic colorations produced by pigments, such as biliverdin and protoporphyrin, but never with cosmetic colorations caused by uropygial secretion. In this thesis it is investigated for the first time if the cosmetic coloration of the eggshells of hoopoes influences the amount of food provided by males (Chapters II and III). In Chapter II a descriptive study of the relationship between the eggshells colour of females and males investment was carried out. In Chapter III, a cross-fostering experiment of clutches between pairs of females was carried out to see if changes in the eggshells colour caused changes in male investment. In both studies hoopoe males invested less in female nests that contained eggshells with more saturated colouration. In addition, eggshell colour saturation was negatively related to the abundance of symbiotic bacteria in the female's secretion (Chapter II). These results are consistent with the hypothesis that the cosmetic coloration of the eggshell due to uropygial secretion would be acting as a post-mating sexual signal of females. This signal would show the antimicrobial potential of female uropygial secretions, which males would use to adjust their parental investment. This is the first experimental demonstration (Chapter III) of the benefits associated with the female cosmetic signal.

III. Chapters IV and V. The coloured secretions of hornbills contain symbiotic bacteria and the ornaments of these birds have evolved to signal their quality in contexts of defense against pathogens.

Until now, it has not been studied whether the uropygial secretions of hornbills are inhabited by bacteria as is the case with those of the hoopoe. In Chapter IV of this thesis, we test this hypothesis for the first time with 13 species of hornbills housed in different zoos in Spain, Portugal and France. For this, samples were collected from different surfaces of their body, including their uropygial gland tuft and their uropygial secretion. Samples were inoculated in culture media, and DNA was extracted and sequenced in order to study the composition of the bacterial communities present. The results showed bacterial growth in the secretion samples in all species of African hornbills and in one Asian species. In addition, several of these species of hornbills had secretions densely populated of bacteria when viewed under microscope. A clear association of particular bacterial taxa with uropygial glands or body areas covered with secretion in several species was also observed. Several of these bacterial taxa are producers of antibiotic substances and are commonly associated with various groups of animals in mutualistic relationships. In addition, in an Asian hornbill species, a group of pigmented carotenoid-producing bacteria was associated with the uropygial gland, and with the stained areas of the body. This evidence suggests that hornbills are a group of birds involved in coevolutive interactions with bacteria that live in or around their uropygial secretions and that some of those symbionts may be responsible for the special properties of their secretions.

The coloured secretions of some species of hornbills could also be dependent on carotenoids obtained in the diet. Carotenoids are responsible for the red, yellow and orange colours. They are important for several biological functions and they are a limited and scarce resource in nature. Thus, carotenoid-based coloration may indicate the access and the ability to obtain nutritional resources, but also other quality traits, such as body condition, antioxidant capacity, parasitic load and its ability to fight against microbial infections. In this way, differences in carotenoid-based coloration among hornbill species could indicate differences in their efficiency in the use of carotenoids and in the importance of signals that reflect their immune response capacity. Many species of

hornbills have, in addition, a conspicuous plumage with wide white areas that contrast with a general black color. These white patches could play an important role in sexual signaling, since, in birds, they are usually dependent on physical condition and phenotypic quality predictors. Hornbills also have a unique keratinized structure located on the beak called casque, which could act as an indicator of sexual maturity, having evolved through sexual selection. The coloration of the casque and beak is very variable among different hornbill species. Since the beak and casque covers are made of keratin, their coloration could indicate the individual quality related to their ability to cope with keratinolytic microorganisms. There are numerous differences between hornbill species in all these ornamental traits. Therefore, they are a group in which a comparative approach among species could allow to study the life history traits or the selective pressures that have been associated with the evolution of these signals. Since all of these traits can be related to selective pressures exerted by bacteria, in Chapter V of this thesis, it is analyzed if their presence and/or size are linked to the load of different bacterial groups.

The main findings of this comparative analysis are, first, that the degree of exaggeration of two ornaments are positively correlated throughout hornbill phylogeny. These are: the surface of white feathers on the body and the coloured surface of the beak (yellow, orange, or / and red). In addition, variation in ornamentation between species is related to bacterial loads of different parts of the body. Finally, the prevalence in the uropygial gland of a particular group of bacteria known for their production of bacteriocins (*enterococci*) was related to the abundance of possible pathogenic (keratinolytic) bacteria in the casque. These results suggest that bacteria have played an important role in the evolution of the complex colour designs of hornbills.

All the results of the thesis support the general hypothesis that in hoopoes and hornbills, cosmetic colorations of the uropygial secretion may be selected as quality signals associated with the possession of symbiotic bacterial communities. In the case of hoopoes, males responded to the changes in the eggshells colour by adjusting their parental investment. In the case of honbills, it is confirmed for the first time, that some species also maintain symbiosis with bacteria in their secretions. Contrary to expectations in our initial hypothesis, bacteria in this group were not present only in species with coloured secretions. However, some symbionts in these species may be responsible for the

special properties of the secretion for their particular biosynthetic abilities. In addition, the comparative study of the ornaments of these species shows associations with the pressure of groups of bacteria, both pathogenic and producing defensive substances, and interactions between these bacterial groups. Together, these findings suggest that hornbills are a new study model of great interest to understand the evolution of mutualistic symbiosis with bacteria in birds, as well as of signals associated with that relationship.

Abstract

INTRODUCCIÓN GENERAL

1) Calidad individual dependiente de bacterias: señalizando el microbioma

En los últimos años se ha descubierto la enorme importancia que tienen los microorganismos. Estos pueden vivir en la superficie o en el interior de otros organismos, desempeñando un papel importante en muchos aspectos de la historia de la vida del hospedador, desde su desarrollo y fisiología, hasta su salud y comportamiento (Archie and Theis 2011, Spor et al. 2011, Ezenwa et al. 2012, McFall-Ngai et al. 2013, Sherwin et al. 2019). Se ha comprobado que en el caso de los humanos, el cuerpo posee la misma cantidad de bacterias que de células humanas, siendo la masa total bacteriana de aproximadamente 0.2 kg (Sender et al. 2016). La composición de las comunidades microbianas residentes (denominadas microbiomas) está influenciada por muchos factores, incluido el sistema inmunitario del hospedador, los factores ambientales (dieta, el ambiente que lo rodea..), las interacciones con los microorganismos no residentes (Spor et al. 2011), e incluso la contaminación (Ruiz-Rodríguez et al. 2016, Dumbrell et al. 2017).

Los microorganismos se caracterizan por su ubicuidad, ya que están presentes en todos los medios donde los animales se desarrollan y se reproducen, interaccionando constantemente con ellos. El término general usado para estas asociaciones es *simbiosis*, y hace referencia a la interacción que ocurre entre distintos organismos (Steinert et al. 2000). Estas interacciones se clasifican en: mutualistas, cuando tanto el simbionte como el hospedador obtienen beneficios; comensalistas, cuando sólo uno de ellos se beneficia mientras que el otro no se ve afectado; y parásitas, cuando uno se beneficia perjudicando al otro (revisado en Rizzo y Lo Giudice 2018). Estas asociaciones pueden ser transitorias o estables. En algunos casos, los microorganismos transitorios pueden no tener ningún efecto en el hospedador, o un efecto muy pequeño. No obstante, incluso los microorganismos más raros pueden tener un papel importante en las funciones ecosistémicas microbianas (revisado en Suzuki 2017).

Las bacterias son capaces de vivir en simbiosis con macroorganismos formando asociaciones parasitarias, o mutualistas (Steinert et al. 2000, Moran 2006, Ley et al. 2008, Haine 2008, McFall-Ngai et al. 2013). Para que se produzca mutualismo, el éxito

reproductor, potencial o real, de cada uno de los que interactúan debe incrementarse por la acción de los demás miembros del sistema (Janzen 1985). Existen numerosos casos de interacciones mutualistas entre animales y bacterias y ejemplos de cómo han evolucionado distintas adaptaciones para poder obtener un beneficio de esas bacterias simbiontes (Douglas 1998, Werren et al. 2008, Moran et al. 2008). Un ejemplo de ello ocurre en casi todas las especies de áfidos. Estos poseen unas células específicas denominadas bacteriocitos, cuya función es alojar bacterias simbiontes que les proporcionan nutrientes esenciales (Shigenobu et al. 2000, Hosokawa et al. 2010).

Las bacterias pueden afectar al comportamiento de los hospedadores como, por ejemplo, a su dinámica durante el emparejamiento (Spicer et al. 2019). Se ha visto cómo las bacterias presentes en los órganos copuladores de la araña *Agelenopsis pennsylvanica* afecta a su comportamiento de cortejo e incluso a la supervivencia de la hembra (Spicer et al. 2019). También se ha encontrado un efecto de la microbiota intestinal comensal de la mosca de la fruta *Drosophyla melanogaster*, en las preferencias de apareamiento de su hospedador (Leftwich et al. 2018).

Entre los posibles beneficios de las interacciones mutualistas entre animales y bacterias, cabe destacar la mejora de la asimilación de nutrientes en el aparato digestivo (Hill 1997, Ley et al. 2008). Una de las funciones beneficiosas mejor estudiadas del microbioma de los mamíferos es su función en la digestión de plantas, especialmente la digestión de la celulosa (revisado en Suzuki 2017). También destacan, entre otros beneficios, la producción de vitaminas (Hill 1997), la desintoxicación de pesticidas (Werren 2012) y el mantenimiento del sistema inmune (Umesaki et al. 1999, Macpherson and Harris 2004). Diversos estudios en mamíferos silvestres han mostrado una correlación entre la inmunidad del hospedador y la composición microbiana (revisado en Suzuki 2017). También se ha documentado su protección frente a microorganismos patógenos (Fons et al. 2000, Dillon et al. 2005, Haine 2008). En este último caso, la simbiosis proporciona protección frente a un amplio rango de patógenos, mediante el aporte de sustancias defensivas por parte de sus simbiontes (Flórez et al. 2015). Se ha sugerido en distintas especies, desde cnidarios a humanos, cómo los virus (bacteriófagos) presentes en las superficies mucosas, ofrecen protección frente a bacterias patógenas (Barr et al. 2013). Esta protección puede ser causada por la producción de metabolitos secundarios, con propiedades antifúngicas y antimicrobianas por parte de las bacterias simbiontes (revisado

en Rizzo y Lo Giudice 2018). Este tipo de simbiosis con microorganismos ha sido descrita tanto en plantas (Heath and Tiffin 2007), como en animales (e.g. Flórez and Kaltenpoth 2017). En cuanto a los animales invertebrados, se han descrito en: isópodos marinos (Lindquist et al. 2005), esponjas (revisado en Rizzo and Lo Giudice 2018), calamares (Heath-Heckman et al. 2013), crustáceos (Gil-Turnes and Fenical 1992), áfidos (Oliver et al. 2003), escarabajos (Scott et al. 2008, Flórez and Kaltenpoth 2017), avispas (Kaltenpoth et al. 2005) y hormigas (Currie et al. 1999). En cuanto a los vertebrados, esta simbiosis es menos conocida. Se han encontrado bacterias aisladas en la piel de los murciélagos con efectos anti-fúngicos frente al hongo *Pseudogymnoascus destructans*, conocido por ser causante de la enfermedad del síndrome de la nariz blanca en esta especie (Warnecke et al. 2012). Los anfibios, a su vez, poseen péptidos antimicrobianos y metabolitos antifúngicos producidos por bacterias simbiontes situadas en su piel, que les proporcionan protección frente a la enfermedad quitridiomicosis, causada por el hongo quitrido de los anfibios, *Batrachochytrium dendrobatidis* (revisado en Rollins-Smith et al. 2011). También ha sido descrita una bacteria antifúngica en la piel de la salamandra *Hemidactylium scutatum*, que inhibe al hongo patógeno de embriones *Mariannaea sp.* (Banning et al. 2008). En cuanto a las aves, esta asociación únicamente ha sido descrita en la abubilla europea *Upupa epops* (Martín-Platero et al. 2006, Soler et al. 2008a, Martín-Vivaldi et al. 2010, Ruiz-Rodríguez et al. 2013) y en la abubilla arbórea *Phoeniculus purpureus* (Law-brown and Meyers 2003) (Figura 1).

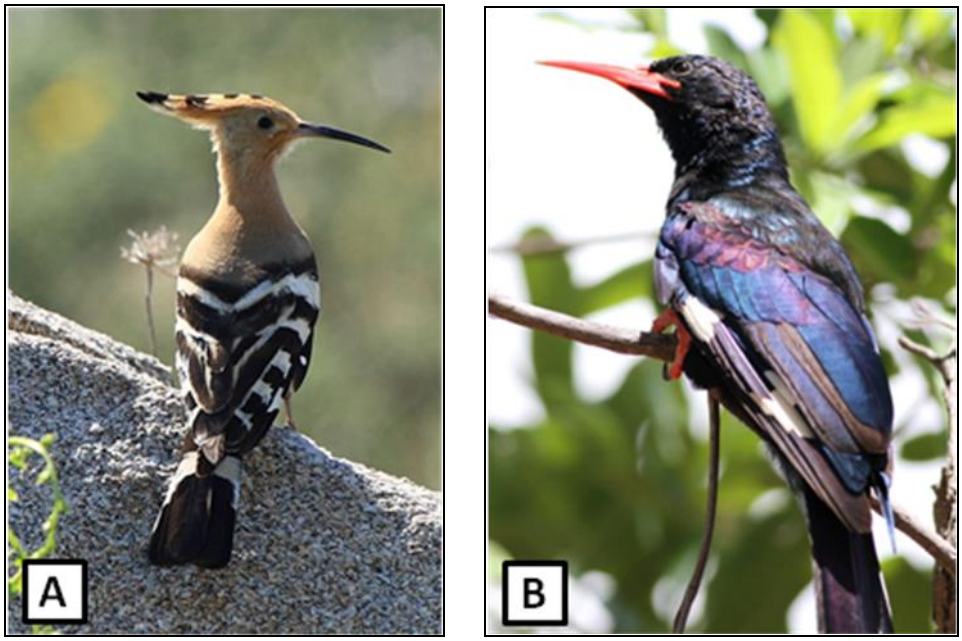

Figura 1. Imágenes de la abubilla europea (A) y la abubilla arbórea (B).

La comunidad microbiana asociada al hospedador puede ser muy variable y ser específica de cada hospedador. De esta manera, el microbioma asociado al hospedador puede variar no sólo entre especies, si no también dentro de una misma especie (revisado en Suzuki 2017). Diversos estudios correlacionales en mamíferos sugieren que esta variación puede tener un rol en la biología del hospedador, en aspectos tales como la digestión, la detoxificación, el sistema inmune, y el comportamiento (revisado en Suzuki 2017). Algunos de los factores que pueden explicar la variación dentro de una misma especie, son los factores morfológicos y genéticos. Esta variación microbiana intra-específica se ha encontrado en distintas especies como en delfines nariz de botella, *Tursiops truncatus* y orcas, *Orcinus* orca (Chiarello et al. 2017), en rodeores, en primates e incluso en humanos (revisado en Suzuki 2017). Recientes estudios han mostrado cómo las especies bacterianas dentro del intestino están compuestas por una multitud de cadenas, y que algunas de ellas pueden ser específicas del hospedador (Ellegaard and Engel 2016). También se han encontrado diferencias en la microbiota en función del sexo en mangostas, una vez alcanzada la madurez sexual (Leclaire et al. 2014), y en el paíño boreal, *Oceanodroma leucorhoa*, cuya microbiota también variaba, además de con el sexo, con la zona del cuerpo (Pearce et al. 2017). Se ha visto en distintos organismos, como la variación

microbiana aumenta la variación fenotípica del hospedador (Henry et al. 2019). Por ejemplo, las diferencias en la comunidad microbiana en *Drosophila* producen un aumento en la variación del tiempo de desarrollo de la larva, en el peso de la pupa y del adulto (Keebaugh et al. 2018). En *Daphnia*, las bacterias tenían una influencia en el tamaño del cuerpo y en el éxito de eclosión (Mushegian et al. 2018). En los peces cebra (*Danio rerio*), diferentes microorganismos se asociaron a variaciones en la respuesta inmune (Rolig et al. 2015). En todos estos casos, los hospedadores eran colonizados por microbios y sus fenotipos eran modificados en respuesta a esa variación microbiana (Henry et al. 2019). Por tanto, el mantenimiento de los fenotipos receptivos a la variación microbiana puede permitir que los hospedadores se ajusten mejor a los cambios ambientales (Alberdi et al. 2016, Shapira 2016). Así, los hospedadores podrían haber evolucionado para mantener asociaciones microbianas variables que ajusten mejor su fenotipo a los entornos que le rodean (revisado en Henry et al. 2019). De esta manera, su fenotipo podría actuar como una señal honesta de los microorganismos que contiene y que le confieren esa ventaja, frente a otros individuos de su misma especie que no poseen ese microbioma (revisado en Henry et al. 2019). Para entender los beneficios evolutivos del microbioma es imprescindible el estudio de cómo los hospedadores adquieren y utilizan los microorganismos del ambiente para ajustar sus fenotipos (revisado en Henry et al. 2019).

Existen evidencias en una gran variedad de animales de cómo los microorganismos también están implicados en la producción de señales (Ezenwa and Williams 2014). Esto es debido a que la calidad de los individuos y los factores que los hacen mejores competidores, mejores supervivientes e incluso mejores parejas, está en gran medida unido al microbioma que alberga (Hamilton and Zuk 1982, Clayton 1991, Folstad and Karter 1992, Kose and Møller 1999, Sharon et al. 2010, Richard 2017). Todos los animales albergan microorganismos beneficiosos. Estos pueden beneficiar al hospedador aumentando la diversidad y eficacia de las sus señales. Una línea interesante de investigación es explorar cómo los microorganismos simbiontes pueden mediar en los sistemas de señales. Por todo ello, los microorganismos son cada vez más tenidos en cuenta en los estudios biológicos, en los marcos evolutivos y ecológicos (Archie and Theis 2011, Ezenwa et al. 2012, McFall-Ngai et al. 2013). Si los microorganismos están involucrados en las señales, y existen diferencias entre individuos en la microbiota que contienen, podría haber evolucionado la señalización de las bacterias simbiontes que

posee como una señal de calidad. De esta manera, estaría influyendo en la evolución del comportamiento social del hospedador al usar su microbiota para facilitar la comunicación entre co-específicos (Whittaker et al. 2010, Leclaire et al. 2017, Javůrková et al. 2019, Sherwin et al. 2019).

Existen distintas posibilidades de señalizar la microbiota, por ejemplo, a través de olores (Ezenwa and Williams 2014, Leclaire et al. 2017, Richard 2017, Maraci et al. 2018, Sherwin et al. 2019). Numerosos estudios se basan en la hipótesis de que las glándulas olorosas de los mamíferos generan metabolitos usados por sus hospedadores para la comunicación y que la variación de esas señales químicas es debida a la variación de las comunidades bacterianas que habitan en esas glándulas (hipótesis de la fermentación). Las secreciones del saco anal pueden ser utilizadas como señales químicas por los animales para comportamientos que van desde la defensa al reconocimiento de especies, a la señalización del estado reproductivo. Los olores juegan un papel importante en la vida de la mayoría de los mamíferos terrestres y pueden transmitir información valiosa sobre los congéneres. Existen varios estudios en distintas especies que han encontrado una relación entre bacterias y comunicación química. Por ejemplo, las hienas rayadas (*Hyaena hyaena*) silvestres presentan comunidades bacterianas simbióticas, más abundantes en los adultos que en los juveniles, que parecen contribuir a los olores de las bolsas situadas en el orificio anal. Además, estas comunidades bacterianas difieren según el sexo y el estado reproductivo del individuo (Theis et al. 2013). Por otra parte, el macho del ciervo almizclero enano (*Moschus berezovskii*) secreta una sustancia a través de su glándula almizclera. Esta posee una gran cantidad de bacterias, causantes del olor de esa sustancia, únicamente en los machos que aún no se han apareado, lo que les permite atraer a más hembras (Li et al. 2016). Otro ejemplo ocurre con la composición de las secreciones axilares de los macacos rhesus (*Macaca mulatta)*. Esta varía en función de la edad y del rango del individuo, proporcionando una gran cantidad de información potencial sobre su portador, a través de los volátiles producidos por la fermentación de bacterias (Weiß et al. 2018). También se han encontrado en anfibios el potencial de las bacterias (*Pseudomonas sp.*) como una fuente de señales químicas, que varían entre sexos (Brunetti et al. 2019). Además de por olores, la composición microbiana puede señalizarse a través de: la ornamentación, como es el caso de las plumas ornamentales del pecho del macho del estornino negro *Sturnus unicolor* (Ruiz-Rodríguez et al. 2015); de la degradación del

plumaje en distintas especies de aves (Gunderson 2008, Ruiz-De-Castañeda et al. 2015, Azcárate-García et al. 2020); y de los colores, tanto del pico de la hembra de estornino negro (Ruiz-Rodríguez et al. in press), como del color de la cáscara de los huevos del carbonero común (Jacob et al. 2015) y de la abubilla (Díaz-Lora et al. en revisión, en revisión.; Soler et al. 2014). De esta manera, la relación mutualista puede influir en los mecanismos de evolución de las señales. Así, esperamos que existan señales que transmitan información sobre los microbiomas presentes en los individuos y que influyan en el comportamiento del receptor, como, por ejemplo, en la elección de pareja.

2) Selección sexual, machos, hembras y sus señales

La selección sexual se define como una selección que depende de la ventaja reproductiva que ciertos individuos tienen sobre otros del mismo sexo y especie, (Darwin 1871). Es un proceso evolutivo clave que ocurre a través de la competencia por parejas y/o por el apareamiento, lo que implican mecanismos de elección de pareja y éxito reproductivo. La elección de pareja es un comportamiento común en el que los miembros de un sexo se exhiben para ser elegidos por el miembro del otro sexo. Estas características fenotípicas que exhiben reflejan su calidad fenotípica o genética incluidas las capacidades reproductivas, que al estar relacionadas con las preferencias de elección de pareja, están seleccionadas sexualmente (Andersson 1994, Andersson and Simmons 2006). Así, solo los individuos de mayor calidad son capaces de mostrar un fenotipo más extravagante (por ejemplo, a través de adornos). De acuerdo con el principio del *handicap*, este fenotipo extravagante se mantiene porque el costo relativo de producir la señal es más bajo en los individuos de mayor calidad (Zahavi y Zahavi 1997). Las expresiones del fenotipo utilizadas en la elección de pareja están correlacionadas con varios parámetros de la condición individual (Andersson 1994). Ya que los microorganismos tienen la capacidad de influir en el fenotipo (revisado en Henry et al. 2019), también podrían influir en las señales utilizadas en la elección de pareja, pudiendo actuar como agentes de selección (Beltran-Bech y Richard 2014).

La selección sexual debida a la competencia entre el mismo sexo explica el desarrollo de caracteres sexuales secundarios en los seres vivos (Darwin 1871). Esta competencia suele ser más común en machos, por lo que sus caracteres sexuales secundarios suelen estar más desarrollados en los machos que en las hembras (Darwin

1871). Sin embargo, estos caracteres pueden aparecen también en las hembras de muchas especies, como son la coloración del plumaje o del pelaje y los ornamentos elaborados (Andersson 1994, Kraaijeveld et al. 2007). La presencia en las hembras de rasgos que los machos exhiben durante el cortejo ha sido tradicionalmente considerada como una consecuencia inevitable de la expresión en los descendientes femeninos de los genes seleccionados sexualmente de sus padres. Además, la expresión atenuada de algunos rasgos de las hembras podría ser la consecuencia evolutiva de las repercusiones negativas del mantenimiento de esos rasgos en su fecundidad. Aunque este escenario evolutivo se ha confirmado en algunas especies, la señalización de las hembras también puede implicar beneficios en la eficacia biológica, en contextos competitivos intra-sexuales o incluso reproductivos (Clutton-Brock 2007, Rosvall 2011). En ocasiones, las hembras pueden poseer caracteres sexuales secundarios que están ausentes en los machos o, incluso mostrar un mayor desarrollo de estos rasgos, cuando están presentes en ambos sexos (Clutton-Brock 2007). Esos caracteres sexuales secundarios pueden actuar como señales, al proporcionar información fiable sobre la calidad del individuo (Zahavi 1975, Andersson 1994, Johnstone 1995, Clutton-Brock 2007, Amundsen 2018). Estas preferencias de elección de pareja se han documentado no solo en hembras, sino también en machos (Amundsen 2000, Kraaijeveld et al. 2007). La elección de pareja por parte del macho ha sido documentada en diversas especies como: insectos, peces, lagartos, aves y mamíferos (revisado en Clutton-Brock 2009). En las especies en las que las hembras presentan ornamentos o una coloración más intensa, se ha visto cómo los machos suelen mostrar una preferencia por las hembras más ornamentadas o con coloraciones más intensas (Griggio et al. 2005, revisado en Clutton-Brock 2009) ya que indican la calidad de la hembra (Møller 1993, Linville et al. 1998). Además, estas preferencias suelen estar ligadas a un aumento del éxito reproductivo (revisado en Clutton-Brock 2009). Por tanto, las señales pueden ser producidas por los machos, por las hembras, o por ambos sexos, y su función puede diferir en función del sexo. Por ejemplo, se ha encontrado en varias especies de aves cómo el brillo del plumaje de las hembras se correlacionaba negativamente con la depredación mientras que en los machos no existía esa relación (Martin and Badyaev 1996).

Las señales pueden mostrar la calidad del hospedador antes o después del apareamiento, influyendo en la inversión parental (Clutton-Brock 1991, Chapman et al.

2003). El cuidado parental es definido como cualquier forma de comportamiento parental que aparece para aumentar la eficacia biológica de los descendientes a costa de la supervivencia y del éxito reproductivo futuro de los padres (Clutton-Brock 1991). Mientras que los beneficios del cuidado parental sobre la eficacia biológica dependen del cuidado total proporcionado por ambos padres, las diferencias sexuales en la inversión reproductiva son de naturaleza generalizada y, por tanto, sus costes asociados dependen del sexo (Trivers 1972, Clutton-Brock 1991). El éxito reproductivo de los machos depende en gran medida de la inversión de la hembra. Por tanto, los machos deberían invertir diferencialmente más cuando están emparejados con hembras que indican una mayor calidad (Clutton-Brock 1991, Chapman et al. 2003). Los rasgos seleccionados sexualmente están típicamente relacionados con el éxito reproductivo, lo cual se explica tanto por el efecto directo de los poseedores, como por la inversión diferencial en la reproducción por parte de la pareja. De esta forma, la inversión diferencial en la reproducción debido a caracteres sexuales podría ser vista como un proceso de selección sexual post-apareamiento que contribuye a la evolución de esos rasgos (Burley 1986, Andersson 1994, Sheldon 2000). Cuando los dos sexos intervienen en el cuidado parental es esperable que ambos sean selectivos a la hora de escoger a su pareja e invertir acordemente a sus señales de calidad. Por tanto, se esperaría la existencia de señales en los dos sexos y también señales post-emparejamiento para poder evaluar la calidad de la descendencia. Estos procesos han sido explorados principalmente en los rasgos sexuales de los machos, al ser normalmente el sexo más ornamentado, en cambio los de las hembras han sido recientemente considerados (Soler et al. 2019).

La calidad fenotípica de las hembras puede ser señalizada antes del apareamiento a través de su plumaje (Møller 1993, Griggio et al. 2005, Morales et al. 2007) o de su habilidad de construcción del nido (Tomás et al. 2006, Soler et al. 2019). Después del apareamiento, puede señalizar su calidad a través del color de la cáscara de sus huevos (Moreno and Osorno 2003, Moreno et al. 2004, 2005, 2006a, Soler et al. 2005, 2018, Siefferman et al. 2006, Krist and Grim 2007, Giordano et al. 2015, Hargitai et al. 2018a). Varios estudios empíricos han sugerido que algunas señales expresadas después del emparejamiento, como el color de los huevo, tienen la intención de inducir una mayor inversión en el cuidado paternal del progenitor masculino (revisado en Lyu et al. 2017). Las explicaciones adaptativas acerca de la variación en la coloración de los huevos, han

intrigado a los investigadores y hoy en día sigue sin haber una única explicación (Kilner 2006). Numerosas hipótesis no exclusivas han sido propuestas para explicar la variación en este rasgo, desde señalizadoras hasta estructurales. Algunas sugieren que su función es minimizar la depredación (Wallace AR 1889, Swynnerton 1916, Solís and de Lope 1995). Otros autores, la han relacionado con el reconocimiento de los huevos dentro de amplias colonias (Gaston et al. 1993) o para evitar el parasitismo de cría (Swynnerton 1918, Victoria 1972, Soler and Møller 1996) o incluso como una adaptación frente a la radiación solar (Lahti 2008). Otras hipótesis se centran únicamente en explicar la existencia de huevos conspicuos. Se ha sugerido también que su función podría ser la de incrementar su visibilidad dentro de las cavidades (Hanley 2012), ya que las condiciones de baja luminosidad dentro de ellas podría reducir su visibilidad y dificultar su evaluación del color de los huevos (Avilés et al. 2006). Otra posible explicación es que los machos proporcionan un mayor cuidado parental para compensar el alto riesgo de depredación de estos huevos conspicuos al ser más fácilmente visibles por los depredadores de nidos (Hanley et al. 2010). La hipótesis que más ha recibido atención en los últimos años es la de la coloración de los huevos seleccionada sexualmente (hipótesis SSEC) (Moreno and Osorno 2003) y en ella nos centraremos en el Capítulo II y III de esta tesis. Esta hipótesis considera la coloración de los huevos, como una posible señal de calidad de las hembras seleccionada sexualmente, y que los machos utilizan para invertir de forma diferencial en las de mejor calidad (Moreno and Osorno 2003), de lo que existen evidencias en varios estudios (e.g. Soler et al. 2005, Moreno et al. 2006a). Esta hipótesis se basa en dos asunciones: en primer lugar que el color de los huevos señaliza la calidad de la hembra, y en segundo lugar, que los machos utilizan el color de los huevos para regular su inversión de acuerdo a la calidad de la hembra. La hipótesis SSEC fue inicialmente propuesta para los huevos con coloraciones azul-verdosas (Moreno and Osorno 2003), debida a la presencia del pigmento biliverdina (Mikšík et al. 1996) que presenta propiedades antioxidantes (Kaur et al. 2003). De esta manera, la intensidad azul-verdosa de la cáscara de los huevos podría señalizar la capacidad antioxidante de la hembra y, por lo tanto, su inmunocompetencia (Morales et al. 2006, 2008, Moreno et al. 2006b, Hanley et al. 2008). Así, los machos ajustarían su esfuerzo reproductivo a la coloración de los huevos, favoreciendo la evolución de estos colores en un proceso de selección sexual post-apareamiento (Moreno and Osorno 2003). Más recientemente, se ha sugerido que la coloración marronácea de la cáscara de los huevos causada por el pigmento protoporfirina,

tiene también un componente seleccionado sexualmente (Martínez-De La Puente et al. 2007, Sanz and García-Navas 2009, Giordano et al. 2015, Poláček et al. 2017, Corti et al. 2018, Hargitai et al. 2018b). Por tanto, hoy en día, se podría asumir de forma generalizada que la coloración de la cáscara de los huevos puede reflejar la calidad fenotípica de las hembras (Moreno and Osorno 2003, Moreno et al. 2004, 2005, 2006a, Soler et al. 2008b, 2018, 2005, Siefferman et al. 2006, Krist and Grim 2007, Giordano et al. 2015, Hargitai et al. 2018a, Holveck et al. 2019); aunque esto no implique necesariamente que sea un rasgo seleccionado sexualmente (Cherry and Gosler 2010). La segunda asunción de la hipótesis ha generado más controversia. Algunos (Soler et al. 2005, 2008a, Moreno et al. 2006a, 2008, Hanley et al. 2008, English and Montgomerie 2011, Poláček et al. 2017, Díaz-Lora et al. 2019 en revisión), pero no todos los estudios (Krist and Grim 2007, Hanley and Doucet 2009, Honza et al. 2011, Johnsen et al. 2011, Stoddard et al. 2012, Bulla et al. 2012, Fronstin et al. 2016), han detectado que los machos invierten más en la reproducción cuando sus nidos contienen huevos intensamente más coloreados o con la presencia de un mayor número de manchas. Además, muchos de estos estudios han investigado el cuidado parental únicamente durante la fase de pollos sin controlar por los rasgos de los pollos (Moreno et al. 2004, 2006a, Krist and Grim 2007, Hanley et al. 2008, Hanley and Doucet 2009, Sanz and García-Navas 2009, Walters and Getty 2010) que podrían estar influyendo en la inversión del macho (revisado en Riehl 2011). Por otra parte, algunos estudios fueron correlacionales (Hanley et al. 2008, Hanley and Doucet 2009, Díaz-Lora et al. 2019 en revisión), por lo que no pueden ofrecer un apoyo total a la hipótesis SSEC, ya que la coloración de la cáscara del huevo podría estar relacionada también con rasgos morfológicos o comportamentales de la hembra que podrían influir en la inversión del macho. Todo ello añadido a que los resultados encontrados son mixtos, hace que sea necesario completar con más estudios experimentales que permitan explorar esta asociación. Para ello habría que investigar la inversión de los padres durante el período de incubación para garantizar que los cambios en la inversión del macho sean causados por el color de la cáscara de los huevos en lugar de por las variables de confusión de los rasgos de los pollos (Riehl 2011).

Una de las posibles ventajas para el macho de utilizar el color de los huevos como señal es que este es indicativo del estado fisiológico actual de la hembra, de su condición física y de su sistema inmunológico en ese mismo momento. Esto no ocurre con otros

posibles rasgos de calidad de la hembra, como son el color del plumaje, ya que éste es indicativo de su calidad durante la fase de muda, que normalmente se encuentra separada de la época de cría por un periodo de tiempo considerable (Montgomerie 2006, Krist and Grim 2007).

Las asociaciones predecibles entre la coloración de la cáscara de los huevos, la condición de la hembra y el esfuerzo de cebas del macho han sido ampliamente investigadas con respecto a la coloración de la cáscara de los huevos producida por pigmentos "intrínsecos", es decir, depositados en el tracto genital femenino durante la construcción de los huevos. Sin embargo, su coloración "extrínseca" (depositadas después de su puesta del huevo) ha empezado a tenerse en cuenta solo recientemente. Este es el caso de las manchas de las cáscaras de los huevos debidas a la actividad de algunos ectoparásitos (López-Rull et al. 2007, Avilés et al. 2009). La presencia y número de manchas marrones debidas a la actividad parasitaria informaría a los machos acerca del ambiente parasitario donde los pollos se criarán, influyendo así en su esfuerzo de ceba, como fue demostrado experimentalmente en el estornino negro (*Sturnus unicolor*) (López-Rull et al. 2007, Avilés et al. 2009). Otro tipo de coloración "extrínseca" de la cáscara de los huevos, nunca antes considerada en esa estructura, implica el uso de la secreción uropigial como cosmético (Soler et al. 2014).

3) Secreciones coloreadas con bacterias para teñir huevos, ¿señales post-emparejamiento en la abubilla?

Las abubillas contienen en su secreción uropigial, bacterias productoras de sustancias antimicrobianas (Martín-Platero et al. 2006, Soler et al. 2008a, Martín-Vivaldi et al. 2010, Ruiz-Rodríguez et al. 2013). Esta secreción es oscura y maloliente y sus glándulas son inusualmente grandes en comparación con el resto de especies (Cramp 1998) (Figura 2). A diferencia de la abubilla arbórea, la cual mantiene esta relación mutualista con bacterias simbiontes durante todo el año, la abubilla únicamente la mantiene en la época reproductora, tanto en las hembras como en los pollos durante su estancia en el nido (Martín-Vivaldi et al. 2009). Este microbioma particular es detectable a través del color de las secreciones ya que se ha demostrado experimentalmente añadiendo antibiótico a la secreción, que su coloración torna a un marrón menos intenso y más rojizo cuando carece de bacterias (Martín-Vivaldi et al. 2009) (Figura 3). Además, su capacidad antimicrobiana

se relaciona negativamente con la saturación (intensidad) del color de la secreción uropigial (Soler et al. 2014).

Figura 2. Imágenes de la glándula uropigial de una abubilla hembra reproductora. A la izquierda: vista lateral de una glándula femenina que muestra una papila llena de secreción oscura, parte de la cual se expulsa a través de la abertura de la papila. A la derecha: vista dorsal de la glándula que muestra una papila llena de secreción oscura (Martín-Vivaldi et al. 2009).

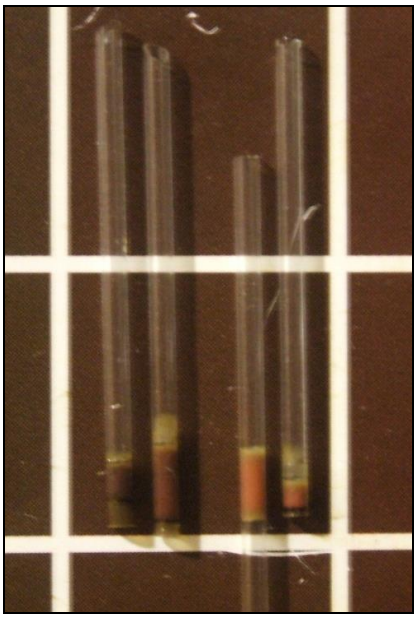

Figura 3. Imagen de cuatro tubos capilares llenos de cuatro secreciones de los pollos de un nido experimental. Los dos de la izquierda pertenecen a la secreción de pollos control. Los dos de la derecha pertenecen a pollos tratados con antibiótico (Martín-Vivaldi et al. 2009).

La microbiota de la secreción uropigial de la abubilla contiene 621 OTUs distribuidos en 68 géneros de cuatro filos (Rodríguez-Ruano et al. 2018) (Figura 4). Durante la época de reproducción, la etapa con mayor riesgo de infección bacteriana, es cuando se alcanzan densidades bacterianas máximas (Rodríguez-Ruano et al. 2018). El 93% de las secuencias analizadas lo forman los *Firmicutes*, mientras que las *Proteobacterias*, *Bacteroidetes* y las *actinobacterias* se encontraron en proporciones más bajas. Se sabe que las bacterias de la clase *Clostridia* son esenciales para mantener la estabilidad de la microbiota intestinal (ej., en humanos (Lopetuso et al. 2013)) y podrían jugar un papel similar en la abubilla. Entre los microorganismos cultivables frecuentemente aislados de estas secreciones, destacan principalmente especies del género *Enterococcus*, que en su gran mayoría son productores de bacteriocinas (Martín-Platero et al. 2006, Ruiz-Rodríguez et al. 2013). Los *enterococos* son cocos Gram positivos, catalasa-negativos, anaerobios resistentes, pertenecientes al filum *Firmicutes*, que habitan generalmente en el intestino de algunos vertebrados (Giard et al. 2001). Se ha demostrado en la abubilla sus beneficios simbióticos debido a la producción de péptidos antimicrobianos (enterocinas) (Soler et al. 2008a, Ruiz-Rodríguez et al. 2009). Se han encontrado siete especies diferentes, siendo las más abundantes *E. faecalis*, *E.faeclum* y *E.mundtii* (Soler et al. 2008a, Ruiz-Rodríguez et al. 2012). Las bacteriocinas son sustancias antimicrobianas de naturaleza peptídica de bajo peso molecular y de síntesis ribosómica, con un espectro de acción variable dependiendo de su clase (Riley and Wertz 2002). Estas pueden ofrecer protección al hospedador frente a infecciones por bacterias patógenas (Haine 2008) e incrementar su éxito reproductor (Martín-Vivaldi et al. 2014a). Más de la mitad de las cepas de *enterococos* aisladas de su secreción de hembras reproductoras y pollos eran productoras de, al menos, una bacteriocina y la mayoría presentaban los genes para las bacteriocinas MR-10 y AS-48 (Ruiz-Rodríguez et al. 2013). Además de los *enterococos*, se han encontrado dos bacterias adicionales productoras de sustancias antimicrobianas que podrían tener una función defensiva: *Vagococcus* y *Parascardovia* (Rodríguez-Ruano et al. 2018) (Figura 4).

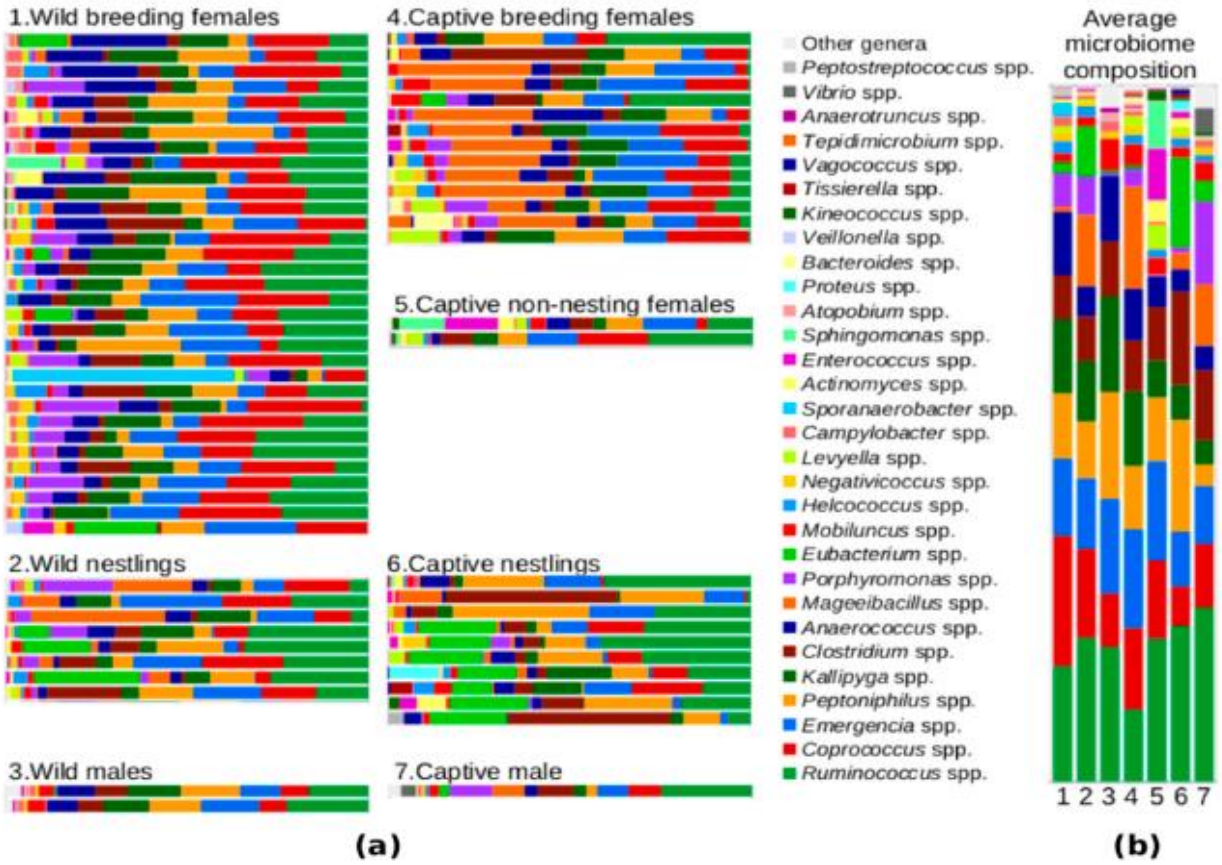

Figura 4. Figura 1 en Rodríguez-Ruano et al. 2018. Abundancia relativa de los géneros encontrados en la microbiota de la secreción uropigial de la abubilla durante la temporada de reproducción. Las muestras se organizan en grupos según las condiciones de vida, el sexo y la fase de reproducción. Se muestran tanto (a) las muestras individuales como (b) la composición promedio para cada grupo.

Uno de los factores clave para el establecimiento y la evolución de la simbiosis, es su modo de transmisión (Bright y Bulgheresi 2010). La transmisión vertical se produce de padre a hijo, y, resulta con el tiempo en una co-especiación entre el hospedador y el simbionte (Moran et al. 2008). Esto conduce a una mayor deriva genética, debido a los cuellos de botella recurrentes de la población durante cada transmisión, y, en última instancia, a una degradación del genoma del simbionte (Bennett y Moran 2015). La transmisión horizontal, en contraste, donde el simbionte se adquiere del ambiente en cada generación del hospedador, facilita la mezcla genética y el cambio de hospedador (Bright y Bulgheresi 2010). La mayoría de los simbiontes se cree que son transmitidos por ambas

vías, incrementando así la eficiencia de la transmisión del simbionte (Kaltz y Koella 2003). En algunos casos, estos simbiontes pueden ser transmitidos por ambos padres (transmisión biparental) (Paz et al. 2017). Un ejemplo de transmisión horizontal ocurre en los peces del orden *Lophiiformes,* que adquieren bacterias simbiontes con propiedades bioluminiscentes a través del medio ambiente (Baker et al. 2019). Algunos insectos plaga también son capaces de adquirir bacterias beneficiosas por el ambiente (del suelo), que le confieren resistencia a los insecticidas (Kikuchi et al. 2012). El estudio de la adquisición de los simbiontes, incluidos los mecanismos genéticos y no genéticos, es importante para comprender la evolución de la interacción entre el hospedador y las bacterias simbiontes (Suzuki 2017). El Capítulo I de esta tesis se centrará en investigar ese mecanismo en la abubilla.

Varios mecanismos han sido propuestos para explicar la compleja comunidad bacteriana de la secreción uropigial de la abubilla (Rodríguez-Ruano et al. 2018). Estas bacterias simbiontes pueden ser transmitidas vertical u horizontalmente (Ruiz-Rodríguez et al. 2014, Rodríguez-Ruano et al. 2015, Martínez-García et al. 2016a, Martín-Vivaldi et al. 2018). Los *enterococos* presentes en su secreción parecen provenir del tracto gastro-intestinal de las hembras (cloaca) por lo que podrían transmitirse a través las heces. Estas se acumulan en el material del nido por lo que una posibilidad es que el material del nido de las abubillas actúe como reservorio de sus bacterias simbiontes. De esta manera, la elección del material del nido podría afectar a la adquisición de estos simbiontes, cuando son transmitidos horizontalmente (Peralta-Sanchez et al. 2010, Martínez-García et al. 2016a, van Veelen et al. 2017) afectando a la eficacia biológica individual. Por tanto, los individuos deberían ser muy selectivos a la hora de escoger un lugar donde criar para maximizar su éxito reproductivo (Refsnider and Janzen 2010). Estos nidos también pueden contener ectoparásitos y patógenos que permanecen inactivos dentro de los residuos del material del nido (Maier et al. 2000), pudiendo afectar al éxito reproductivo de los siguientes individuos que lo usen después (Mazgajski, 2007; Møller et al., 2009). Este aumento en la exposición a microorganismos perjudiciales provenientes del material del nido viejo se podría contrarrestar con el aumento de la diversidad microbiana al incorporar las bacterias simbiontes presentes en ese nido que ha sido previamente usado por una abubilla. Esto permitiría a su sistema inmune funcionar en cooperación con la microbiota para protegerse frente a esos microorganismos. De esta manera, los

compuestos antimicrobianos producidos por las bacterias simbiontes de las abubillas, podrían competir o inhibir la colonización por parte de microorganismos o parásitos situados en el nido, defendiendo y protegiendo al hospedador frente a ellos (Soler et al. 2010, Martín-Vivaldi et al. 2014a). Así, el material viejo de los nidos reusados de abubillas podría actuar como una fuente de bacterias potencialmente beneficiosas durante la época de cría. El ambiente bacteriano del nido predice la composición final del microbioma tanto de los pollos como de la cáscara de los huevos (González-Braojos et al. 2012, Peralta-Sánchez et al. 2012, 2014, Brandl et al. 2014, Grizard et al. 2015, Martínez-García et al. 2016a, Tomás et al. 2018, van Veelen et al. 2018). De esta manera, explorando la carga bacteriana de la cáscara de los huevos en nidos con material viejo o con material artificial nuevo podría ayudar a clarificar el efecto del material del nido en el ambiente bacteriano, uno de los objetivos del Capítulo I de esta tesis. Un estudio previo en la abubilla demostró un efecto del ambiente bacteriano del nido en la comunidad bacteriana de la cáscara de los huevos, pero no encontró ningún efecto en la comunidad bacteriana de la secreción uropigial (Martínez-García et al. 2016a). Sin embargo, este experimento fue llevado a cabo con abubillas mantenidas en cautividad, cuyo microbioma presenta una menor diversidad microbiana que el de las que están en libertad (Martínez-García et al. 2015, Rodríguez-Ruano et al. 2015, 2018). Además, la comunidad bacteriana fue caracterizada a través del análisis del espaciador ribosomal 16S-23S (ARISA fingerprinting), el cual no permite un análisis completo de la diversidad bacteriana total (Bentley et al. 2008). Por otra parte, el material del nido experimental utilizado en el experimento (piedras de aceituna triturada (*Olea europaea*)) tenía propiedades antimicrobianas, por lo que podría haber afectado su composición microbioma (Martínez-García et al. 2016a). Otro de los objetivos del Capítulo I de esta tesis es realizar este experimento con abubillas salvajes, con una técnica mejorada (High Throughput Sequencing) y con un material de nido experimental sin propiedades antimicrobianas.

El hecho de que haya una variación en la coloración de la secreción en nuestra población de estudio podría indicar una diferencia en la concentración de las bacterias simbiontes, en el tipo de comunidad bacteriana que han adquirido y en su capacidad antimicrobiana. Por tanto, podrían haber evolucionado distintos comportamientos y adaptaciones para señalizar a los machos esa capacidad antimicrobiana y que estos sean capaces de detectar esas señales. El color de la secreción no sería detectable por el macho

si esta se mantuviera dentro de la glándula uropigial. Curiosamente, ha evolucionado un comportamiento único en las hembras de abubillas, que no se ha detectado en otra especie. Este consiste en usar activamente su secreción uropigial para cubrir la superficie de sus huevos (Soler et al. 2014). Este comportamiento se produce desde el momento en el que la hembra empieza a incubar, es decir, desde la puesta del primer o segundo huevo. Esto es posible ya que durante el periodo de incubación, la glándula uropigial de las hembras aumenta considerablemente de tamaño (Martín-Vivaldi et al. 2009), permitiéndoles cubrir la superficie de todos los huevos de su puesta. Otra adaptación de esta especie es la estructura externa de la cáscara de sus huevos, muy diferente al resto de aves. Su superficie porosa con cráteres mejora la adherencia de las bacterias simbiontes en la cáscara (Martín-Vivaldi et al. 2014a) (Figura 5). De esta manera, las bacterias simbiontes son transferidas a la superficie de los huevos (Martín-Vivaldi et al. 2014a), determinando la composición de su microbiota (Soler et al. 2016) y aumentando el éxito de eclosión (Martín-Vivaldi et al. 2014a). Los huevos, una vez cubiertos con la secreción, cambian de color, pasando de un azul grisáceo inicial a un verde marronáceo (Soler et al. 2014), correspondiente al color de la secreción cuando contiene bacterias (Martín-Vivaldi et al. 2009) (Figura 6). De esta manera, el color de los huevos podría haber evolucionado como una señal sexual post-apareamiento de la capacidad antimicrobiana de la hembra (Soler et al. 2014), influyendo en el cuidado parental del macho. Dentro de este marco teórico, la coloración cosmética de la cáscara de los huevos de abubilla, podría actuar como una señal post-apareamiento informando al macho de las propiedades de la comunidad simbionte de la hembra. También podría predecir el éxito reproductivo ya que la secreción parece tener un efecto protector en el embrión de infecciones microbianas a través de la cáscara (Martín-Vivaldi et al. 2014a). Además, debido a que los pollos heredan de sus madres la comunidad microbiana de su glándula uropigial, incluyendo aquellas bacterias con potencial antimicrobiano (Ruiz-Rodríguez et al. 2014, Martínez-García et al. 2016b, Martín-Vivaldi et al. 2018), la coloración de los huevos podría reflejar también las características de la microbiota que los pollos van a adquirir, lo cual tendría efectos directos e indirectos en la eficacia biológica en cuanto a la prevención de infecciones microbianas. De esta manera, la inversión diferencial de los machos de abubilla en puestas en función de la coloración de los huevos podría ser una ventaja selectiva (Soler et al. 2014). El hecho de que en esta especie, el macho alimente a la hembra durante el periodo de incubación (Martín-Vivaldi et al. 2014b) permite medir su esfuerzo de cebas durante

ese periodo, pudiendo distinguir si los cambios en la inversión del macho son causados por la coloración de los huevos en vez de por los rasgos de los pollos.

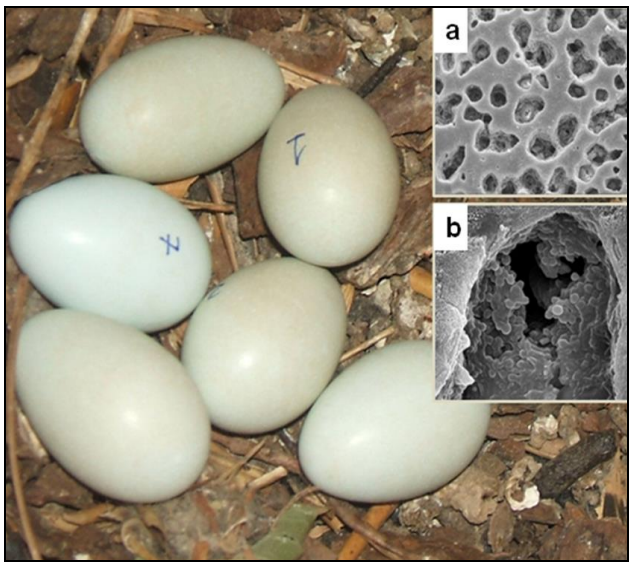

Figura 5. Huevos de abubilla e imágenes que muestran la superficie de las cáscaras de huevo ampliadas mediante microscopio electrónico de barrido. En la imagen *a* se aprecian los cráteres. En la imagen *b*, las bacterias encontradas dentro de la secreción, que llena las criptas de las cáscaras de huevo (Martín-Vivaldi et al. 2014a). También es posible apreciar las diferencias de coloración entre los huevos debido a la cantidad de secreción uropigial que poseen en la cáscara. Aquellos que han sido puestos recientemente (7) tienen una coloración más azulada.

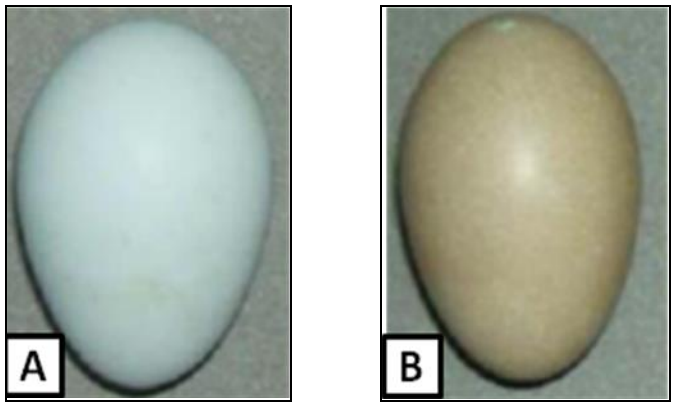

Figura 6. Huevo recién puesto de abubilla, sin presencia de secreción uropigial (A) y huevo cubierto por la secreción uropigial durante la incubación (B).

El efecto del uso cosmético de la secreción uropigial en la coloración se ha investigado principalmente en las plumas (Montgomerie 2006, Delhey et al. 2007, Piault et al. 2008, Amat et al. 2011, Pérez-Rodríguez et al. 2011) en escenarios de selección sexual (Negro et al. 1999, Delhey et al. 2007, López-Rull et al. 2010). Su uso cosmético en la cáscara de los huevos como una posible señal de calidad de la hembra hacia el macho, se testa por primera vez en abubillas en los Capítulos II y III de esta tesis.

4) Más allá de la abubilla, secreciones uropigiales y bacterias en otras aves

Las propiedades simbiontes de la comunidad microbiana de la secreción uropigial de las abubillas podría ser un fenómeno más extendido, pudiendo existir también en otras especies de aves (Soler et al. 2010). Especialmente, en aquellas que tengan olores o colores particulares, beneficiándose de la presencia de esas bacterias por su acción sobre la secreción uropigial. Varios estudios han demostrado cómo varias especies de aves usan sus secreciones uropigiales como marcadores olfativos para la comunicación intraespecífica (Whittaker et al. 2010, 2011, Soini et al. 2013). Sin embargo, sólo se ha puesto de manifiesto experimentalmente en el junco ojioscuro *Junco hyemalis*, el posible papel de las bacterias como mediadores de dicha función mediante la producción de los volátiles utilizados por las aves (Whittaker et al. 2019).

En los últimos años, se han aislado e identificado en la glándula uropigial, nuevas especies bacterianas en diferentes aves, lo que sugiere que es un ambiente especial que causa una especialización de microorganismos. Además de *Enterococcus phoeniculicula*, que se encuentra en la glándula uropigial de la abubilla arbórea (Law-Brown and Meyers 2003), se han encontrado nuevas especies como: *Corynebacterium uropygiale* en el pavo *Meleagris gallopavo* (Braun et al. 2016)*; Corynebacterium heidelbergense* en el ganso egipcio *Alopochena egyptiacus* (Braun et al. 2018a)*; Kocuria uropygialis* y *K. uropygioeca* en el pico picapinos *Dendrocopos major* (Braun et al. 2018b)*;* y *K. tytonicola* y *K. tytonis* en la lechuza común americana *Tyto furcata* (Braun et al. 2019a, b). Aunque la mayoría de los taxones de *Kocuria* se consideran estrictamente aeróbicos, las cepas aisladas de las glándulas uropigiales de la lechuza mostraron un crecimiento reducido, pero no una inhibición en condiciones anaeróbicas (Braun et al. 2019a), lo que sugiere que están adaptadas para vivir dentro de la glándula. En el único caso en el que se estudiaron

los efectos antimicrobianos de estos simbiontes (*C. uropygiale* en el pavo), los resultados indicaron que las bacterias no parecen contribuir a la naturaleza antimicrobiana de la secreción del ave (Braun et al. 2018b). Aunque se desconoce la posible función de esta asociación para el hospedador, todos estos estudios sugieren que la asociación con bacterias dentro de las glándulas uropigiales de las aves puede ser algo más generalizado. Sin embargo, en ninguno de estos casos, las secreciones son visiblemente alteradas por la presencia de bacterias, tal y como sucede en las abubillas europeas y en las abubillas arbóreas. No obstante, el orden *Bucerotiformes*, clado hermano de las abubillas formado por 61 especies de calaos (Gonzalez et al. 2013), posee varias similitudes en cuanto a su secreción uropigial. Algunas de sus especies, tanto machos como hembras, presentan secreciones uropigiales amarillas, naranjas o rojas brillantes, que usan para teñir distintas partes de su cuerpo, posiblemente con una función ornamental (Kemp 2001, Delhey et al. 2007, Poonswad et al. 2013), pudiendo transferir mensajes adicionales con la señal de la coloración. Además, la cáscara de sus huevos también parece contener criptas que podrían favorecer la retención de la secreción (Obs.personal), tal y como se ha descrito en las abubillas europeas (Martín-Vivaldi et al. 2014a). Se desconoce si las secreciones uropigiales pigmentadas de los calaos están vinculadas a la presencia de comunidades microbianas de la glándula uropigial, tal y como se ha descrito en las abubillas europeas y en las abubillas arbóreas. En el Capítulo IV de esta tesis se testará por primera vez esta hipótesis con 13 especies de calaos.

La coloración de las secreciones de los calaos podría estar también relacionada con la incorporación de carotenoides obtenidos en la dieta (Viseshakul et al. 2011, Gamble 2012). Esto se ha comprobado en otros grupos de aves, como los flamencos, que poseen una coloración cosmética derivada de carotenoides (Amat et al. 2011). Los carotenoides son responsables de los colores rojo, amarillo y naranja (Hill 2006). Varios estudios han respaldado la hipótesis de que el acceso a los carotenoides en la dieta puede tener un efecto en la expresión de las señales de color en los vertebrados (Hill 2006). Esto se debe a que la inversión en la coloración dependiente de carotenoides tiene costes fisiológicos, ya que estos compuestos son un recurso limitado y escaso en la naturaleza (Grether et al. 1999) y son importantes para varias funciones biológicas (Olson and Owens 1998, Hill 2000, Amat et al. 2011). De esta manera, las coloraciones más pobres pueden reflejar una menor disponibilidad de carotenoides (Hill 2006). Así, la coloración basada en

carotenoides puede indicar el acceso y la capacidad de obtener recursos nutritivos, pero también otros rasgos de calidad, tales como la condición corporal (Massaro et al. 2003), la capacidad antioxidante (Olson and Owens 1998, Hill 2002, McGraw and Ardia 2003, Searcy and Nowicki 2005), la carga parasitaria (Hill 2002, revisado en Hill 2006) y su capacidad para luchar frente a las infecciones microbianas (Hamilton and Zuk 1982, Hill et al. 2004, Ruiz-Rodríguez et al. under review). Por tanto, solo los de mayor calidad podrían mostrar estructuras más coloridas, evolucionando esta señal. De esta manera, las diferencias en la coloración basada en carotenoides entre especies podrían indicar diferencias fundamentales en su eficiencia en el uso de carotenoides y si se metabolizan o se depositan directamente en el tegumento, ya que la absorción, transporte y deposición de carotenoides requiere un buen estado nutricional (Hill 2006).

Muchas especies de calaos presentan, además de coloraciones rojas, amarillas y/o naranjas en distintas estructuras, un plumaje conspicuo con amplias áreas blancas que contrastan con un color negro general (Figura 7). El porcentaje de plumas blancas en el cuerpo podría reflejar la desventaja de tener plumas blancas en buena condición física. Se sabe en distintas especies como las plumas blancas son menos resistentes a la degradación bacteriana que las melanizadas (Goldstein et al. 2004, Azcárate-García et al. 2020) y a que la degradabilidad de las plumas está relacionada negativamente con la calidad del individuo en el papamoscas cerrojillo *Ficedula hypoleuca* (Ruiz-De-Castañeda et al. 2015). De esta manera, los parches de plumaje blanco, además de la coloración, podrían desempeñar un papel importante en la señalización sexual, ya que son dependientes de la condición física y predictores de la calidad fenotípica (Doucet et al. 2004, revisado en Prum 2006).

Los calaos poseen una estructura queratinizada única situada sobre el pico denominada casco (Gamble 2007). Su forma, color y tamaño varían en función de la especie, aumentando progresivamente de tamaño con la edad y en la madurez sexual (Kemp 2001). Esta estructura se desarrolla de manera diferente en cada sexo, siendo generalmente más grande y más elaborado en los machos. Por lo tanto, podría actuar como un indicador de madurez sexual, habiendo evolucionado mediante selección sexual (Naish 2015). Hoy en día, su función sigue siendo desconocida. Algunas hipótesis incluyen el refuerzo estructural del pico, la mejora de la vocalización a través de la resonancia y actuar como señal de calidad, del estatus social o de madurez sexual (Kemp 2001,

Poonswad et al. 2013). Sin embargo, no se pueden descartar diferentes funciones según la especie (Gamble 2007). Esta estructura es una zona común de lesiones, daños ambientales y enfermedades (Miller et al. 1985, Suedmeyer et al. 2001, Gamble 2007). La coloración del casco y del pico es muy variable entre las distintas especies de calaos. Dado que el pico y las cubiertas del casco están cubiertos de queratina, sus daños físicos y su coloración podrían indicar la calidad individual relacionada con su capacidad para hacer frente a microorganismos queratinolíticos (Gunderson 2008, Shawkey et al. 2009, Ruiz-de-Castañeda et al. 2012).

Figura 7. Patrones de diseño de color de dos especies de calaos: *Tockus deckeni* (A) y *Bucorvus leadbeateri* (B).

Más de un tercio de las especies de calaos se consideran de interés para la conservación en todo el mundo, incluido el 62% de las especies asiáticas, algunas de ellas, en peligro de extinción (Gonzalez et al. 2013). Por esta razón, los estudios de estas especies sobre ecología y biología de la conservación han aumentado recientemente (Gonzalez et al. 2013). Sin embargo, son necesarios más estudios sobre su ecología y comportamiento junto con su filogenia y distribución (Naish, 2015). Los calaos son conocidos por su estrategia de "auto-encarcelamiento", en la que las hembras permanecen en una cavidad durante la época de cría, sellando la entrada y dejando sólo una pequeña

hendidura, por la que el macho le alimenta. Así, se podrían haber seleccionado defensas antimicrobianas en los calaos, para protegerse frente a parásitos del nido, ya que se ha demostrado cómo los nidos viejos pueden incrementar su carga microbiana con el paso de los años (Møller and Erritzoe 1996). Curiosamente, el éxito de la cría en cautividad es bajo para estas especies (Crofoot et al. 2003). Esto sugiere que las hembras pueden responder a las señales de calidad de la pareja, que es primordial para su propia supervivencia dada la extrema dependencia de las hembras de la ayuda del macho durante la temporada de reproducción (Kemp 2001; Poonswad et al. 2013). Por tanto, se esperaría que las hembras tuvieran criterios extremadamente exigentes para la elección de pareja (Kemp 2001). Por todas estas razones y dado que no hay investigaciones sobre la selección sexual en calaos, es importante aumentar los estudios en este área. Existen numerosas diferencias entre las especies de calaos en todos sus caracteres ornamentales, por lo que un enfoque comparativo entre especies podría reflejar cómo estas señales podrían haber evolucionado de manera inter-específica. La posibilidad de que varios de los rasgos ornamentales de los calaos, que además en muchas especies están en los dos sexos, puedan ser señales de calidad relacionadas con los microbiomas albergados, se testará por primera vez en el Capítulo V.

Figura 8. Imagen de una hembra de calao Toco de damara (*Tockus damarensis*) dentro del nido (Fotografía de: Mark Stanback).

OBJETIVOS

En esta tesis se plantean los siguientes objetivos específicos expuestos a continuación:

1) Explorar la preferencia de abubillas por: (1) cajas nido con material en su interior o cajas nido vacías, (2) cajas nido previamente usadas por otra abubilla. Nuestra predicción es que las abubillas prefieren cajas nido con material en vez de cajas nido vacías y específicamente, aquellas con material de nido viejo de abubilla en su interior, es decir, aquellas que han sido previamente usadas por abubillas **(Capítulo I).**

2) Explorar experimentalmente la importancia del material viejo del nido en la elección de las cajas nido por parte de la abubilla investigando los efectos asociados en la carga bacteriana de la superficie de la cáscara los huevos y en la comunidad de la secreción uropigial. Nuestra predicción es que la composición bacteriana de la secreción uropigial y de la cáscara de los huevos diferirá dependiendo del tratamiento experimental (del tipo de material de nido), debido a la incorporación de microorganismos simbiontes procedentes del material de nido viejo de abubilla a las comunidades obtenidas a través de otras fuentes **(Capítulo I)**.

3) Explorar el efecto del material de nido experimental en el éxito de cría en la abubilla. Nuestra predicción es que las hembras que crían en nidos con material antiguo de abubilla tienen un mayor éxito reproductivo que aquellas que crían en nidos sin él debido a una mayor diversidad en la composición de la comunidad bacteriana en la glándula uropigial **(Capítulo I)**.

4) Explorar la asociación entre la coloración de la cáscara de los huevos tras ser manchados con la secreción uropigial por la hembra de abubilla, y el esfuerzo de cebas del macho. Así, por primera vez en abubillas, se pretende comprobar correlacional **(Capítulo II)** y experimentalmente **(Capítulo III)** la hipótesis de la coloración de los huevos seleccionada sexualmente (hipótesis SSEC) con una coloración cosmética de los huevos. Nuestra predicción es que los machos deberían invertir más en puestas que contengan huevos con una menor saturación ya que esta se relaciona negativamente con la capacidad antimicrobiana de la secreción.

5) Testar la hipótesis de que los calaos albergan bacterias simbióticas en sus glándulas uropigiales, y que las coloraciones especiales que se encuentran en las secreciones de algunas especies del linaje asiático podrían estar relacionadas con la presencia de estos simbiontes. Si ese es el caso, se deberían encontrar que las secreciones uropigiales obtenidas en condiciones estériles están cargadas de bacterias, y que las de las especies con coloración cosmética tienen taxones bacterianos que producen metabolitos coloreados **(Capítulo IV)**.

6) Comprobar si los diseños de color blanco / negro, el tamaño del casco y las áreas corporales coloreadas con carotenoides y / o con coloración cosmética pueden haber evolucionado como señales de calidad individual relacionadas con la capacidad de hacer frente a los patógenos en los calaos. Con este fin, se realizó un estudio comparativo de la posible evolución de estas señales en calaos vinculados a su interacción con las bacterias. Para ello se van a estudiar 13 especies de calaos con información disponible sobre variables morfológicas y de color y carga bacteriana de diferentes partes del cuerpo. Esperamos encontrar: (1) una relación positiva entre el color de las diferentes partes del cuerpo o el tamaño de la casco y la extensión del plumaje blanco. (2) Una relación positiva entre la presencia de secreciones coloreadas y el área de superficie rojo-naranja-amarillo en diferentes estructuras corporales, si manchan su cuerpo con ella. (3) Una relación entre la carga bacteriana de diferentes partes del cuerpo y la coloración corporal como una posible señal de calidad relacionada con la capacidad de hacer frente a los patógenos. (4) Dado que algunas especies de calaos tienen secreciones uropigiales coloreadas, se pretende comprobar si, como en las abubillas, existe una relación entre las secreciones coloreadas y su carga bacteriana, concretamente en el penacho de la glándula uropigial. Finalmente, (5) se predice una relación entre las bacterias del penacho de la glándula uropigial y las bacterias de diferentes estructuras corporales, ya que las utilizan para teñir su cuerpo. Tanto una relación positiva (si hay más bacterias en la glándula en esa zona) como una relación negativa (si elimina otras bacterias) es esperada dependiendo de los grupos de bacterias.

MATERIAL Y MÉTODOS

1) ESPECIES DE ESTUDIO

- **Abubilla**

La abubilla euroasiática (*Upupa epops epops* Linnaeus, 1758) es un ave de la familia *Upupidae*, cercana filogenéticamente a las familias *Bucerotidae* (calaos) y *Phoeniculidae* (abubilla arbórea) (Sibley and Ahlquist, 1990; Hackett et al., 2008). Es un ave migratoria que se distribuye por Europa, África y Asia. En áreas con pocas diferencias estacionales en el clima existen poblaciones parcialmente migratorias, como la analizada en este estudio, en la que se reproducen tanto individuos sedentarios como migrantes (Reichlin et al. 2013, van Wijk et al. 2018). En general se trata de una especie solitaria, pero pueden formar grupos durante la migración (Cramp 1998).

Es un ave de medio tamaño (26-28 cm), de color marrón anaranjado y con un diseño de bandas blancas y negras en las plumas del ala y de la cola y una llamativa cresta naranja desplegable con puntos negros en los extremos de las plumas (Fig. 1A). Posee un largo y pico fino de 5-6 cm de longitud, curvado ligeramente hacia abajo (Cramp 1998) con el que se alimenta principalmente artrópodos y de pequeños vertebrados de hábitos subterráneos.

Generalmente habita en zonas de campo abierto, con parches de vegetación baja o pastizal o en terrenos de cultivo donde puedan tener acceso al suelo desnudo para la búsqueda de alimento. Anida en distintos tipos de cavidades (de árboles, paredes rocosas, muros..) (Cramp 1998) y en cajas nido, cuando éstas tienen las dimensiones adecuadas (Arlettaz et al. 2010). Suele reutilizar los nidos tanto entre distintos años como entre distintas puestas (Martín-Vivaldi et al. 1999, Hoffmann et al. 2015). No transportan material al nido, pero sí utilizan el material blando disponible en la cavidad seleccionada y excavan una ligera depresión donde ponen los huevos (Martín-Vivaldi et al. 2014b). Este material puede estar formado por restos de presas y excrementos de reproducciones previas de abubillas o de otras especies, nidos viejos de otras especies, tierra y/o madera descompuesta.

En nuestra área de estudio, las hembras realizan una o dos puestas de entre seis y ocho huevos entre los meses de Febrero y Julio (Martín-Vivaldi et al., 1999; Plard et al.,

2018). Empiezan a incubar antes de terminar la puesta, muchas veces con el primer huevo, causando su eclosión asincrónica y por tanto una marcada jerarquía de tamaños entre los pollos (Cramp 1998). Esta diferencia de tamaños generalmente provoca la reducción de la nidada por muerte de los más pequeños (Martín-Vivaldi et al. 2014b, Hildebrandt y Shaub 2017)

Las hembras permanecen dentro del nido desde el comienzo de la incubación (desde que ponen el primer o segundo huevo), hasta que el primer pollo tiene unos 8 días. Durante todo este tiempo, el macho se encarga de alimentar a la hembra y a los pollos. Una vez que la hembra sale del nido, ambos se encargan de alimentar a los pollos hasta que estos dejan el nido a los 24-30 días (Martín-Vivaldi et al. 2014b).

Los individuos de nuestra población de estudio poseen una variación notable en el color de la secreción uropigial y, por tanto, también en la cáscara de los huevos, al ser manchados activamente por la hembra con su secreción (Soler et al. 2014).

- **Calaos**

Los calaos pertenecen al orden *Bucerotiformes* y lo forman 61 especies pertenecientes a 15 géneros, que se distribuyen a lo largo de África subsahariana y Asia tropical, y también por el Oriente Medio y Australasia. Poco se sabe de ellos ya que son increíblemente esquivos, dificultando su estudio (Naish 2015). Poseen rasgos anatómicos únicos, tales como el casco (Fig. 7B). En la mayoría de los casos esta estructura es hueca, por lo que una de sus posibles funciones sería la de actuar como cámara de resonancia acústica utilizada para transmitir las vocalizaciones territoriales (Alexander et al. 1994). En las especies en las que el casco es macizo, como el calao de yelmo (*Rhinoplax vigil*), en peligro crítico de extinción debido a la caza furtiva por el marfil de su casco, se ha sugerido que lo podrían usar como martillo. Poseen pestañas extraordinariamente largas y planas en los párpados (Kemp 2001, Gonzalez et al. 2013). También presentan una gran variación en el tamaño, alcanzando algunas especies una envergadura de más de 1.5 metros y un peso de 6 kilogramos (como es el caso del calao terrestre sureño africano, *Bucorvus leadbeateri*). Esta especie, junto con otras, como el calao bicorne, *Buceros bicornis*, pueden vivir más de 60-70 años y alcanzan su madurez sexual a los 4 o 5 años (Naish 2015). El grado y distribución de su dimorfismo sexual es muy variado, casi no existiendo en algunas especies, como, por ejemplo, en *Buceros rhinoceros* ni en *Buceros bicornis*. En cambio, en otras el macho puede alcanzar casi el doble de tamaño que la

hembra (Kinnaird and O'Brien 2007). Algunas de sus especies poseen, en ambos sexos, un plumaje con bandas blancas y negras (sobre todo en la cola y el ala) con tonalidades brillantes en la piel del cuello y de la cara y emiten llamadas ruidosas. Se ha observado como algunas especies pintan su cuerpo (plumas, pico y casco) con las secreciones coloreadas de su glándula uropigial (Naish 2015).

Dentro de los calaos, se distinguen dos líneas evolutivas: los calaos asiáticos y los africanos (González et al. 2013). Los primeros habitan en su mayoría en bosques tropicales y son predominantemente frugívoros. Son considerados especies clave en la dispersión de semillas a larga distancia (Kemp 2001, Kinnaird and O'Brien 2007, Trail 2007), teniendo una influencia incluso en la expansión histórica de los bosques Paleotropicales (Viseshakul et al. 2011). El género *Rhyticeros* es considerado el más dispersivo, pudiendo volar entre 10 y 15 km en un sólo día (Kinnaird and O'Brien 2007). Los calaos africanos, a diferencia de los asiáticos, son predominantemente carnívoros (a excepción del grupo que incluye los géneros *Ceratogymna* y *Bycanistes*) y habitan en la sabana. Son especialmente sensibles a la pérdida y fragmentación del hábitat debido a su necesidad de extensas áreas de forrajeo, que junto con la caza y el comercio internacional (Trail 2007), los convierte en uno de los grupos más amenazados de los ecosistemas tropicales (Kinnaird and O'Brien 2007). Más de un tercio de las especies de calaos se consideran de interés para la conservación a nivel mundial, incluido el 62% de las especies asiáticas, algunas de las cuales (ej: *Anthracoceros montani*, *Aceros waldeni*), se encuentran en peligro de extinción (Gonzalez et al. 2013).

2) ÁREA DE ESTUDIO

- **Abubillas**

El estudio con las abubillas se llevó a cabo entre los meses de Marzo y Julio de 2015 y 2016, en una población silvestre que nidifica en cajas nido en la Hoya de Guadix (37°18′N, 38°11′W), Granada, España (Figura 9). Esta población lleva siendo estudiada durante los últimos 25 años. Las cajas nido están hechas de corcho, y tienen las siguientes dimensiones: 35 × 18 × 21 cm (altura × anchura × profundidad), 24 cm (altura desde la entrada hasta el fondo de la caja) y 5.5 cm (diámetro de la entrada) (Figura 13).

Figura 9. Situación en el mapa de la zona de campo de las abubillas, la Hoya de Guadix (37°18′N, 38°11′W), en Granada, España.

- **Calaos**

El estudio de los calaos se realizó con individuos mantenidos en cautividad en instalaciones de parques zoológicos de distintos países de Europa (Figura 11). Se muestrearon un total de 53 ejemplares pertenecientes a 13 especies y 6 géneros. Durante los años 2015 y 2016, 32 individuos pertenecientes a 11 especies y 6 géneros, fueron muestreados en España y Portugal en las siguientes instalaciones colaboradoras: Zoológico de Barcelona, Parque temático Oasys de Tabernas (Almería), Zoológico de Lourosa (Portugal), Palmitos Park (Las Palmas de Gran Canaria) y Jungle Park (Santa Cruz de Tenerife). Durante los meses de Noviembre y Diciembre de 2017, se muestrearon 22 individuos pertenecientes a 9 especies y 6 géneros en los siguientes zoológicos de Francia: Parc zoologique de Fréjus, Zoo de la Boissiére du Doré, Zoo d´Upie, Zoo Parc de Beauval y Zoo du Bois d'Attilly (Figura 11 y Tabla 1 del Capítulo V).

Figura 10. Distintas zonas de muestreo en la Hoya de Guadix, Granada. En la figura de arriba se muestran dehesas de encina. En la figura de abajo, pinares de repoblación, mezclados con encinas dispersas.

Figura 11. Localización de los distintos parques zoológicos muestreados en España, Portugal y Francia.

3) MÉTODOS DE MUESTREO EN EL CAMPO

3.1. Seguimiento y biometría

En la población silvestre de abubillas, las cajas nido se visitaron cada 5 días, desde principios de marzo hasta finales de julio. Se consideró fecha de puesta aquella en la que la hembra ponía su primer huevo, asumiendo que un huevo era puesto diariamente (Cramp 1998), y como fecha de eclosión, aquella en la que eclosionaba el primer huevo. Las hembras se capturaron a mano dentro de las cajas nido 15 días después de la fecha de puesta y, durante la temporada del 2015, también se capturaron 5 días después de la fecha

de eclosión. Los pollos se muestrearon 19-20 días después de la fecha de eclosión. Se tomaron las siguientes medidas morfológicas: se midió la longitud del pico y del tarso mediante un calibre (precisión 1mm) y el peso mediante una balanza (Pesola 0-100 g, precisión 1 g). Se extrajo la secreción de la glándula uropigial mediante el uso de micropipetas de volúmenes de 1-10 µl (ver Material y Métodos, apartado 4).

Durante la temporada de 2016 (Capítulo III) también se midió el tamaño (largo y ancho) de todos los huevos de la puesta mediante un calibre. Cuando fue posible, también se capturaron los machos mediante redes invisibles colocadas delante de la caja nido, tomándoles las mismas medidas y muestras que a las hembras.

Todos los individuos fueron marcados con anillas metálicas numeradas (Instituto Español para la Conservación Natural, ICONA) y los adultos, además, con una combinación única de anillas de colores. Las hembras y los pollos fueron liberados dentro de la caja nido después de la toma de muestras y los machos en el mismo sitio en el que fueron capturados. Para evitar la contaminación entre los nidos, se usaron guantes de látex previamente limpiados con etanol (96%) en todas las manipulaciones.

La condición física de la hembra fue calculada a través de los residuos del peso y de la longitud del tarso elevado al cubo (Senar and Pascual 1997, Peig and Green 2010, Labocha and Hayes 2012).

Las variables morfológicas o de color de los calaos, utilizadas en los análisis del Capítulo V, fueron las siguientes: proporción de la superficie coloreada de color amarillo naranja o rojo en (1) el pico, (2) el casco, (3) suma de proporciones coloreadas en el pico y en el casco, (4) tamaño del casco, (4) superficie de plumas blancas en el cuerpo, (5) número de superficies coloreadas en el cuerpo en esas tonalidades y (6) coloración cosmética (sí o no). Estas variables fueron extraídas de Poonswad et al. 2013 y Kemp 2001 y de las fotografías de los tubos de microcentrífuga (Figura 12). Las proporciones coloreadas del pico y del casco se calcularon en función de la cantidad de superficie coloreada de estas partes (amarillo, naranja y / o rojo), bien mediante carotenoides o bien mediante el uso de la secreción uropigial como cosmético. En cuanto al porcentaje blanco de plumas, la superficie del cuerpo se dividió en 5 partes, desde la cabeza hasta la punta de la cola: (1) cabeza y cuello, (2) alas superiores y pecho (3) alas inferiores y abdomen, (4) base del ala y (5) punta del ala. Se agregó la superficie blanca total de cada parte y el resultado se dividió entre 5. Valores de 0 corresponderían a individuos sin plumas blancas en todo su cuerpo y 1 a individuos completamente blancos. Se consideró como coloración

corporal de cada especie como el número de estructuras coloreadas (rojo, naranja y/o amarillo). Cuando tenían la secreción uropigial coloreada, que utilizaban como cosmético en el cuerpo, se tuvo en cuenta la glándula uropigial como una estructura coloreada más. Cuando algunas de estas variables variaban entre sexos, se tuvo en cuenta los datos del sexo más ornamentado. Para el análisis comparativo, se calculó la media de cada variable por especie al comprobar que existían variaciones en la carga bacteriana entre especies.

3.2. Estimas de color

Para la estimación del color de los huevos se usó un espectrómetro (Ocean Optics S2000 conectado a una luz halógena de deuterio (D2-W, Mini)). Durante la temporada de 2015 se tomó una única medida del color de los huevos el día 15 desde la fecha de puesta para el estudio descriptivo (Capítulo II). En cambio, durante la temporada de 2016 se midió tanto antes como después del experimento del intercambio de huevos (ver Figura 1. Capítulo III). Para estandarizar las condiciones de luz ambiental, se usó una bolsa de tela negra en el que se introducía la punta de la fibra óptica y el huevo que iba a ser muestreado. Antes de empezar las mediciones en cada puesta, el espectrómetro se calibró utilizando un blanco estándar (WS-2) y un negro de referencia. Se obtuvieron valores de reflectancia cada 10 nm, desde 300 nm hasta 700 nm para todos los huevos de cada puesta (aunque para el experimento del 2016 sólo se usaron los datos de color de los 6 huevos intercambiados). El color se midió en 5 puntos equidistantes sobre una línea aleatoria a lo largo de un eje desde el ápice hasta la base del huevo. Para estimar la repetibilidad, cada zona se midió tres veces.

3.3. Muestreo de cebas

Para cuantificar la inversión parental de la abubilla, se grabaron las visitas a los nidos por los padres durante la fase de incubación (una grabación durante la temporada de 2015 y dos grabaciones durante la temporada de 2016: antes y después del intercambio) y durante la fase de pollos (cuando el primer pollo tenía 3 y 11 días). Para ello se usaron cámaras de video digitales (Sony Handycam DCR-SR55 and DCR SR190) situadas varios metros alejadas del nido, camufladas con vegetación, piedras y troncos o situadas en el interior de pequeñas cajas nido camufladas. Las grabaciones se iniciaron alrededor de las 16 h y

grabaron un periodo de aproximadamente tres horas. Los individuos se identificaron mediante su combinación de anillas de colores y mediante otras características distinguibles de su patrón de coloración del plumaje o de su cuerpo.

3.4. Muestreo de comunidades bacterianas

Para detectar si el tipo de material de nido tenía un efecto en la abundancia relativa de bacterias cultivables en la cáscara de los huevos de las abubillas silvestres (Capítulo I), se muestreó un huevo por puesta, escogido aleatoriamente, el día 15 desde que la hembra puso su primer huevo.

Para testar la diferencia en la composición bacteriana que poseen los calaos en función de la zona del cuerpo (con mayor o menor acceso a la secreción) y del color del plumaje (posible coloración cosmética) así como la composición bacteriana de la secreción de la glándula uropigial, se tomaron muestras en ejemplares mantenidos en cautividad en las siguientes zonas: plumaje (zona blanca y negra del ala, zona blanca y negra de la cola y zona de la nuca), pico, casco, penacho que rodeaba a la glándula uropigial y, cuando fue posible, su secreción. Para detectar de forma visual alguna diferencia en la coloración de las muestras en función de la zona del cuerpo se fotografiaron las muestras (los tubos de microcentrífuga con el hisopo en su interior) sobre una tabla de color (Figura 12).

Para el muestreo de las comunidades bacterianas en huevos de abubillas, y en las distintas zonas del cuerpo de los calaos, se limpió la superficie a muestrear con hisopos estériles (EUROTUBO® DeltaLab) previamente humedecidos en tampón fosfato estéril (PBS, 0.2M; pH = 7.2). A continuación se introdujo el hisopo en un tubo estéril de microcentrífuga con 1000 µl de PBS. Las muestras se mantuvieron refrigeradas a a 4 ºC hasta su procesado, en un máximo de tres días. Después de sembrarlas se mantuvieron congeladas hasta su extracción de ADN (ver Material y Métodos, apartado 5).

Figura 12. Tubos de microcentrífuga con los hisopos muestreados en su interior donde es posible apreciar una coloración cosmética en las muestras del penacho de la glándula uropigial, del pico y del casco (2ª, 5ª y 8ª de izquierda a derecha). Las muestras corresponden a un macho de *Buceros rhinoceros*, del Zoo de Beauval.

Para extraer la secreción de la glándula uropigial de las abubillas, en primer lugar, se limpió la zona próxima a la papila de la glándula mediante un algodón humedecido en etanol 96%. A continuación, se succionó parte de la secreción mediante la introducción suave de una punta estéril con una micropipeta de 1-10 µl y se introdujo en un tubo estéril de microcentrífuga. Este proceso se repitió varias veces hasta extraer toda la secreción contenida dentro de la glándula. Las muestras se mantuvieron refrigeradas en una nevera portátil hasta ser procesadas posteriormente en el laboratorio en ese mismo día.

Para extraer la secreción de la glándula uropigial de las distintas especies de calaos muestreadas, una vez muestreado el penacho de plumas que rodeaba a la glándula con el hisopo, se cortó en lo posible el penacho de plumas, y posteriormente, se limpió la zona de alrededor de la glándula con un algodón previamente humedecido en etanol 96%. A continuación, se estimuló la producción de la secreción presionando ligeramente la glándula en distintas zonas. Cuando se consiguió extraer su secreción, esta se recogió con ayuda de capilares que fueron posteriormente introducidos en tubos estériles de microcentrífuga. Estas muestras se mantuvieron refrigeradas hasta su procesado, en un máximo de 3 días.

3.5. Diseño experimental

Para comprobar si el material viejo del nido afecta a la selección de cavidad para la nidificación y a la comunidad bacteriana desarrollada en la glándula (Capítulo I), se instalaron cajas nido nuevas en el área de estudio al principio de la primavera del 2015 (Figura 13). También se limpiaron las cajas nido antiguas (instaladas en las temporadas anteriores), dejándolas vacías a principios de la temporada de cría (Marzo del 2015). En el caso de las cajas nido que fueron usadas por abubillas la temporada anterior (14 nidos en el 2014), se recogió el material del nido y se mantuvo en bolsas de plástico para utilizarlo en el experimento. Se conservó a temperatura ambiente y con las bolsas agujereadas para permitir el intercambio de aire, hasta ser añadido a las cajas experimentales una o dos semanas después. Las cajas nido experimentales fueron situadas al azar en pares, con una distancia entre ambas de unos 25 metros, mezcladas con las cajas nido antiguas instaladas los años anteriores (Figuras S1 y S2 en material suplementario del Capítulo I). Para investigar el posible efecto del material antiguo, una de las cajas nido emparejadas se rellenó con una capa de unos 3 cm de serrín (Allspan® Animal bedding, wood shavings; nidos control) y la otra con el mismo volumen, consistente en una mezcla que contenía la mitad de serrín y la otra mitad de material de un nido viejo de abubilla del año anterior (material viejo) (Figura 14). Este procedimiento se llevó a cabo usando guantes de látex para cada caja nido, evitando así la contaminación de las cajas controles (con sólo serrín). Se colocaron un total de 49 pares de nuevas cajas nido experimentales tanto en zonas donde había cajas nido antiguas como en nuevas superficies de plantaciones de pino en el borde del área usada en años anteriores. Para evaluar si había preferencia entre las cajas nido nuevas con material frente a las cajas nido nuevas sin material, se instalaron 88 nuevas cajas nido vacías, situadas en el mismo árbol que las cajas nido antiguas (limpiadas al principio de la temporada de campo) (Figuras S1 y S2 en material suplementario del Capítulo I).

Figura 13. Cajas nido nuevas instaladas durante la temporada de campo del 2016.

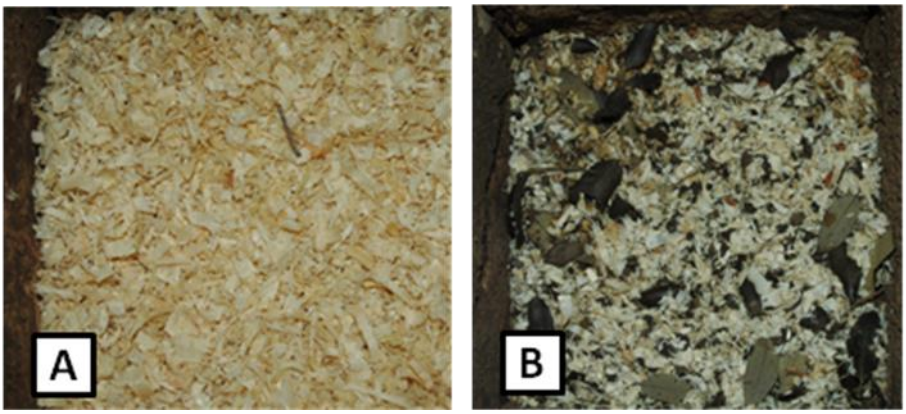

Figura 14. Material experimental introducido dentro de las cajas nido nuevas. A la izquierda, serrín solo, y a la derecha, serrín mezclado con restos de un nido viejo de abubilla.

Para determinar si la coloración de los huevos afecta a la contribución de cebas del macho (Capítulo III) se realizó un experimento durante la temporada de cría de 2016 manipulando el color de los huevos. Para ello se intercambiaron 6 huevos entre parejas de puestas con una diferencia en la fecha de puesta menor a 4 días. El intercambio se realizó cuando habían transcurrido al menos dos días desde que el sexto huevo había sido puesto (para dar tiempo suficiente a la hembra a que manchase los huevos con su secreción uropigial) (ver Figura 1 del Capítulo III). De esta forma, los machos fueron expuestos a dos tipos de puestas: a sus huevos iniciales manchados con la secreción uropigial de su hembra, y aquellos de otra hembra que finalizó la incubación al mismo tiempo aproximadamente.

Para el intercambio se escogieron los 6 huevos de cada puesta que estaban más cubiertos de secreción, es decir, aquellos que con más probabilidad eran los seis primeros en la secuencia de puesta. El resto de huevos se retiraron y se mantuvieron en una incubadora a 37.5ºC (Covatutto 24 Eco, Novital) hasta su eclosión. Una vez que eclosionaron, se alimentaron a mano con larvas previamente congeladas y se introdujeron ese mismo día en nidos que tenían pollos de su misma edad.

4) MÉTODOS DE LABORATORIO

- **Medios de cultivo**

Para el cultivo de los microorganismos se emplearon los medios indicados a continuación:

Tabla 1. Composición del medio de cultivo TSA

TSA *(Tryptic Soy Agar.* Scharlau Chemie S.A., Barcelona)	
Digerido pancreático de caseína	17 g
Digerido enzimático de soja	3 g
D-Glucosa	2.5 g
NaCl	5 g
K_2HPO_4	2.5 g
Agar	18 g
Agua destilada	Hasta 1 L

El pH del medio se encuentra ajustado a 7.3 por el fabricante. Autoclave a 121ºC durante 15 min.

Tabla 2. Composición del medio de cultivo FMA

FMA (*Feather Meal Agar*)	
Plumas previamente lavadas y trituradas	15 g
KH_2PO_4	0.4 g
Cicloheximida	0.1 g
NaCl	0.5 g
K_2HPO_4	0.3 g
Agar	15 g
Agua destilada	Hasta 1 L

Autoclave a 121ºC durante 10 min.

Tabla 3. Composición del medio de cultivo KF

KF Agar *(Kenner Fecal Agar.* Scharlau Chemie S.A., Barcelona)	
Proteosa peptona	10 g
Extracto de levadura	10 g
NaCl	5 g
Glycerofosfato sódico	10 g
Maltosa	20 g
Lactosa	1 g
Azida de sodio	0.4g
Púrpura de bromocresol	0.015 g
Agar	15 g
Agua destilada	Hasta 1 L

El pH del medio se encuentra ajustado a 7.2 por el fabricante. Después del autoclave a 121ºC durante 10 min, se añadieron 8mL de Triphenyl Tetrazolium Chloride (TTC) al 1%.

Tabla 4. Composición del medio de cultivo VJ

VJ Agar *(Vogel Johnson Agar.* Scharlau Chemie S.A., Barcelona)	
Digerido pancreático de caseína	10 g
Extracto de levadura	5 g
Manitol	10 g
K_2HPO_4	5 g
LiCl	5 g
Glicina	10 g
Rojo de fenol	0.025 g
Agar	15 g
Agua destilada	Hasta 1 L

El pH del medio se encuentra ajustado a 7.2 por el fabricante. Después del autoclave a 121ºC durante 15 min, se añadieron 16mL de solución estéril ***Telurito potásico*** *al 1%.*

Tabla 5. Composición del medio de cultivo HK

HK Agar *(Hektoen Enteric Agar*. Scharlau Chemie S.A., Barcelona)	
Extracto de levadura	3 g
Sales biliares	9 g
Lactosa	12 g
Sacarosa	12 g
Salicina	2 g
NaCl	5 g
Tiosulfato de sodio	5 g
Citrato de amonio férrico	1.5 g
Fucsina ácida	0.10 g
Azul de bromotimol	0.06 g
Agar	15 g
Agua destilada	Hasta 1 L

El pH del medio se encuentra ajustado a 7.5 por el fabricante. Se llevó a ebullición en el microondas.

Los tubos de microcentrífuga fueron agitados vigorosamente en un agitatubos para facilitar la transmisión de las bacterias situadas en el hisopo hacia el PBS, y para homogeneizar la muestra.

Para las muestras de hisopos de huevos de abubilla se realizaron diluciones seriadas decimales hasta 10^{-6}. Finalmente se sembraron 100 µl de cada una de las diluciones en los siguientes medios de cultivo sólido: TSA, ampliamente utilizado para el crecimiento de bacterias heterotróficas mesófilas; KF, selectivo y diferencial para especies del género *Enterococcus*, y dos medios específicos y diferenciales para bacterias potencialmente patógenas: *Enterobacteriaceae* (HK) y *Staphylococcus* (VJ). Las placas se incubaron aeróbicamente a 37 ºC entre 24-72 horas, y hasta la aparición de colonias. La estimación de la carga bacteriana se estandarizó a número de unidades formadoras de colonias (UFC) por mililitro (Nº colonias * $10^{\text{factor de dilución}}$) / ml inoculado).

- **Determinación de mesófilos**

Se utilizaron 5 µl de la secreción de la abubilla y se homogeneizaron con 45 µl de PBS en un tubo estéril de microcentrífuga. A partir de esta suspensión inicial se realizaron diluciones seriadas en solución salina (10^{-4}) en los medios TSA, KF, HK y VJ, sembrándose 100 µl por placa y por triplicado. Las placas se incubaron aeróbicamente a 37 ºC durante 24 h (medios TSA y HK) y 48-72 horas (medios KF y VH). Las estimas de carga bacteriana se estandarizaron a UFC/ml.

En cuanto la secreción de los calaos, se usó el extremo de los capilares con secreción en su interior y en sus alrededores para sembrar en estría en TSA y se anotó la presencia o ausencia de crecimiento a las 24 horas en condiciones aerobias a 37ºC. Las muestras del penacho de plumas de rodeaban a la glándula uropigial se sembraron en los siguientes medios: TSA, KF, HK, VJ y FMA.

Para estimar la abundancia de distintos grupos de bacterias en las distintas zonas del cuerpo de los calaos, tanto las muestras del plumaje (ala, cola y nuca) como las del casco, se sembraron en los medios TSA y FMA. Para las muestras del pico se usaron los medios TSA, KF, HK, VJ. A las 72 horas se procedió al conteo del número de UFC/ml en cada uno de los medios de cultivos utilizados.

- **Estudio microscópico de la secreción**

En algunos casos se depositó también una gota de secreción de calaos en un portaobjetos de microscopio de adhesión de polisina (Thermo Scientific, Waltham, MA, EE. UU). Esta gota se extendió con ayuda de un segundo portaobjetos limpio para formar una capa lo más delgada posible. A continuación se fijó en paraformaldehído al 4%; lavado en agua destilada estéril; deshidratado en sucesivos baños de etanol al 50%, 80% y 96%; aire seco; y almacenado en oscuridad a 4 ° C hasta su observación microscópica. Se dio prioridad al muestreo para el cultivo de bacterias y, con frecuencia, no se obtuvo secreción, por lo tanto, las muestras para el cultivo / estudio microscópico de las secreciones no siempre estaban disponibles (Tabla x).

Las secreciones fijadas en portaobjetos se utilizaron para estudiar la presencia y abundancia de bacterias mediante varios métodos diferentes. Algunos de ellos se tiñeron con cristal violeta durante dos minutos después de la re-fijación en etanol-cloroformo, se lavaron en etanol 96º y se secaron al aire. El resto se marcaron para observación al microscopio de fluorescencia. Para ello, las paredes celulares se permeabilizaron

agregando 10 µL de solución de lisozima (100 mM Tris-HCl 50 mM EDTA, pH 8) 2 mg / ml de lisozima (USB Corporation, Cleveland, OH, EE. UU) y se incubó a 37 ° C durante 60 minutos en atmósfera húmeda. Después, se lavaron los portaobjetos con agua destilada filtrada; aire seco; y deshidratado en sucesivos baños de etanol al 50%, 80% y 96%. El marcado fluorescente de ADN se realizó cubriendo las secreciones fijas con una mezcla de hibridación (20 mMTris-HCl; 0.9 M NaCl; SDS 0.1%; 25% formamide, Sigma-Aldrich; pH 7.2–7.4) y 10 µL de Hoechst (10 µg / mL, Sigma-Aldricht, St. Louis, MO, EE. UU). Se mantuvo durante 30 minutos en una atmósfera húmeda. A continuación se lavó con solución salina tamponada con fosfato (PBS, 0.2 M; pH = 7.08) durante 2 minutos y se secó a temperatura ambiente. Finalmente, se montaron los portaobjetos aplicando una gota de solución *antifade* (Vectashield Mounting Medium H-1000, Vector Laboratories, Burlingame, CA, EE. UU.) y un cubreobjetos, y luego se sellaron con barniz. Las observaciones se realizaron a través de un microscopio de fluorescencia Olympus BX51 con un aumento de 100x con filtro DAPI (azul). Todo el proceso se realizó manteniendo los portaobjetos en oscuridad para evitar la decoloración del colorante Hoechst, y se almacenaron en cajas oscuras a 4 ° C hasta su visualización bajo microscopía de fluorescencia.

- **Extracción de ADN total de las comunidades bacterianas**

Para la extracción del ADN genómico de la comunidad bacteriana de la glándula uropigial de las abubillas se utilizó el kit comercial FavorPrep Blood Genomic DNA Extraction Mini Kit (Favorgen Biotech), combinando con un tratamiento previo de la muestra con lisozima (10 mg/ml, 37 ºC durante 30 minutos) (Rodríguez-Ruano et al. 2018), para asegurar la rotura de las paredes celulares bacterianas, y posteriormente siguiendo las instrucciones del fabricante. Las extracciones se realizaron a partir de unos volúmenes de 10-50 µl de secreción, procedentes de animales capturados en el 2015 (Capítulo I). La suspensión con el ADN se almacenó en tubos de microcentrífuga a -20 ºC hasta su uso.

En el caso de las muestras de calaos, se siguió el mismo proceso que en abubillas para su extracción de ADN, pero con ligeras modificaciones. La suspensión de los hisopos se sonicó previamente en un baño de ultrasonidos durante 90 segundos para separar la muestra del hisopo y facilitar su dispersión en el sobrenadante. Posteriormente las muestras se centrifugaron durante 5 minutos en una microfuga a máxima velocidad.

Finalmente, el sedimento se trató con lisozima antes de ser procesado con el kit comercial Favorgen Biotech.

El ADN se almacenó a -20ºC hasta que se procesó para la secuenciación. Paralelamente a las muestras de calaos, se realizó el mismo protocolo para controles negativos para analizar posibles fuentes de contaminación. Las muestras se procesaron en dos lotes diferentes desde la extracción de ADN hasta su secuenciación, incluyendo controles para ambos lotes.

1. **Electroforesis en gel de agarosa**

La visualización y comprobación de las extracciones de ADN y de los productos de PCR se llevó a cabo mediante electroforesis en gel de agarosa. Se realizaron electroforesis sumergidas horizontales en gel de agarosa, formado por tampón TAE (1x), preparado a partir del tampón TAE 50x (Tabla 6) y una concentración de agarosa variable en función del tamaño del ADN (0,75% para la comprobación de las extracciones de ADN total y 1% para los productos de PCR). Los pocillos se cargaron con 10 µL de la mezcla de producto de PCR y solución de carga (1/10; Glicerol 50% (v/v); azul de bromofenol 0,25 %; TE (10 mM Tris- HCl pH 8; EDTA 1mM) 49,75 %). Al gel con las muestras de ADN y los marcadores se le aplicó un voltaje constante de 80-90 V hasta que el frente alcanzó el borde del gel.

Una vez terminada la electroforesis, el gel se sumergió en una solución de bromuro de etidio (1 µg/mL en agua destilada) durante 15 minutos. Posteriormente se lavó con agua abundante mediante inmersión para eliminar el exceso de bromuro de etidio y se visualizaron las bandas mediante el sistema Gel Doc XR (Bio-Rad), que expone al gel a luz ultravioleta de 302 nm. Finalmente, el gel se fotografió con una cámara CCD de 1.4 Megapixels (1360x1024).

Tabla 6. Composición del tampón TAE 50x utilizado en la preparación de geles de agarosa y posterior electroforesis.

Tampón TAE	
Tris base	242,0 g
Ácido acético glacial	57,1 mL
EDTA-Na_2 O,5 M, pH 8	100 mL
Agua destilada	Completar hasta 1L

2. Identificación de las comunidades bacterianas mediante secuenciación masiva: secuenciación mediante tecnología MiSeq/HiSeq (Illumina)

En primer lugar, se estimó la concentración de ADN mediante su absorción a 260 nm en un nanodrop. Las muestras se estandarizaron a una concentración de 3 ng/ µL para igualar la concentración de ADN. La preparación de librerías se llevó a cabo mediante amplificación por PCR del gen del ARN ribosomal 16S procariota (16S rDNA). Este gen contiene aproximadamente 1.500 pares de bases (pb) de longitud y contiene 9 regiones variables, intercaladas entre las conservadas. En nuestro caso se amplificaron las regiones hipervariables V6-V8 de este gen.

El procedimiento consiste en 2 amplificaciones consecutivas con purificación de las muestras tras cada amplificación. Todas las amplificaciones de ADN se realizaron utilizando un termociclador iCycler 170-8720 (BioRad) o en un Mastercycler (Eppendorf).

Para la primera amplificación de la región V6-V8 se utilizaron cebadores que incluían adaptadores de Illumina en su extremo 5'. Los cebadores usados fueron B969F (5´ AATGATACGGCGACCACCGAGATCTACAC-NNNNNNNN-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACGCGHNRAACCTTACC-3´)) y BA1406R (5´-CAAGCAGAAGACGGCATACGAGAT-NNNNNNNN GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACGGGCRGTGWGTRCAA-3´), que incluyen los adaptadores estándar de Illumina Nextera y 8 pb de adaptadores específicos (barcodes) (series S500 + N700). Los barcodes están representados por NNNNNNNN, y las regiones cebadoras específicas están subrayadas. Los componentes de la PCR se ajustaron a 25 µL (Tabla 7) y las condiciones de la amplificación fueron:

desnaturalización inicial a 98ºC durante 30 s, 25 ciclos de desnaturalización a 98ºC durante 10 s, anillamiento a 52ºC durante 20 s y extensión a 72ºC durante 10 s, y una extensión final a 72ºC durante 5 min. El producto de PCR se purificó mediante el uso de micropartículas magnéticas comerciales (DNA Purification SPRI Magnetic Beads - Canvax), siguiendo las recomendaciones del fabricante y con ligeras modificaciones:

- ✓ Se mezcló 25 µL de suspensión de nanopartículas magnéticas en los microtubos con un volumen similar del producto de PCR a purificar. Esta suspensión de mantuvo unos 5 minutos a temperatura ambiente, mezclándose de forma ocasional.
- ✓ Pasado ese tiempo, se adaptó la placa magnetizada bajo la placa que contiene la mezcla; tras 3 minutos se retiró el sobrenadante de forma cuidadosa.
- ✓ Una vez desechado el sobrenadante, se realizaron dos lavados con 200 µL de etanol 80% recién preparado, esperando 30 segundos antes de retirar de nuevo el sobrenadante y secándose posteriormente durante 5-10 minutos en la campana de flujo laminar.

Se retiró la placa magnetizada y el ADN retenido en las bolas se eluyó con 30 µL de tampón de elución. Los productos amplificados se almacenaron hasta su uso a -20º. Posteriormente se separó el ADN de las macropartículas magnéticas por elución en agua milliQ o TE. La concentración de ADN se midió usando un fluorímetro Qubit® 3.0 (Invitrogen ™) y se estandarizaron para obtener 25 ng de ADN por muestra en mezclas de secuenciación. Cuando las muestras o los controles mostraron concentraciones de ADN muy bajas no detectables, se utilizaron 25 µl de su solución almacenada. La secuenciación de alto rendimiento se llevó a cabo en la plataforma IlluminaMiSeq en el Instituto de Parasitología y Biomedicina López Neyra (CSIC, Granada, España).

Tabla 7. Composición de la solución de PCR para la amplificación de la región V6-V8 como primer paso para la secuenciación masiva mediante la plataforma Illumina MiSeq. Los cebadores Mi_U515 y Mi_E786 incluyen los adaptadores de Illumina.

Solución PCR (1)	
ADN molde (3ng/ µL)	5 µL
B976F (3pm/µL)	2,5 µL
BA1406R (3 pm/µL)	2,5 µL
Master Mix (iProof HF Bio-Rad)	12,5 µL
H_2O	2,5 µL

A continuación, se llevó a cabo una segunda PCR en la cual se introdujeron barcodes específicos para cada muestra. Los cebadores utilizados en esta PCR solapan parcialmente con los adaptadores de Illumina incluidos en la primera PCR. No es posible incluir la secuencia de estos cebadores debido a protección por patente. El producto de PCR se purificó de igual forma que los de la primera PCR. La cantidad total se ajustó a 25 µL del volumen final (Tabla 8) y las condiciones de la amplificación con los cebadores fueron las siguientes: desnaturalización inicial a 98ºC durante 30 s, 8 ciclos de desnaturalización a 98ºC durante 10 s, anillamiento a 55ºC durante 20 s y extensión a 72ºC durante 10 s, y una extensión final a 72ºC durante 5 min. Todas las amplificaciones se realizaron por duplicado.

Tabla 8. Composición de la solución de PCR para la amplificación de la región V6-V8 como segundo paso para la secuenciación masiva mediante la plataforma Illumina MiSeq. Los cebadores N70 y S50 incluyen los barcodes específicos para cada muestra.

Solución PCR (2)	
ADN molde (3ng/ µL)	5 µL
N70(X) (3pm/µL)	3,3 µL
S50(X) (3 pm/µL)	3,3 µL
Master Mix (iProof HF Bio-Rad)	12,5 µL
H_2O	2,5 µL

Por último, la integridad de las librerías se confirmó mediante una tercera PCR utilizando cebadores complementarios a los adaptadores incorporados en la segunda PCR (Tabla 9). No es posible incluir la secuencia de estos cebadores debido a protección por patente. Las condiciones de amplificación en este caso fueron las siguientes: desnaturalización inicial a 98ºC durante 30 s, 29 ciclos de desnaturalización a 98ºC durante 10 s, anillamiento a 55ºC durante 20 s, y una extensión a 72ºC durante 10 s, y una extensión final a 72ºC durante 5min.

Tabla 9. Composición de la solución de PCR para la amplificación de la región V6-V8, como tercer paso para la comprobación de una correcta amplificación antes de enviar las muestras a secuenciar mediante la plataforma Illumina MiSeq.

Solución PCR (3)	
ADN molde (2ng/ µL)	5 µL
MIFINAL F (3pm/µL)	2,5 µL
MIFINAL R (3 pm/µL)	2,5 µL
Master Mix (iProof HF Bio-Rad)	12,5 µL
H_2O	2,5 µL

Las librerías se secuenciaron mediante MiSeq de Illumina (2 x 300 bp output mode), una secuenciación de segunda generación. Este proceso se llevó a cabo en el Integrated Microbiome Resource, Centre for Comparative Genomics and Evolutionary Bioinformatics (CGEB), University of Dalhousie (Canadá), o bien en el servicio de secuenciación masiva del Instituto de Parasitología y Biomedicina López Neyra (CSIC, Granada).

3. Estudio de la capacidad antagonista de la secreción uropigial de abubillas

La actividad antagonista de la secreción uropigial de las abubillas se estimó frente a la cepa indicadora D13 de *Bacillus licheniformis*, un microorganismo potencialmente patógeno por su capacidad de degradación de plumas en las aves (Ruiz-Rodríguez et al. 2009). Para la preparación de las placas de antagonismo, se utilizaron tubos de agar en sobrefusión (1,5 % TSB-0,75 % agar, 0,1 M tampón fosfato pH 7,2) previamente

inoculados con 100 µl de un cultivo de una noche de la cepa indicadora, vertiéndolo directamente sobre una placa de agar base de TSA. A continuación, se ensayaron 5 µl de la secreción uropigial de la abubilla sobre la superficie de la placa solidificada de antagonismo. Como control de la actividad antibacteriana se ensayó en paralelo 2 µl de amoxicilina (714 µg/ml) diluida en PBS. Las placas fueron incubadas a 28ºC durante 12 horas. La actividad antimicrobiana de la secreción se detectó mediante la aparición de un halo claro que mostraba la inhibición del crecimiento bacteriano alrededor de la gota de la secreción uropigial. Se midió tanto el diámetro del halo como el diámetro de la gota, restando esta última medida sobre el diámetro del halo para obtener el ancho de la zona de inhibición.

4. Cuantificación de parámetros de color

Para analizar los datos de espectrometría se utilizó el programa Avicol V.6 (Gómez 2006). Antes de realizar los análisis, se realizaron las siguientes modificaciones a los datos del espectro: se ajustaron a cero los valores negativos, se interpolaron los datos en columnas y se disminuyó el ruido de las curvas de reflectancia usando una corrección triangular (Gómez 2006).

En contextos de señalización, es preferible analizar los datos de color de una manera que sea apropiada para la visión del animal (Endler 1990, Renoult et al. 2017). En el caso de las aves, estas tienen cuatro tipos de conos en sus retinas, a diferencia de los humanos, que tienen tres. Estas diferencias hacen que nuestra visión y nuestra percepción de los colores sea muy diferente de la que perciben los que poseen una visión tetracromática (Ödeen and Håstad 2003). Además, su visión del color se puede categorizar en dos clases: con sensibilidad a longitudes de onda corta sesgadas hacia el violeta (VS) o sesgadas hacia el ultravioleta (UVS) (Ödeen and Håstad 2003). Los primeros tienen una absorbancia máxima en sus conos que oscila entre 402 y 426nm mientras que los segundos oscila entre 355 y 380 nm (Hart 2001). La abubilla posee un sistema visual sensible al violeta, que no le permite captar longitudes de onda cercanas al rango UV (Ödeen and Håstad 2003). Debido a que aún no hay datos de la sensibilidad espectral de la abubilla, se usaron los datos del pavo real (*Pavo cristatus*) como valores de referencia del sistema VS en la abubilla (Hart 2002). Para estimar sus parámetros visuales se utilizó un modelo fisiológico para visión tetracromática en

Avicol. Concretamente se usó el modelo propuesto por Endler y Mielke (2005) con algunas modificaciones propuestas por Stoddard y Prum (2008), tales como, la no transformación logarítmica de los valores de respuesta de los fotorreceptores y la corrección para los colores oscuros. Se obtuvieron dos variables con coordenadas esféricas: tetha (θ) y phi (φ) y las variables chroma (r) y qrQ. θ mide el ángulo (entre -180º y 180º) en el plano rojo-verde-azul y φ (entre -90º y 90 º) en el rango sensible UV/V (Endler and Mielke 2005). θ y φ aportan información del tono, qrQ de la luminosidad y r de la saturación del color (Stoddard and Prum 2008, Saino et al. 2013). Debido a que el espacio de color es un tetraedro y no una esfera, el máximo potencial chroma (r_{max}) depende de los valores del tono. Por este motivo, en nuestros análisis se usó *achieved chroma*(rA) calculado como: $rA=r/ r_{max}$ (Stoddard and Prum 2008). De esta manera, los datos obtenidos tienen en cuenta la sensibilidad y la estimulación de cada cono del sistema visual de las aves, permitiéndonos medir el color de una manera relevante a como lo aprecian las aves (Endler 1990, Saino et al. 2013). Sin embargo, poco se sabe acerca de los procesos neuronales, es decir, de cómo los ojos y el cerebro procesan los patrones de color (Stoddard and Osorio 2019). Por este motivo, se deben interpretar con precaución los resultados obtenidos en los modelos visuales fisiológicos de las aves.

5. Estima de la inversión parental

Para estimar la cantidad de comida llevada por cada individuo, se consideró el número de presas llevadas por hora (tasa de cebas) y la media del tamaño relativo de cada presa, la cual se estimó con una escala ordinal de 1 a 3 en función del tamaño de la presa comparándolo con el tamaño del pico. Así, se consideró 1 cuando el tamaño de la presa era menor que un cuarto del tamaño del pico, 2 cuando era entre un cuarto y la mitad del pico, y 3 cuando era mayor que la mitad del pico (Martín-Vivaldi et al. 1999). Se multiplicaron estas dos medidas para obtener un índice de aporte. Cuando no se pudo apreciar el tamaño de las cebas debido a la mala calidad del vídeo o a que la orientación del macho durante la ceba impedía su observación, se consideró que su aporte era la media del aporte del resto de machos durante esa fase. Para la visualización de los vídeos se utilizó el programa VLC Media Player (versión 2.2.6). Sólo las primeras puestas fueron consideradas en el análisis de los vídeos del 2015, analizando un total de 39 nidos (Capítulo II). En cuanto a los vídeos de la temporada de 2016, se descartaron aquellos que sólo tenían una grabación, al ser depredados o

abandonados después del intercambio de huevos, analizando un total de 61 nidos (Capítulo III).

5) ANÁLISIS DE SECUENCIAS

Tras la secuenciación, las secuencias obtenidas se analizaron utilizando los paquetes informáticos QIIME v1.9.1 y QIIME2 v2019.4 (Quantitative Insights In Microbial Ecology). QIIME es un software de análisis del microbioma, extensible y abierto que permite comenzar los análisis con secuencias de ADN en bruto y finalizar con cifras de calidad, perfiles taxonómicos y filogenéticos, así como comparaciones de las muestras de estudio, análisis estadísticos y visualizaciones gráficas de los datos (Caporaso et al. 2010). Las tablas de OTUs (Operational Taxonomic Units) y el procesado de datos genómicos se completaron además con las recomendaciones propuestas por diversos autores ((Navas-Molina et al. 2013, Dumbrell et al. 2017, Knight et al. 2018).

QIIME utiliza secuencias obtenidas a partir de una o más tecnologías de secuenciación como Sanger, Roche/454 o Illumina (Kuczynski et al. 2012). Este software integra herramientas ("plug-in"), en concreto "envuelve" los algoritmos y herramientas producidos por investigadores en una única línea para el análisis de secuencias (Navas-Molina et al. 2013). Algunas de estas herramientas que utiliza QIIME son:

- *USEARCH* (Edgar 2010): usado para el OTU picking y la eliminación de quimeras;
- *GreenGenes Database* (DeSantis et al. 2006): proporciona una base de datos de referencia para asignar taxonomía y para selección de OTU basado en referencias;
- *PyNAST* (Caporaso et al. 2010): se utiliza para alineamiento de secuencias multiples;
- *Deblur* (Amir et al. 2017): programa que permite la clasificación de secuencias en sub-OTUs;
- *Fragment-insert*: algoritmo que permite el alineamiento de secuencias y la preparación de estas para la construcción de árboles filogenéticos (en proceso de publicación);

- *UniFrac* (Ley et al. 2005): utilizado para análisis de diversidad beta;

Además, QIIME utiliza otros paquetes como IPython y R, lo que permite realizar análisis adicionales.

El proceso mediante el cual opera QIIME se puede dividir en dos etapas, denominadas "aguas arriba" y "aguas abajo" (Navas-Molina et al. 2013). "Aguas arriba" parte de las secuencias en bruto en formato *fastq* y genera archivos clave como la tabla de OTUs y el árbol filogenético. "Aguas abajo" utiliza la tabla de OTUs y el árbol filogenético generado en el paso anterior para realizar análisis de diversidad, estadísticas y visualizaciones interactivas de los datos.

- **Abubillas (Capítulo I)**

Para obtener la tabla de OTUs se utilizó el programa QIIME v1.9.1 (Capítulo 1) con las siguientes recomendaciones en el procesado de datos genómicos (Navas-Molina et al. 2013, Dumbrell et al. 2017, Knight et al. 2018).

Es conveniente realizar un solapamiento o "join" de las lecturas Forward (directa) y Reverse (reversa) puesto que el secuenciador toma lecturas de >250 pb tanto del extremo 3' como 5', usando el método *fastq-join* (Aronesty 2011), con un mínimo de solapamiento de 100 pb y un 10% de diferencia máxima en la región de solapamiento. A continuación, se procedió a la asignación de secuencias a cada muestra (*demultiplexing*) y al filtrado de calidad de las secuencias (at Phred >= Q20), recortando las secuencias a 400 pb con *Usearch*. La tabla de OTUs se generó mediante el método de referencia abierta (*open-reference OTU picking procedure* (Rideout et al. 2014)), agrupando las secuencias que tenían una similitud del 97% con las secuencias de referencia de la base de datos *Greengenes* versión 13_8 (DeSantis et al. 2006, McDonald et al. 2012), con un tamaño mínimo de 10 secuencias por OTU. De esta manera, la tabla de OTUs se filtró para eliminar secuencias de Arqueas, cloroplastos, mitocondrias, OTUs sin filo asignado, secuencias únicas (*singletons*) y OTUs con una frecuencia menor al 0.005% del total de secuencias (Bokulich et al. 2013). Después del filtrado, se obtuvieron un total de 1.696.144 secuencias válidas (Capítulo I). Con el fin de controlar e igualar el esfuerzo de secuenciación, se realizó una rarefacción múltiple (10 repeticiones aleatorias) de 1500 secuencias y se realizaron análisis con cada uno de los archivos rarificados para obtener la

media de los parámetros de los modelos estadísticos aplicados. Para los análisis y las figuras se usó el número normalizado de secuencias por OTU después de la rarefacción como una estimación de su abundancia rarificada (de 1.500 secuencias).

Una de las cuestiones más discutidas en el procedimiento de los datos HTS, es el método para el alineamiento de secuencias y agrupamiento (*clustering*) y la asignación de secuencias a OTUs. Tanto es así que cualquier enfoque puede ser cuestionado y, por tanto, debería ser justificado. El método de referencia abierta ha demostrado producir una asignación de OTUs más estable que sus alternativas de-*novo* o de referencia cerrada (He et al. 2015). Además, las etiquetas (*labels*) de los métodos basados en las referencias se pueden comparar entre estudios, a diferencia de con la aproximación *de-novo* (Callahan et al. 2017). A pesar de que los métodos basados en las referencias están sujetos a más errores cuando se asignan las taxonomías a OTUs que las aproximaciones *de-novo*, los métodos recientes de agrupamiento mejoran la precisión de la asignación de OTUs y preservan la diversidad estimada, también para los enfoques basados en referencias, y muchos de ellos están implementados en QIIME (Kopylova et al. 2016). Cualquier herramienta bioinformática usada para el análisis del microbioma tiene sus propias ventajas y desventajas, y sorprendentemente, todos tienen una baja precisión en la asignación taxonómica, ya que pequeños cambios en los parámetros utilizados pueden afectar profundamente a los resultados (Golob et al. 2017). Estas herramientas están siendo mejoradas continuamente y los mejores enfoques disponibles se modifican dependiendo de la versión (Callahan et al. 2017, Knight et al. 2018). De esta manera, los investigadores deberían evaluar las herramientas que han escogido para el procesado de los datos y compilaciones de los mismos (*pipelines*) para confirmar si puede responder adecuadamente a la hipótesis planteada así como proporcionar los detalles de sus métodos (Golob et al. 2017). Así, creemos que la aproximación del OTU basado en la referencia abierta usada en el Capítulo I, con los filtros de abundancia y calidad aplicados, reduce la posibilidad de falsos positivos (Bokulich et al. 2013, Golob et al. 2017), y es adecuado para la comparación del microbioma de cada individuo de la misma especie de ave, a nivel de OTU para bacterias.

- **Calaos (Capítulo IV)**

El procesamiento de las secuencias obtenidas de IlluminaMiSeq se llevó a cabo con QIIME2 v2019.4 para obtener los resultados de los dos lotes juntos. Primero, el recorte del

cebador se realizó usando el complemento *cutadapt* con parámetros predeterminados (Martin 2011). Las lecturas directas e inversas (*forward* y *reverse*) se unieron usando VSEARCH (Rognes et al. 2016). El filtrado de calidad se realizó utilizando parámetros predeterminados, y a continuación, se usó el programa *Deblur*, un enfoque de unidad taxonómica sub-operacional (sOTU), para crear la tabla de sOTUs y eliminar los errores de secuenciación (Amir et al. 2017). Las secuencias que pasaron los filtros de calidad se truncaron a 380 pb, utilizando 20 como límite de criterio de calidad, dando un conjunto de 11,430,874 lecturas totales. Después se usó el complemento de inserción de fragmentos, un *script* que realiza la alineación de secuencias y que crea el árbol bacteriano filogenético (Janssen et al., 2018). La asignación taxonómica se basó en *Greengenes* 13.08 con una similitud del 99% (DeSantis et al., 2006). Finalmente, los cloroplastos, las mitocondrias y el ADN no bacteriano se eliminaron de la tabla de sOTUs, conservando solo las secuencias del ARNr 16S bacteriano.

La tabla de sOTUs para todo el conjunto de muestras y controles incluyó 31,225 sOTUs. Un gran porcentaje de esos sOTUs mostró una prevalencia muy baja en las muestras, incluidas las muestras control (prevalencia = 1 (13,691); prevalencia = 2 (9,256), prevalencia = 3 (6,852), prevalencia = 4 (5,396)). Para varios tipos de análisis de secuencias, se recomienda trabajar solo con sOTUs sobre una prevalencia mínima (Morton et al. 2017), por lo que se decidió retener solo las presentes en al menos 5 muestras. Además, varios de nuestros controles negativos mostraron concentraciones de ADN detectables, y los lotes de secuenciación presentaron diferentes perfiles taxonómicos, con algunas sOTUs individuales que mostraron altas abundancias a lo largo del lote. Estas evidencias sugieren la existencia de contaminantes en nuestra lista de sOTUs. Hoy en día es ampliamente aceptado que incluso siguiendo protocolos estrictos de muestreo y procesamiento de laboratorio (Eisenhofer et al. 2019, Hornung et al. 2019), es inevitable cierta contaminación en los productos de secuenciación (de Goffau et al. 2018, Eisenhofer et al. 2019). Los contaminantes pueden provenir de diferentes fuentes, incluidos los reactivos, los procedimientos de muestreo o incluso los kits de extracción comerciales (Minich et al. 2018, Eisenhofer et al. 2019, Zinter et al. 2019) y también es posible la contaminación cruzada entre muestras (Wright and Vetsigian 2016, Wang et al. 2017, Edgar 2017, Larsson et al. 2018). Por lo tanto, aunque la eliminación de todos los contaminantes no es posible y la descontaminación puede eliminar algunas características válidas de las muestras (Karstens et al. 2019), se han propuesto varios algoritmos de post-

secuenciación para mejorar la calidad de los datos utilizados para los análisis aguas abajo (*downstream*) (Jervis-Bardy et al. 2015, Davis et al. 2018, Zinter et al. 2019, Caruso et al. 2019, McKnight et al. 2019, Karstens et al. 2019). Para limpiar los posibles contaminantes de nuestra tabla de OTUs, se utilizó *Decontam* (Davis et al. 2018). Primero, se usó el método de frecuencia para descartar sOTUs que mostraban relaciones negativas entre la abundancia y la concentración de ADN de la muestra, con un umbral pFreq = 0.1. En segundo lugar, se utilizó el método de prevalencia para descartar los sOTUs que mostraban una mayor prevalencia en los controles negativos que en las muestras, con el umbral pPrev = 0.5. En ambos casos se usó la opción *per_batch* para que la identificación de contaminantes se realizase en cada lote por separado. Al usar ambos métodos, se identificaron 741 sOTUs como probables contaminantes que se descartaron de las muestras. De esta forma, se retuvo un total de 4.515 sOTUs para los análisis posteriores. Después de examinar el total de lecturas disponibles por muestra, se rarificaron las muestras a 1100 lecturas para estimar la diversidad y comparar la composición de los tipos de muestra.

6) ANÁLISIS ESTADÍSTICOS

Las predicciones asociadas a los distintos objetivos se han testado mediante el uso de los siguientes análisis estadísticos:

- Análisis de Componentes Principales (ACP): Para verificar que la coloración de la cáscara del huevo era más variable entre nidos que dentro de un mismo nido, se calculó la repetibilidad para cada zona del huevo y entre los huevos de una misma puesta. Para resumir la información del color se usó un Análisis de Componentes Principales (ACP) y se calculó la repetibilidad en base a los tres primeros componentes del ACP. Finalmente, se usó el valor medio de los parámetros de color para cada huevo y después para cada nido. Para evitar el abandono, todas las medidas se realizaron cerca de la caja nido y lo más rápido posible.

- Modelos Lineales Generales (GLM): Se utilizaron para explorar el efecto del material viejo del nido en la carga bacteriana y en el éxito reproductivo (Capítulo I). Cuando se analizaban variables de los pollos, se consideró la identidad del nido como factor random.

Para explorar el efecto del color de los huevos (rA) en el aporte del macho (Capítulo II) se realizó un GLM Multivariante incluyendo como predictores el tamaño de puesta, la fecha de puesta y la condición física de la hembra además de rA. Para testar el efecto de estos predictores en cada fase de forma individual (incubación, primer pollo con 3 y 11 días) se realizó un GLM Univariante. Para la representación de los colores de la cáscara de los huevos dentro del tetraedro se usó el paquete colourvision de R versión 3.5.3 (Gawryszewski 2018) (Figure S1. Material suplementario del Capítulo II).

- Modelos de Regresión General (GRM): Se utilizaron para obtener la mejor combinación de variables de color del huevo que se relacionan con la carga bacteriana de la secreción de la hembra (UFC/ml) (Capítulo II). Se incluyó UFC/ml como variable dependiente y las variables de color (θ, φ, rA, qrQ) como predictores. Los valores de las variables esféricas θ y φ, al localizarse dentro del mismo cuadrante (valores de θ entre 4.45 y 90.42; valores de φ entre -86.65 y -66.27), se pudieron usar en escalas lineales. De esta manera, los valores crecientes siempre implicaron cambios en la misma dirección. Además, la variable que explicó la carga bacteriana y el aporte del macho, rA, (ver resultado, capítulo II), tiene una naturaleza lineal.

Para verificar que el color inicial de la puesta causado por la secreción uropigial de la hembra inicial se mantenía después del experimento de intercambio de huevos, se realizó otro GRM. Para ello se utilizó el color de después del intercambio como variable dependiente y el color inicial de esos mismos huevos antes del intercambio y el de los huevos de su hembra adoptiva como predictores. Si los huevos mantuvieron su color después del intercambio, el principal predictor debería ser su color inicial (Capítulo III).

Para testar el efecto del experimento en el aporte del macho (Capítulo III) se hizo un GRM relacionando las diferencias entre el aporte con las diferencias entre el color de los huevos. Las diferencias se calcularon restando los valores de antes del experimento a los valores obtenidos después del intercambio. Además se controló por las siguientes variables: fecha de puesta, número de puesta (si era la primera o segunda puesta o intermedia), condición física de la hembra, tamaño de puesta, y las diferencias entre el tamaño de los huevos de después y antes de su intercambio.

Para testar el mejor modelo en todos estos casos se utilizó el índice Mallow (Mallows 1973), equivalente al Criterio de Información Akaike (AIC) (Boisbunon et al. 2013).

- Análisis de la comunidad bacteriana: se usó QIIME para generar estimaciones de alfa-diversidad de las muestras (riqueza de sOTUs e índice Shannon), y las matrices de distancias de beta-diversidad entre las muestras (distancias ponderadas y no ponderadas), teniendo en cuenta el índice/distancia *UniFrac* (Lozupone and Knight 2005). Para comparar entre tipos de muestras se usó Primer 7.0.13 (PRIMER-e) y Statistica 7.1. Para comparar el índice de Shannon y el número de sOTUs se utilizaron test no paramétricos *Kruskal-Wallis*, ya que la distribución de los valores difería de la distribución gaussiana (prueba de Kosmogorov-Smirnov $p < 0.01$). No obstante, para poder realizar modelos complejos con factores random, se utilizaron GLMs. Para comparar la composición de la comunidad bacteriana entre los tipos de muestras se usó PERMANOVA en Primer 7.0.13, utilizando matrices de distancia ponderada de *UniFrac*, generadas con QIIME a nivel de sOTU. Para visualizar la posición relativa de los dos tipos de muestras en un espacio multidimensional en función de la composición de la comunidad bacteriana se realizó un Análisis de Coordenadas Principales (PCoA).

Abubillas:

Para determinar qué OTUs se vieron afectados por el experimento del material del nido (Capítulo I), se usaron 2 aproximaciones diferentes:

(1) Un análisis discriminante lineal, basado en el efecto del tamaño (LEfSe LDA Effect size, Segata et al. 2011), el cual determina los taxones que podrían explicar con mayor probabilidad las diferencias entre los grupos de muestras. Este método se basa en tres pasos para testar la significanción estadística: suma de rango (Kruskal Wallis entre clases), consistencia (test no pareado de suma de rangos de Wilcoxon entre subclases) y efecto relevancia (análisis discriminante lineal, basado en el efecto del tamaño). Estos análisis se llevaron a cabo en la plataforma *Galaxy* (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=testtoolshed.g2.bx.psu.edu/repos/george-weingart/lefse/LEfSe_run/1.0) utilizando los parámetros por defecto (normalización por muestra de los *counts* brutos a 1M, umbral de puntuación LDA = 2.0 y nivel de significancia 0.05). Se usó como tratamiento "clase", tipo de muestra (hembra o pollo) como "subclase", y muestra como "sujeto". Ambos análisis se llevaron a cabo tanto

(a) restringiendo las comparaciones de Wilcoxon con la misma subclase como (b) haciendo todas las comparaciones posibles dos a dos entre subclases. Estas dos maneras de comparar subclases difirieron solo en un taxón (perdido en el segundo análisis), por lo que se incluyeron sólo los resultados del primer análisis para mostrar todo el conjunto de posibles biomarcadores de grupos, detectados mediante LEfSe.

(2) Test t no paramétricos comparando entre tratamientos la abundancia rarificada de todos los OTUs o de niveles taxonómicos más altos presentes. Para controlar por la consistencia de los resultados, se repitieron los tests con diez rarefacciones y se calculó el valor promedio de los valores de t y p (incluyendo la corrección por repetición de tests mediante *false discovery rate* (FDR).

Para comprobar si la abundancia relativa de distintos OTUs está relacionada, evitando los errores causados por la naturaleza composicional de los datos de abundancia obtenidos de las secuenciaciones de microbiomas (Friedman and Alm 2012), se realizaron análisis de co-ocurrencia de OTUs en la misma muestra, mediante medias de correlaciones *SparCC* (Sparse Correlations for Compositional data). *SparCC* estima la correlación lineal de Pearson entre los componentes transformados a logaritmo (abundancias de OTUs), basándose en la transformación *log-ratio* de las fracciones de OTUs estimadas desde los conteos observados de secuencias mediante una aproximación bayesiana (Friedman and Alm 2012). De esta manera, se preprocesaron los conteos de OTUs sumando 0.1 a todos los conteos para evitar los valores de cero (Friedman and Alm 2012). Finalmente, *SparCC* generó una matriz de correlaciones inferidas y p-valores estimados mediante un proceso *bootstrap* para todos los pares de OTUs.

Por último, para seleccionar la combinación de OTUs que mejor explicaba el éxito de eclosión de las puestas, se realizó un análisis de regresión por pasos (BEST Stepwise) sobre la comunidad bacteriana de las secreciones uropigiales de la hembra (100 reinicios). Este tipo de análisis selecciona las variables ambientales o las especies que mejor explican el patrón de la comunidad, maximizando una correlación de rango entre sus respectivas matrices de semejanza. Para ello, se utilizó una matriz de distancias Euclídeas en el éxito de eclosión entre las hembras (tanto incluyendo como sin incluir los huevos no eclosionados sin embrión) como matriz de semejanza para los análisis. Los OTUs incluidos en el mejor modelo fueron testados mediante correlaciones bivariadas de Spearman con el éxito de eclosión. Además de las cajas nido experimentales (con y sin material viejo de abubilla), se analizaron todas las cajas nido que fueron usadas durante

esa temporada de cría. Se excluyeron tres puestas que no tenían información sobre la presencia de embriones en los huevos no eclosionados.

Finalmente se presentaron los análisis principales que se llevaron a cabo de forma independiente con las 10 tablas de los OTU rarificados, y los valores de las medias (Error Estándar) de los parámetros. Sin embargo, los análisis *post-hoc* se llevaron a cabo sólo con la primera rarefacción, y para los análisis del subconjunto de OTUs que mejor explicaban el éxito de eclosión, se usó la media de la abundancia de OTUs a través de las diez rarefacciones.

Calaos:

Para determinar qué sOTUs particulares o niveles taxonómicos más altos diferían entre los grupos de muestras de calaos (Capítulo IV), se usó el análisis de *Gneiss* (Morton et al. 2017). Este método analiza las asociaciones (por medio de la regresión ordinal de mínimos cuadrados (ols) o la regresión lineal de efectos mixtos (lme)) entre variables que identifican factores ambientales, y los valores de *balances* entre grupos de sOTUs determinados por un gradiente conocido, o por su co-ocurrencia. Al usar *balances*, el análisis evita errores relacionados con la interdependencia de las abundancias relativas de diferentes sOTUs, causadas por la naturaleza composicional de los datos de secuenciación (Morton et al. 2017). Todos los análisis se realizaron utilizando el método de regresión OLS para un factor multinivel cada vez. Los análisis se consideraron válidos cuando la *mse* predicha era más pequeña que la *mse* del modelo. Para cada análisis de Gneiss, se exploró el *heatmap* que muestra las abundancias de los sOTUs por muestra, así como los valores de p corregidos por FDR para todos los *balances* para los diferentes niveles de factor testado. Debido a que nuestro principal interés era determinar si las glándulas de calaos están asociadas a comunidades bacterianas especiales, se prestó atención no solo a los coeficientes significativos en los primeros nueve *balances*, sino también a los significativos para las glándulas después de y_9. Para los *balances* que incluyen coeficientes significativos para los niveles de interés de los factores, se examinó la dirección de las diferencias y la identificación de los sOTUs en el denominador, infiriendo así los sOTUs que son comparativamente más abundantes en los *balances*.

- Tablas de contingencia y la prueba Chi cuadrado: se utilizaron para testar la preferencia por las cajas nido en función de su presencia y el tipo de material (Capítulo I). Para estos análisis se tuvo en cuenta sólo la primera elección del par de cajas nido experimentales.

- Análisis no paramétricos: cuando las variables no seguían una distribución normal, ni siquiera después de transformarlas a escala logarítmica, se usó el test de Mann Whitney (Capítulo I).

- Análisis filogenéticos: Para realizar el estudio comparativo en las especies muestreadas de calaos se realizaron análisis filogenéticos (Capítulo V). El mejor subconjunto de filogenia para construcciones de árboles se obtuvo a través de la herramienta *Bird Tree* (http://www.birdtree.org) (Jetz et al. 2012, Rubolini et al. 2015). Se descargaron 10.000 árboles con las 13 especies de cálaos muestreados en los Zoológicos. A continuación, se creó un árbol de consenso a través del programa MESQUITE versión 3.6 (Maddison and Maddison 2018).

- Modelos Lineales Generales Filogenéticos (PGLM): Las variables de color y morfológicas asignadas a las diferentes especies, o las estimaciones de carga bacteriana de las diferentes partes del cuerpo no pueden considerarse datos estadísticamente independientes ya que las especies que están estrechamente relacionadas filogenéticamente debido a una ascendencia común, también tienen rasgos de historia de vida más similares (Harvey y Pagel 1991). Por tanto, para controlar los posibles efectos de la ascendencia común, se realizaron PGLMs (Pagel 1997, 1999) (Capítulo V). PGLM es un modelo de regresión lineal que incorpora información filogenética en una matriz de error, controlando los rasgos de historia evolutiva compartidos entre las especies (Harvey y Pagel 1991, Martins y Hansen 1997).

Las diferencias en el esfuerzo de muestreo pueden ser fuentes de sesgo ya que las diferentes estimaciones no se estiman con una precisión similar (Garamszegi y Møller 2010, 2011). Por tanto, se analizaron los modelos PGLM pesados y no pesados por el tamaño de muestra en los casos en los que la variable dependiente tenía un tamaño de muestra diferente en función de la especie.

Todos los análisis estadísticos se realizaron en la versión R 3.5.3 (R Core Team 2019) con paquetes de complementos "ape" y la función pglm (Paradis et al. 2004). Las

figuras se realizaron con el software Statistica 7 (Statsoft 2006) sin tener en cuenta las distancias filogenéticas ya que los pglm no permiten la presentación gráfica de los modelos.

CAPÍTULOS

I. Experimental old nest material predicts hoopoe *Upupa epops* eggshell and uropygial gland microbiota

Silvia Díaz-Lora, Manuel Martín-Vivaldi, Natalia Juárez García-Pelayo, Manuel Azcárate García, Sonia M. Rodríguez-Ruano, Manuel Martínez-Bueno & Juan José Soler. *Journal of Avian Biology, 50(9)* (2019).
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II. Hoopoe (*Upupa epops*) male feeding effort is related to female cosmetic egg coloration

Silvia Díaz Lora, Tomás Pérez-Contreras, Manuel Azcárate-García, Manuel Martínez Bueno, Juan José Soler & Manuel Martín-Vivaldi
Journal of Avian Biology (submitted)

III. Cosmetic coloration of cross-fostered eggs affects male parental investment in the Hoopoe (*Upupa epops*)

Silvia Díaz-Lora, Tomás Pérez-Contreras, Manuel Azcárate-García, Juan Manuel Peralta-Sánchez, Manuel Martínez-Bueno, Juan José Soler & Manuel Martín-Vivaldi

IV. Symbioses with bacteria in hornbill (Bucerotiformes) uropygial glands

Manuel Martín-Vivaldi, Silvia Díaz-Lora, Magdalena Ruiz-Rodríguez, Manuel Azcárate- García, Miguel Rabelo-Ruiz, Juan José Soler, Manuel Martínez-Bueno, and Anders Pape Møller

V. Colouration and bacterial load of uropygial secretion and hornbills' teguments

Silvia Díaz-Lora, Magdalena Ruiz-Rodriguez, Juan Manuel Peralta, Manuel Azcárate-García, Juan J Soler, Manuel Martínez-Bueno, Manuel Martín-Vivaldi, and Anders Pape Møller[5]

CAPÍTULO I

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Experimental old nest material predicts hoopoe *Upupa epops* eggshell and uropygial gland microbiota

Silvia Díaz-Lora, Manuel Martín-Vivaldi, Natalia Juárez García-Pelayo, Manuel Azcárate García, Sonia M. Rodríguez-Ruano, Manuel Martínez-Bueno & Juan José Soler. *Journal of Avian Biology, 50(9)* (2019). https://doi.org/10.1111/jav.02083

ABSTRACT

Nest re-use in birds has the potential cost of infection by parasites and pathogens but may also be a source of beneficial symbiotic bacteria transmitted horizontally. Eurasian hoopoes (*Upupa epops*) host antibiotic-producing bacteria in their uropygial gland but only while breeding, which suggests that the nest-hole may be a source of those symbionts. Interestingly, hoopoes do not build nests, thus might prefer for reproduction nest holes with soft materials from previous reproductions. Here, we tested experimentally this preference by installing in the field new nest boxes that were left empty or filled with either sawdust or a mixture of sawdust and hoopoe´s nest material from the previous year. We explored the experimental effect on the composition of the uropygial secretion bacterial community, on eggshell bacterial loads, and on several proxies of reproductive success. Hoopoes bred significantly more often in nest boxes with nest material than in empty ones, but the type of nest material did not affect nest box occupancy. Eggs in nest boxes with old-soft material harbored higher bacterial density on their shells, and the microbiota of the uropygial secretion of nestlings and females in these nest boxes differed from those in nest boxes without old-soft material. Moreover, although the experiment did

not affect breeding success or related proxies, several Operational Taxonomic Units from female uropygial secretions were positively associated with hatching success. This is the first experimental evidence showing that re-used nest material affects the bacterial community of the uropygial secretions of hoopoe females. This suggests that the nest material can be a source of strains for their incorporation to both the uropygial gland and eggshell communities, highlighting a possible advantage of nest re-use previously unconsidered.

Keywords: Symbiotic bacteria, nest material re-use, horizontal transmission, uropygial gland secretion, eggshell, *Upupa epops*.

INTRODUCTION

Territory choice affects individual fitness, so individuals should be very selective when choosing the place to breed to maximize reproductive success (Refsnider and Janzen 2010). This choice might for instance affect probability of parasitism or availability of resources (Sergio and Newton 2003). In addition to territory choice, the choice of the nesting site and, specifically, the choice of nest material is also very important and might even affect acquisition of symbiotic bacteria when they are transmitted horizontally (Peralta-Sanchez et al. 2010, Martínez-García et al. 2016a, van Veelen et al. 2017). In hole-nesting species that do not add any material to the nest, the presence and quality of soft material inside the cavity could be an important nest-site choice criterion, because it could for instance improve the incubation efficiency by increasing thermal insulation and reducing egg heat loss (Mazgajski 2007, Mainwaring et al. 2014, Podofillini et al. 2018). Moreover, nest material could act as a physical barrier against bacterial contamination

from the bottom of the nest or, due to their antimicrobial properties, prevent offspring infection (Gwinner and Berger 2005, D'Alba and Shawkey 2015, Ruiz-Castellano et al. 2016, Soler et al. 2017). Secondary hole nesters usually re-use nests from previous breeding seasons (Mazgajski 2007). These nests can contain nest dwelling ectoparasites and pathogens that remain quiescent within old nest material residues (Maier et al. 2000), and that will affect reproductive success of future users (Mazgajski, 2007; Møller et al., 2009). Moreover, it can contain remains of previous reproduction such as faeces, discarded food and even dead nestlings that enhance bacterial growth but that might also inform visitors of whether nestlings survived during their nest stage in previous reproductive events (Erckmann et al. 1990, Olsson and Allander 1995, Sumasgutner et al. 2014). This information is especially important in migratory birds, because they have less time to evaluate the quality of the territory before the start of the breeding season (Mazgajski 2007). Many studies have investigated the preference for nest boxes with old nest material in birds (reviewed in Mazgajski 2007). However, they have only focused on the presence or absence of nest material *per se*, independently of whether it comes from previous reproductions or from artificial sources.

Parental and breeding activity, as well as nest material and ectoparasites, determine bacterial environment of nesting cavities and of eggshells (González-Braojos et al. 2012; Peralta-Sánchez et al. 2012; Peralta-Sánchez et al. 2014; Grizard et al. 2015, Tomás et al. 2018). These bacteria can have positive, negative, or no effects on the offspring (Singleton and Harper 1996, Moreno et al. 2003, González-Braojos et al. 2012, Soler et al. 2017, Devaynes et al. 2018). For example, some symbiotic bacteria can produce chemicals, such as antimicrobial compounds, that might outcompete or inhibit the colonization by pathogenic microorganisms or parasites, defending and protecting the host against them (Soler et al. 2010, Martín-Vivaldi et al. 2014a). Bacteria on the eggshells and offspring are

partly from the nest materials (Brandl et al. 2014, Martínez-García et al. 2016a, van Veelen et al. 2018) and, thus, old nest materials might be the source of potentially beneficial bacteria for breeding birds. Exploring the bacterial load of eggshells in nests with old or artificial new material would therefore help to clarify the effect of nest material on nest bacterial environments.

Here we explore the importance of old nest materials in nest box choice for breeding in a population of Eurasian hoopoes (*Upupa epops*). This species is a secondary hole-nester where a mutualistic relationship with bacteria growing in their uropygial gland has been described particularly in breeding females and nestlings (Martín-Platero et al. 2006, Soler et al. 2008a, Martín-Vivaldi et al. 2010, Ruiz-Rodríguez et al. 2013). These mutualistic bacteria reach the egg surface when females smear their uropygial secretion on them soon after laying, increasing hatching success (Martín-Vivaldi et al. 2014a, Soler et al. 2014). Even though uropygial gland bacteria have been also described in dark-eyed juncos (*Junco hyemalis)* and red-billed woodhoopoe (*Phoeniculus purpureus*) (Law-Brown and Meyers 2003, Whittaker and Theis 2016), the behaviour of smearing the eggs has only been demonstrated in hoopoes (Soler et al. 2014). Several sources and mechanisms have been proposed to explain the complex bacterial community of hoopoe uropygial secretion (Rodríguez-Ruano et al. 2018). These bacteria can be transmitted vertically from mother to offspring but others are obtained possibly, from their own gut microbiota (cloaca) and from the nest environment (e.g., nest materials) (Ruiz-Rodríguez et al. 2014, Rodríguez-Ruano et al. 2015, Martínez-García et al. 2016a, Martín-Vivaldi et al. 2018). Hoopoes do not carry nest materials to the nest (Martín-Vivaldi et al. 2014b). Therefore, by re-using nests, the new female could increase the diversity of strains of her uropygial gland symbiotic bacterial community. In addition to other advantages, this could be one of the explanations of why hoopoes frequently re-use the same holes for many

years (Cramp 1998), and why the first individuals arriving to the breeding grounds preferentially occupy previously used nest boxes (van Wijk et al. 2017). A previous study demonstrated an effect of nest bacterial environment on hoopoe eggshells bacterial communities, but no effect on the bacterial community of the uropygial secretion was found (Martínez-García et al. 2016a). However, that experiment was carried out with captive hoopoes, whose microbiome is slightly poorer than that of wild ones (Martínez-García et al. 2015, Rodríguez-Ruano et al. 2015, 2018) and bacterial community was characterized by means of ARISA fingerprinting technique, that is not able to detect the whole diversity of bacteria (Bentley et al. 2008). Moreover, the experimental nest material used (commercial crushed and mashed olive (*Olea europaea*) stones) had antimicrobial properties, which could have affected the composition of microbiomes (Martínez-García et al. 2016a).

Within the theoretical background exposed above, and considering previous knowledge on the symbiotic association between hoopoes and bacteria of their uropygial secretion, we experimentally explored nest box choice by wild hoopoes and associated effects on the bacterial load of eggshell and the bacterial community of the secretion using high throughput sequencing. We tested whether hoopoes prefer to breed in (1) nest boxes with available soft material instead of in empty ones, (2) mainly those used in previous reproduction (i.e., with old hoopoes´ nest material inside). We also (3) explored the effect of experimental old material on the bacterial community of the uropygial gland secretion and on the bacterial load of the eggshells of hoopoes. Finally, (4) we explored possible fitness effects of the experimental nest materials in terms of breeding success. Our prediction is that hoopoes should prefer nest boxes with material instead of empty ones and specifically those with old hoopoe nest material. In this way, we expect that the bacterial composition of uropygial secretion and of eggshell will differ depending on the

experimental treatment because of the incorporation of the symbionts from old nest material to the communities obtained from other sources (Ruiz-Rodríguez et al. 2014, Martínez-García et al. 2016a, Martín-Vivaldi et al. 2018, Rodríguez-Ruano et al. 2018). Finally, because of a greater diversity of the composition of the bacterial community in the gland, we expect that females that breed in nests with old material have higher breeding success than those breeding in nests without it.

MATERIAL AND METHODS

Ethics statement

The study was conducted according to relevant Spanish national (Decreto 105/2011, 19 de abril) and regional guidelines. The protocol was approved by the ethics committee of the University of Granada (Comité de Ética en Experimentación Animal, CEEA, Ref.: 785), and all necessary permits for hoopoe´s manipulations were provided by Consejería de Medio Ambiente de la Junta de Andalucía, Spain (Ref: SGYB/FOA/AFR/CFS and SGMN/GyB/JMIF). Our study area is not protected, but privately owned, and the owners allowed us to work in their properties. The time spent in each hoopoe nest was the minimum necessary for the experiment.

Study species

The hoopoe is a cavity nester that readily breeds in nest boxes. This species is a migratory bird distributed throughout Europe, Africa and Asia. There can be partial migrant populations in areas with low seasonal differences, like in our study population, where sedentary and migratory specimens reproduce (Reichlin et al. 2013, van Wijk et al. 2018). Hoopoes do not carry nest materials, but if soft materials are present in the cavity selected, females excavate a slight depression where they lay the eggs (Martín-Vivaldi et al.

2014b). These soft materials are usually remains of previous reproduction, soil and/or decomposed wood. Females lay one or two clutches of 6 to 8 eggs between February and July (Martín-Vivaldi et al., 1999; Plard et al., 2018). Incubation starts with the first eggs, resulting in asynchronous hatching, generating a marked size hierarchy within the brood (Cramp 1998). Females stay inside the nest from the start of incubation (which lasts 17 days from the laying of the first or second egg), until nestlings are about 8 days old. Nestlings leave the nest after 24-30 days (Martín-Vivaldi et al., 2014a).

Study area and general procedures

The fieldwork was carried out during the 2015 breeding season in the Hoya de Guadix (37 ºC 18´N, 11´W), Granada (southern Spain). In this area, hoopoes breed in nest boxes situated in trees and in natural cavities. Nest boxes were made in cork with the following dimensions: 35 × 18 × 21 cm (internal height × width × depth), 24 cm (bottom-to-hole height) and 5.5 cm (entrance diameter).

Nest boxes were visited every five days from early March to the end of July. Hoopoes lay one egg per day (Cramp 1998) and, thus, this frequency of nest visiting allowed us to estimate laying and hatching dates, clutch size and number of fledglings that left the nest. Laying date was defined as the day when female laid the first egg. Females were captured by hand inside the nest boxes twice, 15 days after laying the first egg and again when the first nestling was five days old. Nestlings were sampled 19-20 days after hatching of the first egg. We measured the bill, the tarsus and the uropygial gland size using a calliper (accuracy 1mm), the wing using a metallic ruler, and the body mass with a hanging scale (Pesola 0-100 g, accuracy 1 g). We also extracted uropygial gland secretion by automatic 1-10 µl micropipettes (see below for more details) and collected blood samples in heparinized capillary tubes by puncturing the brachial vein. Individuals were marked with numbered aluminum rings (Spanish Institute for Nature Conservation,

ICONA). Afterwards, they were released into their nest box. All the manipulation was made wearing disposable latex gloves previously cleaned with 96% ethanol to prevent contamination among nests.

As proxies of breeding success, we used the following variables: clutch size, hatching success (proportion of eggs hatched), number of fledglings (number of nestlings that survived 20 days in the nest), fledging success (proportion of hatchlings that survived until fledging in successful nests), and clutch productivity (proportion of eggs that produced a fledgling in successful nests). Some nests were predated or deserted before hatching or during the nestling stage and, thus, sample sizes differ depending on the considered variable (see degrees of freedom in Table 1).

Body condition was estimated as the residuals of body mass on tarsus length^3 (Senar and Pascual 1997).

Experimental design

The experiment was performed by installing new nest boxes in the study area at the beginning of spring of 2015. This hoopoe population breeding in nest boxes has been studied for many years and we knew the fate of the nesting attempts of the previous breeding seasons. Old nest boxes in the study area were cleaned of nest material at the beginning of March of 2015 and, in the case of those where hoopoes successfully bred in 2014 (14 nest boxes), materials were kept in plastic bags shortly before added to the experimental nest boxes (one or two weeks later). During this short period of time, they were kept at room temperature and with a small opening in the bag to allow air exchange. Experimental new nest boxes were geographically placed randomly in pairs with a distance from each other of about 25 m, more or less spatially mixed with old nest boxes installed in previous years (Fig. S1 and S2 in supplementary material). To investigate the possible effects of old material, one of the nest box in the pair was filled with a 3 cm layer

of only commercial sawdust (Allspan® Animal bedding, wood shavings; control nests), and the other with the same volume of 50% mixture of sawdust and hoopoe´s nest material from the previous year (old nest material). This procedure was performed wearing different new latex gloves for each nest box to avoid contamination of the clean sawdust of controls. A total of 49 pairs of new experimental nest boxes were scattered within the area with old nest boxes, or installed in new surfaces of pine plantations at the edge of this area. To test if there is a preference between new nest boxes with material versus new empty nest boxes, we also installed new empty nest boxes. The new empty nest boxes were placed on the same tree, paired with an old empty (cleaned) nest box (a total of 88 new empty nest boxes) (Fig. S1 in supplementary material).

Microbiological study

Eggshell bacterial loads

To estimate the abundance of cultivable bacteria on the eggshells, the 15th day after incubation started, we cleaned the complete surface of one randomly chosen egg per clutch with a sterile swab (EUROTUBO® DeltaLab) previously moistened with sterile sodium phosphate buffer (PBS, 0.2M; pH = 7.2). Afterwards, the swab was introduced into a sterile microcentrifuge tube with phosphate buffer and transported in a portable refrigerator at 4-6 ºC to the laboratory. Samples were stored at 4 ºC until being processed within the next three days. The microcentrifuge tubes were gently vortexed to facilitate the transmission of bacteria from swabs to the phosphate buffer, as well as its homogenization. Serial tenfold dilutions to 10^{-6} were cultured. Briefly, 100 µl of sample of each serial dilution was spread onto four different solid culture media (Scharlau Chemie S.A., Barcelona): Tryptone Soya Agar (TSA), a broadly used general medium to grow heterotrophic bacteria; Kenner Fecal Agar (KF), a selective medium for *Enterococcus*, and two specific media for potentially pathogenic bacteria: *Enterobacteriaceae* (Hektoen

Enteric Agar, HK) and *Staphylococcus* (Vogel Johnson Agar, VJ). In these two last specific media we hardly found growth (only in 1 of 19 nests in both cases) so we will show the results of the first two media (TSA and KF). The plates were incubated aerobically at 37 ºC for 24-72 hours before colony counting. Estimates of bacterial loads were standardized to number of colony forming units (CFU) per milliliter (Nº colonies * $10^{\text{dilution factor}}$) / ml spread).

Uropygial secretion bacterial loads

The available uropygial gland secretion was collected using automatic 1-10 µl micropipettes. First, with gloves previously disinfected with ethanol, we cleaned the surroundings of the gland with a cotton swab soaked in 96% ethanol. Second, we gently introduced a previously autoclaved tip into the opening of the papilla of the uropygial gland and directly pipetted the secretion. Finally, we introduced the secretion into a sterile microcentrifuge tube. This procedure was repeated until the papilla got empty. The samples were kept cold in a portable fridge after collection until storage in a fridge in the lab. The samples were processed within the following 24h to estimate bacterial loads and afterwards stored in a freezer until DNA extraction. Briefly, 5 µl of the secretion were homogenized with 45 µl of PBS in a sterile microcentrifuge tube and 5 µl of the mixture of each serial dilution (tenfold dilutions to 10^{-4}) was spread onto TSA and KF media. The plates were incubated aerobically at 37 ºC and colonies were counted after 24 hours (TSA plates) and 72 hours (KF plates). Estimates of bacterial loads were standardized to CFU/ml as explained before.

Nest material effect on hoopoe bacterial community

For the study of the composition of the bacterial community of uropygial secretions, we used Illumina High-throughput sequencing (HTS). The total DNA was extracted from 80 samples (20 µL) using the FavorPrep Genomic DNA extraction kit (Favorgen Biotech,

Ping-Tung, Taiwan) according to manufacturer's instructions, adding a lysozyme treatment (10 mg/ml, at 37 °C for 30 min).

The libraries for sequencing were obtained amplifying a fragment of approximately 400 bp of the 16S ribosomal DNA (rDNA) V6-V8 hypervariable regions. The universal primers for that region, B969F and BA1406R, were modified to include the standard Illumina Nextera adapters and 8 bp barcodes (S500+N700 series) in the forward and reverse primers to allow for dual-indexing, as follows: B969F (5´ AATGATACGGCGACCACCGAGATCTACAC-NNNNNNNN-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**ACGCGHNRAACCTTACC**-3´) and BA1406R (5´-CAAGCAGAAGACGGCATACGAGAT-NNNNNNNN-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**ACGGGCRGTGWGTRCAA**-3´), where the Nextera adaptors (L and R arm) are in normal font (to either side of the barcodes), the barcodes are represented by NNNNNNNN, and the specific primer regions are in bold and underlined. Then the libraries were sequenced in a single run of Illumina MiSeq (2 x 300 bp output mode) sequencer. All this processing was carried out at the Integrated Microbiome Resource, Centre for Comparative Genomics and Evolutionary Bioinformatics (CGEB), University of Dalhousie (Canada).

Original sequences are available in NCBI RSA (accession: http:xxxxxxx). Sequence processing to get an Operational Taxonomic Units (OTU) table was performed using QIIME software v1.9.1 (Quantitative Insights In Microbial Ecology; (Caporaso et al. 2010)) and following recommendations on genomic data processing (Navas-Molina et al. 2013, Dumbrell et al. 2017, Knight et al. 2018). Briefly, sequences of paired-read amplicon libraries were paired-end aligned using fastq-join method (Aronesty 2011), with a minimum overlap of 100 base-pairs and maximum 10% difference in the overlapping region. Then, demultiplexing and quality filtering (at Phred >= Q20) was performed, and

sequences trimmed to 400 base-pairs with Usearch. The subsampled open-reference OTU picking procedure (Rideout et al. 2014) was applied to generate an OTU table, clustering sequences against Greengenes database version 13_8 at 97% similarity (DeSantis et al. 2006, McDonald et al. 2012), with a minimum OTU size of 10 sequences, the reverse-strand-match option enabled, and suppressing step 4 (a second round of de-novo picking after the first one). Subsequently, the OTU table was filtered to remove Archaea, chloroplast, mitochondria, non-phylum assigned OTUs, singletons and OTUs with frequency lower than 0.005% of the total sequence count (Bokulich et al. 2013). After filtering we obtained a total of 1696144 valid sequences (number of sequences per sample, n samples = 80, Mean(Min, Max) = 21201.8(1116, 38458)). In order to control for the sequencing effort, we performed a multiple rarefaction (10 random repetitions) at 1500 sequences (this caused discarding one sample from a non-experimental female), and performed analyses with each of the rarefied files to obtain mean(SE) estimates of the parameters of the statistical models applied. The normalized number of sequences per OTU after rarefaction was used as an estimate of their rarefied abundance (out of 1500 sequences) for the analyses and graphs.

One of the most discussed issues on the processing of HTS data, is the method for clustering and assigning sequences to OTUs, so that any approach may be questioned and therefore, should be justified. The open-reference picking method has been shown to produce more stable OTU assignation that the alternatives de-novo or closed reference (He et al. 2015) and the labels of reference-based methods are comparable among studies, while this is not possible with the de-novo approach (Callahan et al. 2017). Moreover, although reference-based methods are subject to more errors when assigning taxonomies to OTUs than de-novo approaches (Westcott and Schloss 2015), recent clustering methods improve the accuracy of OTU assignations and preserve estimated diversity also for

reference based approaches, and several of them are implemented in QIIME (Kopylova et al. 2016). Any bioinformatic tool used in microbiome analyses has its own advantages and disadvantages and, surprisingly, all have low accuracy in the assignation of taxonomies, with small changes in the parameters used profoundly affecting results (Golob et al. 2017). Such tools are being improved continuously and the best available approaches change from year to year (Callahan et al. 2017, Knight et al. 2018), being difficult to work updated. In this situation, researchers should evaluate their chosen pipeline and settings to confirm if it can adequately answer the research question, as well as provide the details of their methods (Golob et al. 2017). In this sense we believe that the open-reference OTU based approach used here with the pertinent quality and abundance filtering applied, that reduces the possibility of false positives (Bokulich et al. 2013, Golob et al. 2017), is adequate for the comparison of the microbiomes of individuals of the same bird species at the OTU level for bacteria.

Antagonistic activity of uropygial secretion

The antagonistic activity of uropygial secretion was estimated against *Bacillus licheniformis* D13, a very common feather degrading bacteria. To test the antimicrobial activity, 5 µl of secretion was deposited on the surface of pre-inoculated BHA plates (prepared the same morning), directly after extraction from the bird, in the field. We deposited also 2 µl of amoxyciline (714 µg/ml) diluted in sterile sodium phosphate buffer as positive control. Plates were incubated at 28 °C for 12 hours. The antimicrobial activity was detected by the appearance of clear growth-inhibition halos around the drop of uropygial secretion. Then, the external diameter of the halo and that of the drop of secretion were measured. The diameter of the secretion was subtracted from the diameter of the halo to obtain the width of the inhibition zone.

Statistical procedures

Nest box preference

For these analyses, we used only the first time that a nest box of each experimental pair was occupied (i.e. first female choice).

Hoopoe preference for breeding in nest boxes under different experimental treatments (with vs without soft nest material, or with vs without old nest material) were analysed by means of contingency tables and two-tailed Chi-square tests.

Effect on breeding parameters

For these analyses we used only the first clutch of each female.

The variables that approximately followed a normal distribution were: antagonistic activity (nestlings) and all the variables of breeding success except hatching success. The variables that followed a normal distribution after log10 transformation + 0.1 were: CFU/ml of eggshells in TSA medium, antagonistic activity (in females) and CFU/ml in the uropygial secretion in TSA. The variables that were not normally distributed, even after logarithmic transformation, were: CFU/ml of eggshells and of secretion in KF medium and hatching success (Table 1).

To explore the effect of hoopoe old nests material on bacterial load and breeding success, we used ANOVA with experimental treatment (type of nest material) as fixed factor and laying date as covariable (when it was not statistically significant, it was eliminated from the model). When considering information from individual nestlings, we considered nest identity as a random factor. Whenever laying date explained a significant proportion of variance, the statistical model does not allow to include nest identity as random factor (i.e., laying date does not vary within nest identity). In these cases we calculated F-values from models, but df were adjusted to the number of nests considered in the analyses to estimate p-values. When variables were not normally distributed, even after transforming them, we used non-parametric analysis (Mann Whitney tests).

All these statistical tests were performed with STATISTICA 7 (StatSoft).

Bacterial community composition

QIIME was used to generate alpha-diversity estimates for samples (Shannon index), and beta-diversity matrices of distances among samples (weighted and unweighted UniFrac distances (Lozupone and Knight 2005)). For this approach, the phylogenetic tree built in the subsampled open-reference OTU picking procedure (Rideout et al. 2014) by means of FastTree (Price et al. 2009) was used to estimate branch lengths (see the phylogeny obtained in Fig. 1). Comparisons among types of samples were performed with Primer 7.0.13 (PRIMER-e) and Statistica 7.1. Comparison of Shannon index was performed by means of ANOVA, since the distribution of values did not differ from Gaussian distribution (Kosmogorov Smirnov test $p > 0.2$) and there were homogeneity of variances among groups (Levene test, Shannon index $F(1,34) = 1.47$, $p = 0.236$). The composition of bacterial community were compared among types of samples by means of PERMANOVA in Primer 7.0.13 using weighed UniFrac distance matrices generated with QIIME for the OTU level. Principal Coordinates analyses (PCoA) were used to visualize the relative position of the two types of samples in the multidimensional space of bacterial community composition.

In order to determine which particular OTUs or higher taxonomic level were affected by the experiment, we used two different approaches: (1) A Linear Discriminant Effect Size analysis (LEfSe, Segata et al. 2011) which determines the taxa most likely to explain differences between groups of samples (possible "biomarkers"). This method relies on three steps testing statistical significance (Kruskal Wallis sum-rank test among classes), consistence (unpaired Wilcoxon rank-sum test among subclasses) and effect relevance (Linear Discriminant effect size). The LEfSe analyses was performed in the Galaxy platform

(http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=testtoolshed.g2.bx.psu.edu/repos/george-weingart/lefse/LEfSe_run/1.0) with the default parameters (per sample normalization of raw counts to 1M, threshold of LDA score = 2.0 and significant level 0.05). We used treatment as "class", type of sample (female or nestling) as "subclass", and sample as "subject". The analysis was performed both (a) restraining Wilcoxon comparisons to the same subclass and (b) letting all-two-all comparisons. The two ways of comparing subclasses only differed in one taxon (lost in the second approach), so we have included results only of the first one to show the whole set of possible biomarkers of groups detected by LEfSe. (2) Non-parametric t-tests comparing between treatments the rarefied abundance (counts rarified to 1500 sequences per sample, see above), of all OTUs or higher order levels present. In order to control for the consistence of the results, the tests were repeated with ten rarefactions and t and p values were averaged. Additionally, we included a false discovery rate correction for multiple tests.

Analyses of the co-occurrence of OTUs in the same sample were performed by means of SparCC correlations (Sparse Correlations for Compositional data) to avoid compositional bias (Friedman and Alm 2012). SparCC estimates the linear Pearson correlations between the log-transformed components (OTUs abundances), based on the log-ratio transformation of the fractions of OTUs estimated from observed counts of sequences by a bayesian approach (Friedman and Alm 2012). Therefore, for the analysis we preprocessed OTU counts by summing 0.1 to all counts to avoid zero values (Friedman and Alm 2012). SparCC generates a matrix of inferred correlations and p-values estimated by a bootstrap procedure for all pairs of OTUs.

Finally, a Stepwise BEST analysis on bacterial community of female uropygial secretions (100 restarts) was used to select the best combination of OTUs explaining hatching success of clutches. The BEST analysis selects environmental variables, or

species "best explaining" community pattern, by maximizing a rank correlation between their respective resemblance matrices. For this, a matrix of Euclidean distances in hatching success among females (both including and not-including un-hatched non-embryonated eggs) was used as the resemblance matrix for the analysis. The OTUs included in the best model were tested for bivariate Spearman correlations with hatching success. In addition to the experimental nest boxes (with and without old hoopoe nest material), we analyzed all the nest boxes that were used during that breeding season (all nests). For these analyses we excluded three clutches without information on the presence of embryos for un-hatched eggs.

The main analyses were conducted independently with the 10 rarefied OTU tables, and mean(SE) values of parameters are presented. However, the post-hoc analyses were performed only with the first rarefaction, and for the analysis of best subset of OTUs explaining hatching success, the mean abundance of OTUs across the ten rarefactions was used.

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.8574ft0 > (Díaz-Lora et al. 2019).

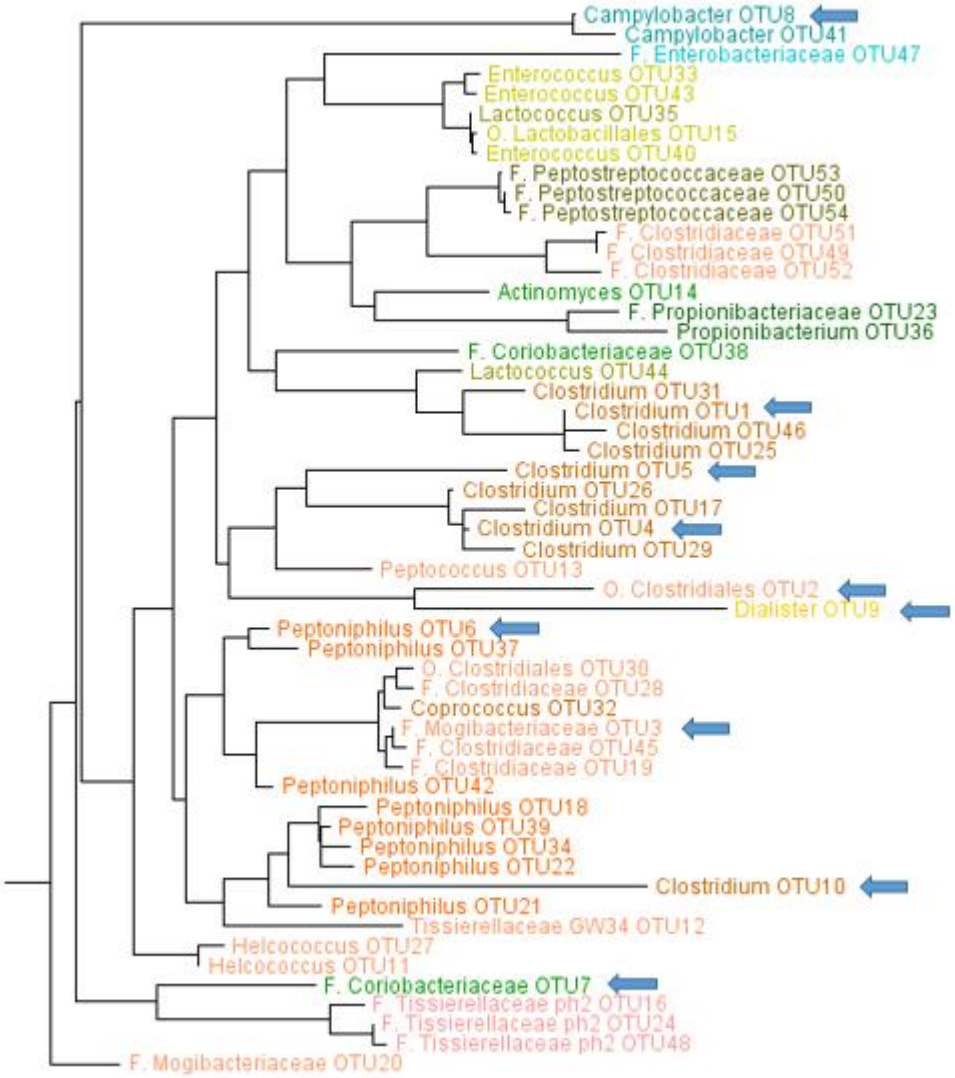


Fig 1. Phylogenetic tree reflecting the similarities of the 16S sequences for the 54 OTUs identified by Illumina HTS in the uropygial secretions of female and nestling hoopoes. Names in tips reflect the taxonomic level of identification reached by comparison with Greengenes database, and OTUs are numbered in decreasing abundance as for Fig 4. Blue arrows mark the ten OTUs with the highest abundances. Taxa in blue characters are Proteobacteria, those in dark green are Actinobacteria, and the remaining are *Firmicutes*.

RESULTS

Nest box preference

Hoopoes significantly preferred to breed in nest boxes with material over empty nest boxes (Chi-square test: $\chi^2 = 16.49$, df = 1, $p < 0.001$, Fig.2). However, there was no preference for the nest boxes with hoopoe old nest material experimentally added over those only with sawdust (Goodness of fit Chi-square: $\chi^2 = 0.182$; df = 1; p = 0.670; occupied nests with sawdust = 12; occupied nests with sawdust and hoopoe old nest material = 10; total nest boxes = 98 (49 pairs of nest boxes)).

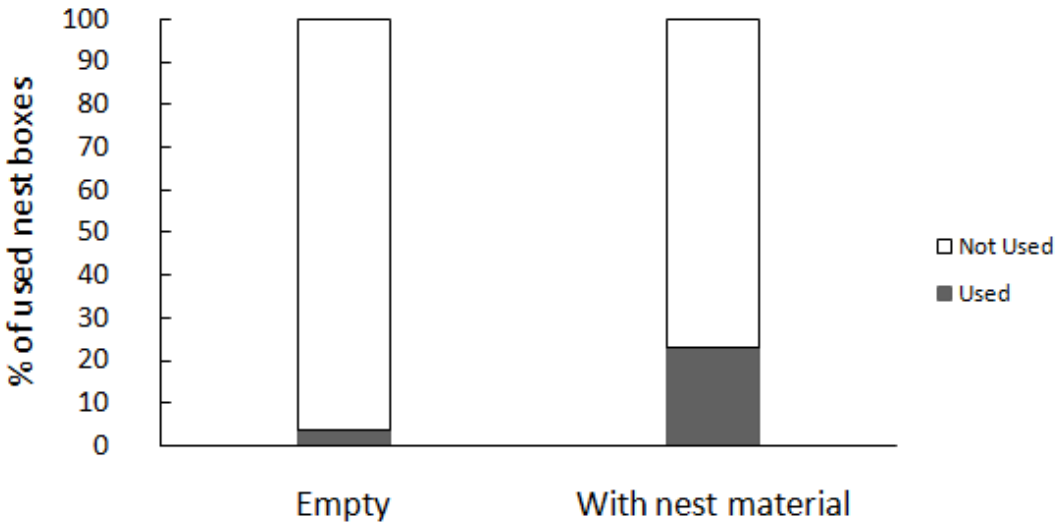


Fig 2. Differences in the frequency of use by hoopoes of new nest boxes depending on the presence (n = 100, 23% occupation) or absence (n = 105, 3.81% occupation) of experimental nest material.

Microbial load and uropygial secretion´s properties

Eggshell

Eggshells in nest boxes with hoopoe old nest material harboured higher mesophilic bacterial density than those in nests with only sawdust (Fig.3, Table 1). No differences were detected for *enterococci* (Table 1).

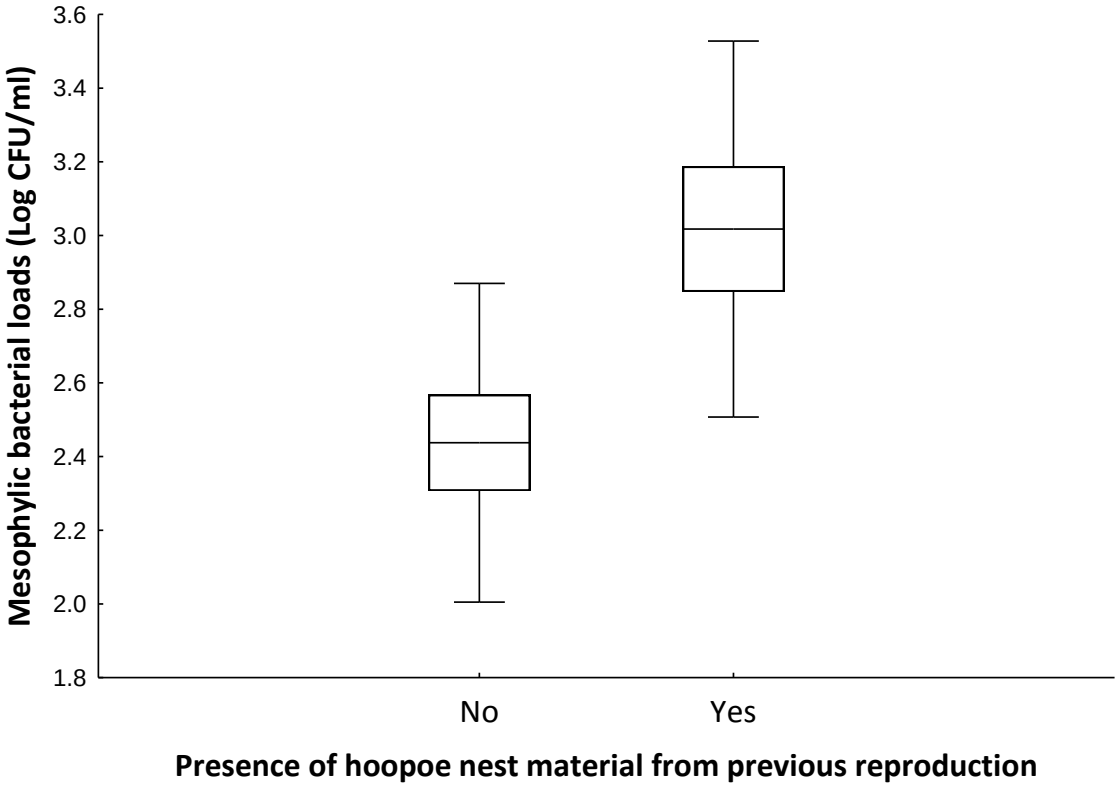


Fig 3. Mean ± 95% CI of bacterial growth (log(CFU/ml)) in TSA medium of samples from hoopoe eggshells, depending on type of experimental nest material added to nest boxes.

Table 1. Results from ANOVA (F-values) or Mann Whitney tests (Z-values in parentheses), means and standard error (SE) explaining the influence of experimental nest material (sawdust or a mixture of sawdust and old hoopoe´s nest material) on several dependent variables. We include only the co-variables that explained additional significance variance to those explained by the experimental treatments. Significant P-values are in bold.

	Type of nest material				**Comparisons**		
	Sawdust		**Old hoopoe´s nest**				
	Mean (SE)	**N**	**Mean (SE)**	**N**	**df**	**F or (Z)**	**P**
MICROBIAL LOAD AND ANTAGONISM:							
Log CFU/ml eggshell TSA							
- Nest material	**2.438(0.141)**	**11**	**3.018(0.156)**	**9**	**1,18**	**7.58**	**0.013**
CFU/ml eggshell KF							
- Nest material	113.64(51.013)	11	93.33(34.521)	9		(-0.43)	0.669
Log antagonistic activity secretion of females							
- Nest material	-0.690(0.046)	10	-0.636(0.048)	9	1,16	0.66	0.426

- Laying date					**1,16**	**5.55**	**0.032**
Antagonistic activity secretion of nestlings							
- Nest material	0.216(0.032)	32	0.197(0.026)	41	1,57	0.09	0.773
- Nest (random)					**14,57**	**2.48**	**0.008**
Log CFU/ml secretion TSA of females							
- Nest material	3.443(1.096)	8	1.810(1.386)	5	1,11	0.85	0.375
Log CFU/ml secretion TSA of nestlings							
- Nest material	4.753(0.629)	20	4.516(0.586)	23	1,11	0.08	0.787
CFU/ml secretion KF of females							
- Nest material	$84x10^5$(19636700)	10	0.000(0.000)	5		(-1.03)	0.301
BREEDING SUCCESS:							
Clutch size							
- Nest material	7.000(0.385)	11	7.900(0.403)	10	1,19	2.61	0.123
Hatching success							

- Nest material	0.901(0.175)	8	0.901(0.151)	8		(0.00)	1.000
Number of fledglings							
- Nest material	4.787(0.305)	7	5.312(0.285)	8	1,12	1.51	0.242
- Laying date					**1,12**	**10.12**	**0.008**
Fledglings success							
- Nest material	0.740(0.058)	7	0.818(0.054)	8	1,13	0.97	0.342
Clutch productivity							
- Nest material	0.639(0.061)	7	0.735(0.057)	8	1,13	1.32	0.272
Body condition of nestlings							
- Nest material	-1.040(1.367)	34	0.804(1.190)	44	1,12	0.96	0.346
- Laying date					**1,12**	**7.83**	**0.015**
- Number of nestlings					**1,12**	**5.10**	**0.043**

Uropygial gland secretion

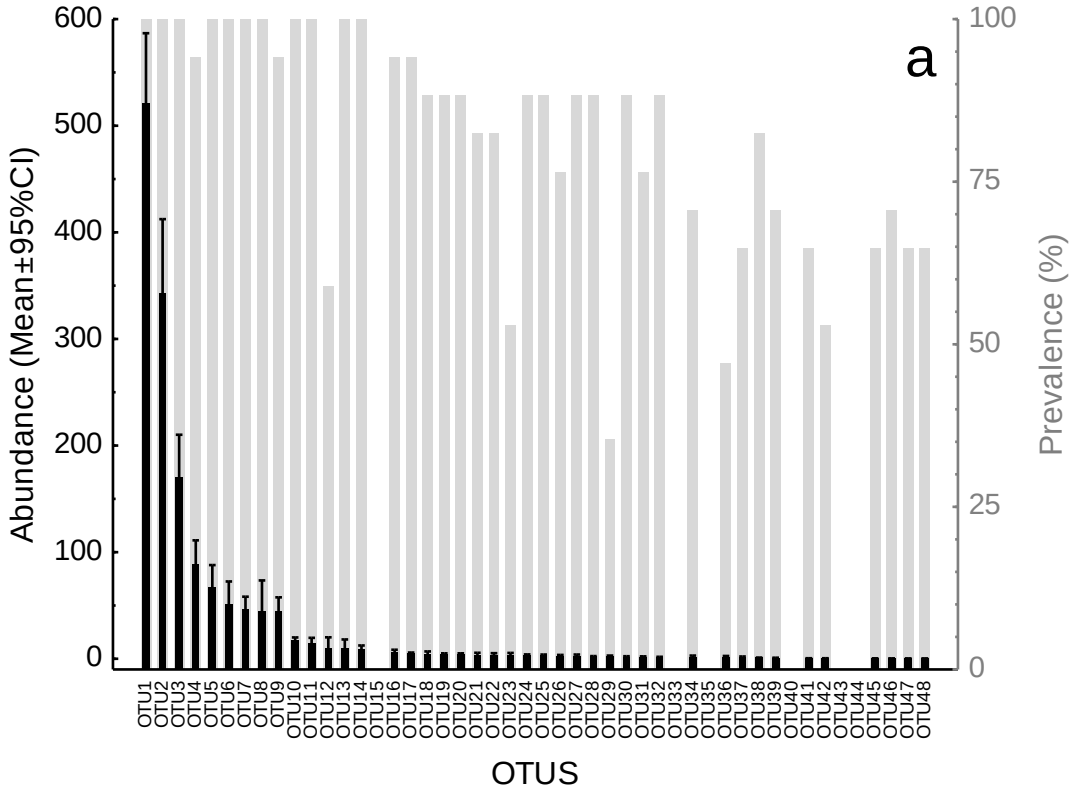
Neither in females nor in nestlings did the antagonistic activity of secretions or their bacterial loads (mesophilic bacteria and *enterococci*) depend on experimental treatment. The antagonistic activity of secretions was negatively related to laying date in females (Table 1).

Breeding success

Experimental treatment did not affect any of the variables used as proxy of breeding success of hoopoes. Laying date explained a significant proportion of variance only in the case of number of fledglings and body condition of nestlings, that decreased significantly with it (Table 1).

Uropygial gland bacterial community

When considering the two types of nest material together (with and without old hoopoe nest material), the uropygial secretion bacterial community consisted of 54 different OTUs. The bacterial community of uropygial secretion of incubating females and nestlings were very similar except for the presence in nestlings of six OTUs not present in females (Fig. 4a and 4b respectively). In both kinds of secretions, the same three OTUs clearly dominated the community, comprising together 70% of the analysed sequences per sample (Fig. 4).


a
Abundance (Mean±95%CI)
Prevalence (%)
OTUS
0
100
200
300
400
500
600
0
25
50
75
100
OTU1 OTU2 OTU3 OTU4 OTU5 OTU6 OTU7 OTU8 OTU9 OTU10 OTU11 OTU12 OTU13 OTU14 OTU15 OTU16 OTU17 OTU18 OTU19 OTU20 OTU21 OTU22 OTU23 OTU24 OTU25 OTU26 OTU27 OTU28 OTU29 OTU30 OTU31 OTU32 OTU33 OTU34 OTU35 OTU36 OTU37 OTU38 OTU39 OTU40 OTU41 OTU42 OTU43 OTU44 OTU45 OTU46 OTU47 OTU48

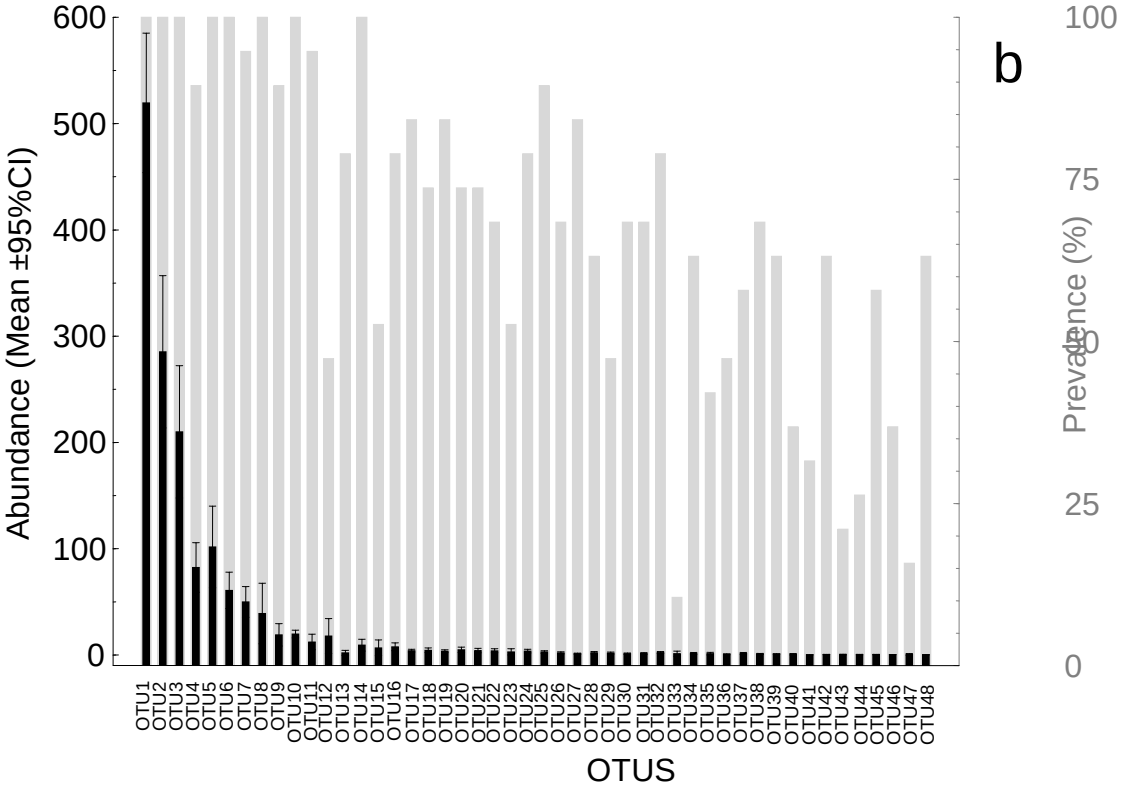

b
Abundance (Mean ±95%CI)
Prevalence (%)
OTUS
0
100
200
300
400
500
600
0
25
50
75
100
OTU1 OTU2 OTU3 OTU4 OTU5 OTU6 OTU7 OTU8 OTU9 OTU10 OTU11 OTU12 OTU13 OTU14 OTU15 OTU16 OTU17 OTU18 OTU19 OTU20 OTU21 OTU22 OTU23 OTU24 OTU25 OTU26 OTU27 OTU28 OTU29 OTU30 OTU31 OTU32 OTU33 OTU34 OTU35 OTU36 OTU37 OTU38 OTU39 OTU40 OTU41 OTU42 OTU43 OTU44 OTU45 OTU46 OTU47 OTU48

Fig 4. Rarefied abundance (number of sequences out of 1500 per sample after rarefaction, mean ± 95% CI; black bars for the left Y axis) and prevalence (percentage of samples where a target OTU was detected; grey bars for the right Y axis) of the OTUs detected by Illumina HTS in the uropygial gland secretions of hoopoes. Both types of nest material (with and without old hoopoe nest material) are considered together. Only the 48 (out of 54) most prevalent OTUs (those present in at least 3 individuals) are included in the graphs. (a) Data for females. (b) Data for nestlings.

When we distinguish between the two types of nest material, the experimental addition of hoopoe old nest material did not affect the alfa-diversity of the bacterial community inside hoopoe glands (Shannon index, ANOVAs mean(SE) across 10 rarefactions, F (1,34) = 1.45(0.10), p = 0.243(0.017), sawdust: mean(SE) = 2.89(0.08), n = 16, sawdust + old nest material: mean(SE) = 2.77(0.06), n = 20). However, the composition of the bacterial community in these two kinds of samples was different. The experiment manipulating the presence of hoopoe old nest material in nest boxes affected the bacterial community established in female glands, both considering weighted and unweighted Unifrac distances (Permanovas averaged across 10 rarefactions, weighted Unifrac: mean(SE), pseudoF(1,15) = 4.51(0.10), p = 0.0036(0.0007), Fig. 5a; unweighted Unifrac: mean(SE), pseudoF(1,15) = 2.78(0.14), p = 0.023(0.007). On the other hand, the communities present in nestling glands did not differ between treatments (Permanovas averaged across 10 rarefactions, after controlling for nest identity as a random factor, weighted Unifrac: mean(SE), pseudoF(1,17) = 1.07(0.21), p = 0.3791(0.0097), Fig.5b; unweighted Unifrac: mean(SE), pseudoF(1,17) = 0.89(0.11), p = 0.502(0.066)).

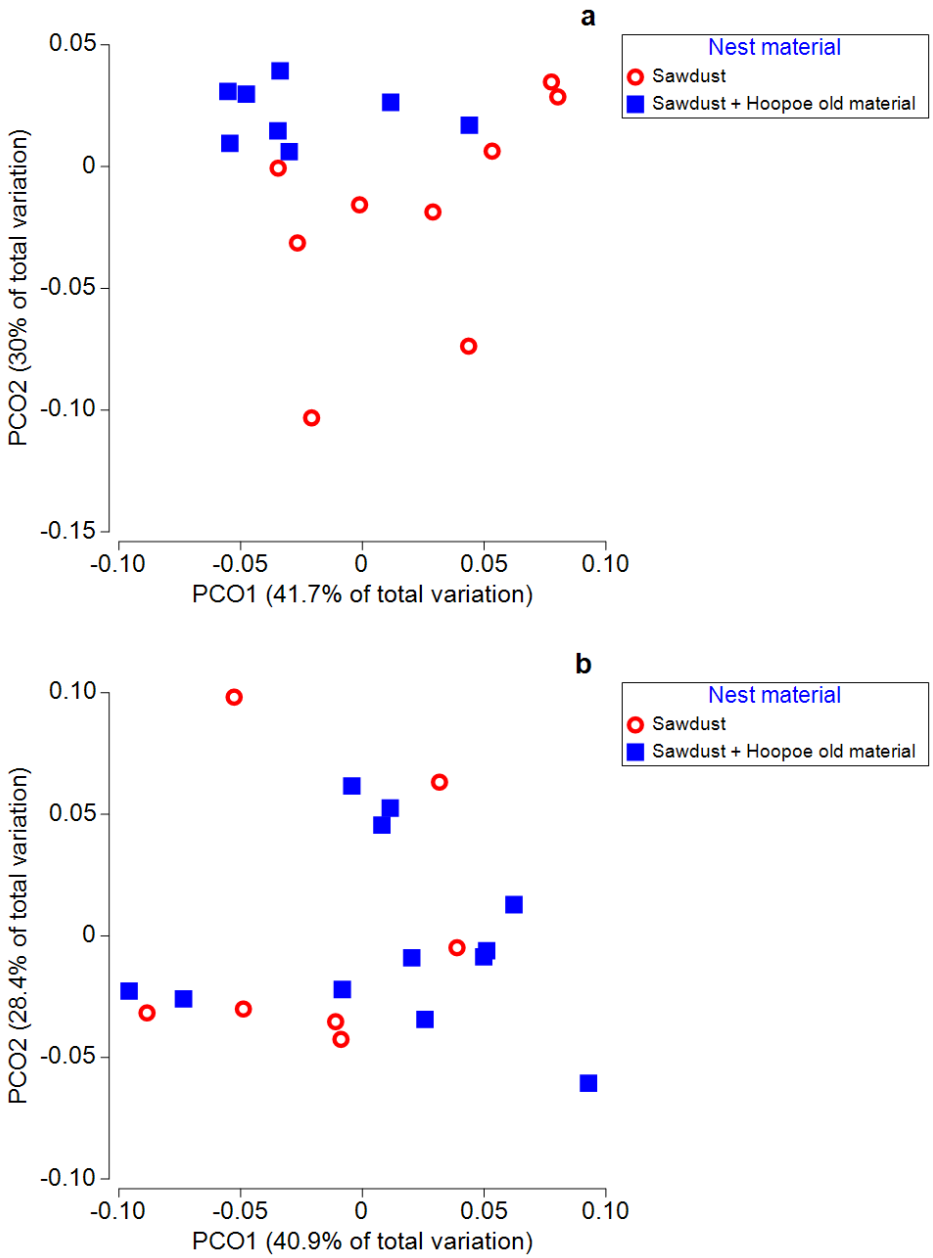


Fig 5. Comparison, based on weighted unifrac distances, between the microbial communities found in the uropygial glands of hoopoes breeding in nest-boxes with sawdust or a mix of sawdust and old hoopoe nest material. (a) females and (b) nestlings.

The LefSe analysis identified several markers for the two experimental groups of samples (Fig. 6). Two OTUs (OTU29 and OTU37) were markers for the group of samples from nests with old hoopoe nest material, while other three were markers of the control group

only with sawdust in the nest (OTU12, OTU13 and OTU23). At higher taxonomic levels, the Fam. Propionibacteriaceae including OTU23+OTU38, and the whole Class Actinobacteria including additionally OTU14, resulted more typical of the control group. On the other hand, the use of non-parametric t-tests on samples rarified to 1500 reads, resulted in non- significant differences between experimental groups for the Fam. Propionibacteriaceae (Non-parametric t (mean(SE)), t = -1.31(0.10), p = 0.234(0.041), FDR-p across all classes = 0.778(0.043)) and for Actinobacteria (Non-parametric t (mean(SE)), t = -0.96(0.07), p = 0.409(0.270), FDR-p across all classes = 0.758(0.596)). With this approach, differences in rarefied abundance between groups only appeared for two OTUs: OTU29 and OTU12 (Fig. 7a, both females and nestlings together to increase the power of the analyses), with mean p-values across 10 rarefactions > 0.1 for the other three OTUs identified as biomarkers by the LeFse analysis (data not presented). OTU29 (unidentified *Clostridium*) was completely absent from samples from nest boxes without old nest material, while it appeared at high prevalence in the experimental group with it (prevalence: experimental nests = 75%, n = 16; control nests = 0%, n = 20, comparison between control and experimental nests, two-tailed Fisher exact test, p < 0.001; rarefied abundance: Non-parametric t(mean(SE)), t = 4.27(0.09), p = 0.0010(0.0000)FDR-p across all OTUs p = 0.051(0,007)). Moreover, OTU12 (identified as *Tissierellaceae* GW-34) was more prevalent and abundant in samples from nests without experimental old nest material (prevalence: experimental nests = 35%, control nests = 75%, comparison between control and experimental nests, two-tailed Fisher exact test, p = 0.023; rarefied abundance: Non-parametric t (mean(SE)), t = -2.65(0.04), p = 0.0020(0.0003), FDR-p across all OTUs = 0.054(0.008)). Interestingly, these two OTUs were never simultaneously present in the same sample (Fig. 7b). When we analyzed the relationships among abundances of OTUs in samples with SparCC, these two OTUs resulted significantly correlated with several

other components of the community, including some of those retained in the best subset of the components of female secretion bacterial community that explained hatching success with the BEST method (Table 2). Moreover, the abundance of OTU29 was positively related to that of three of the ten most abundant OTUs of the community, and OTU12 to one of them.

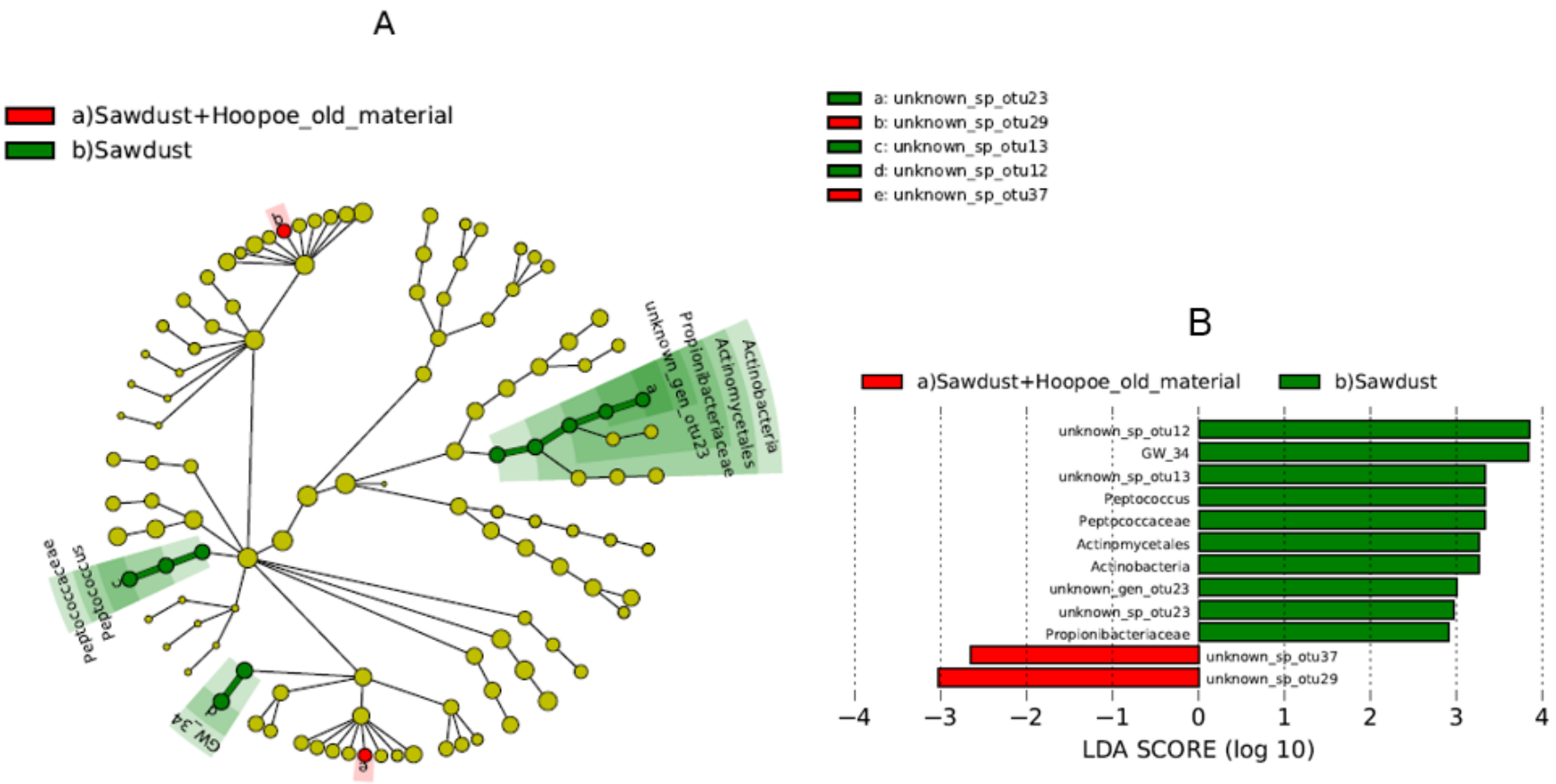


Fig 6. Results of the LefSe analysis comparing the bacterial microbiome of the hoopoe of the hoopoe uropygial secretion samples of individuals from nests with sawdust vs sawdust+hoopoe nest material added. A) Cladogram of the 54 OTUs found in hoopoe uropygial secretions highlighting in colors the bacteria identified by the LefSe method as biomarkers of the two experimental groups (those with a LDA (linear discriminant analysis effect size)>2). B) LDA scores for the different biomarkers.

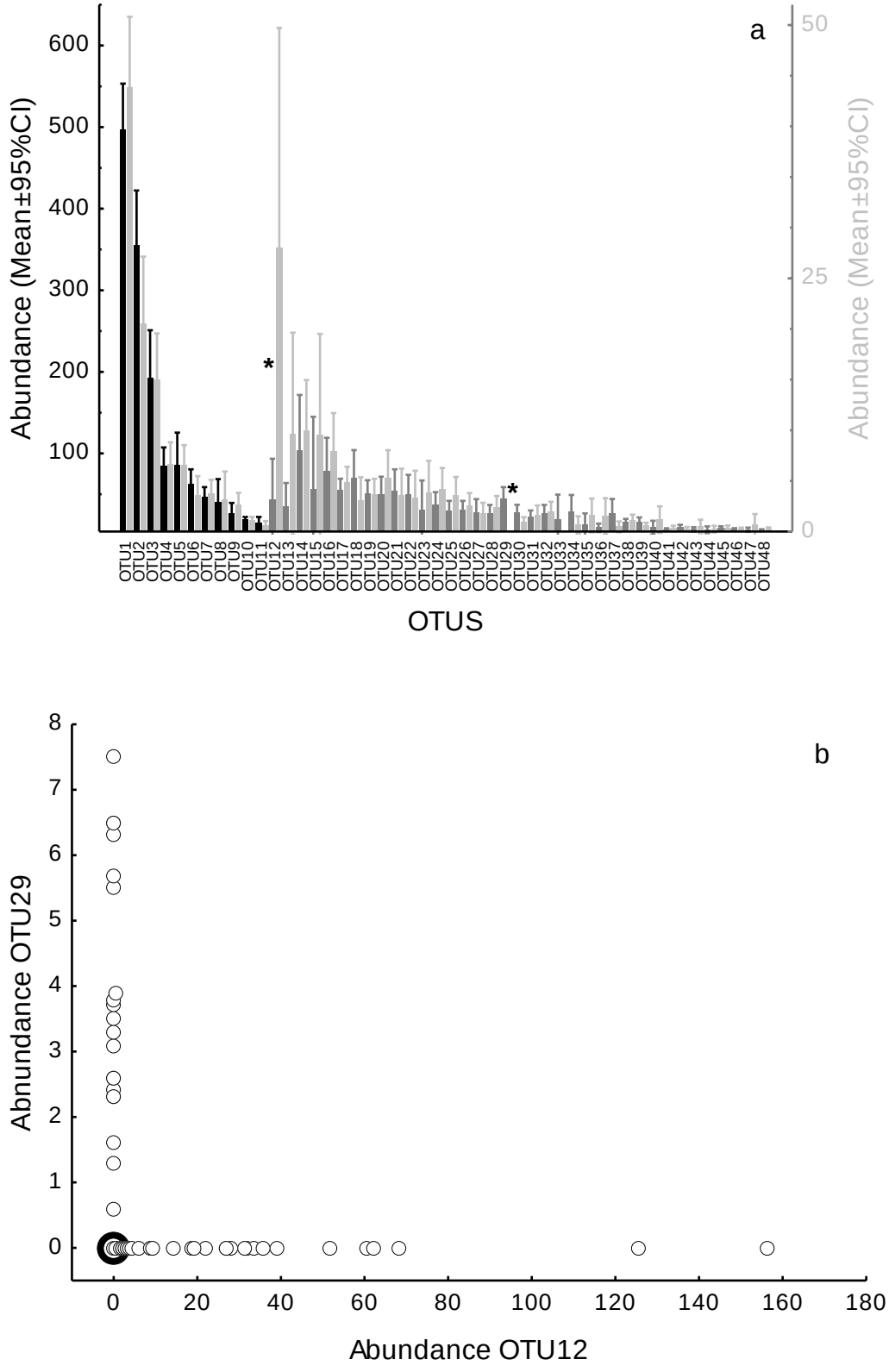


Fig 7. Influence of presence of hoopoe old nest material in nest boxes on the rarefied abundance (number of sequences out of a total 1500 counts per sample) of bacterial OTUs

in the uropygial secretion of hoopoes (female and nestling samples together). (a) Comparison of the abundance of OTUs between nests with sawdust + hoopoe nest material (black and dark gray bars for the left and right y-axes, respectively) and nests only with sawdust (light grey bars). Significant comparisons for a particular OTU (Non-parametric t-tests after FDR correction) are marked with asterisks. The bars from OTU12 to OTU48 are scaled for the right y-axis. Only the 48 (out of 54) most prevalent OTUs (those present in at least 3 individuals) are presented in the graph. (b) Relationship between the rarefied abundance of OTU29 and OTU12 in the same hoopoe uropygial secretion samples, including all types of nest boxes. Point sizes are in log-scale.

The BEST analysis also identified several female secretion OTUs positively correlated with the hatching success of embryonated eggs in their clutches. These included an unidentified Coriobacteriaceae (Ph. Actinobacteria), five closely related *Firmicutes* in a clade including one *Coprococcus* and one *Peptoniphilus*, another Mogibacteriaceae (probably also *Peptoniphilus*) and *Enterococcus* strains (Table 2, Fig 1).

Table 2. List of the significant relationships between the two OTUs affected by the nest material experiment (OTU12 and OTU29) and other OTUs of the hoopoe secretion microbiota (SparCC sig. correlations, only for those with $r \geq 0.20$ and $p < 0.05$, the sign of the relationship is indicated). The groups of OTUs from female secretions best explaining the hatching success of their clutches are shown (BEST stepwise search with Primer7, R value of the final model indicated in the columns), with the corresponding bivariate Spearman correlations (rs(p)) with hatching success for each OTU. Significant p-values are in bold.

	(SparCC sig. correlations)		Best subsets explaining hatching success				Identification
	OTU12	**OTU29**	All nests (R=0.220, n=29) r_s(p)	All nests, embrionated eggs (R=0.486, n=29) r_s(p)	Exper. nests (R=0.516, n=13) r_s(p)	Exper. nests embrionated eggs (R=0.546, n=13) r_s(p)	
OTU30	-						(Clostridiales)
OTU28				**0.42**(0.024)			(Clostridiaceae)
OTU32	-			**0.42**(0.025)		0.52(0.066)	*Coprococcus*
OTU19		-	0.14(0.469)	**0.40**(0.025)		**0.59**(0.035)	(*Clostridiaceae*)
OTU45				-0.10(0.619)		0.20(0.512)	(*Clostridiaceae*)
OTU42				0.11(0.569)	0.54(0.055)	0.47(0.102)	*Peptoniphilus*
OTU20			0.05(0.795)	**0.46**(0.012)		0.51(0.078)	(*Mogibacteriaceae*)
OTU38			0.30(0.118)	**0.47**(0.011)		**0.57**(0.041)	(*Coriobacteriaceae*)

OTU40					0.37(0.211)	0.23(0.449)	*Enterococcus*
OTU43					0.24(0.435)	0.29(0.330)	*Enterococcus*
OTU27			-0.26(0.181)	-0.11(0.569)		-0.47(0.106)	*Helcococcus*
OTU46			-0.23(0.235)				*Clostridium*
OTU39						-0.02(0.956)	*Peptoniphilus*
OTU12		-					*Tissierellaceae*
OTU24		-					*Tissierellaceae*
OTU41		-					*Campylobacter*
OTU6		+					*Peptoniphilus*
OTU2		+					(Clostridiales)
OTU31		+					*Clostridium*
OTU26		+					*Clostridium*
OTU17		+					*Clostridium*
OTU4		+					*Clostridium*
OTU29	-			-0.32(0.094)			*Clostridium*
OTU37	-						*Peptoniphilus*
OTU8	+						*Campylobacter*
OTU47	+						(Enterobacteriales)
OTU36	+						*Propionibacterium*

DISCUSSION

Our results indicate that hoopoes preferred for reproduction nest boxes with experimental nest material regardless of whether the material added came from previously used hoopoe nests or not. Characteristics of nest material did not affect reproductive success of hoopoes but affected the bacterial loads of the eggshell and the uropygial gland bacterial community composition. Several OTUs from female uropygial secretions were positively associated with hatching success. These results suggest that a main function of the uropygial secretion of females is to protect the eggs from infection as previously mentioned in Martín-Vivaldi et al. (2014a) and Soler et al. (2014).

As predicted, hoopoes prefer nest boxes with material instead of empty ones. Associated advantages of reusing nest boxes should be even more important for this species that do not built nests because old nest material may help to improve water absorption, thermo-insulation and, thus, incubation efficiency (Mazgajski 2007, Mainwaring et al. 2014, Podofillini et al. 2018). In addition, it could minimize the risk of egg breakage (Podofillini et al. 2018) and the rest of previous breeding activities within the nest material could act as an indicator that it is a good nesting place (Orell et al. 1993).

We hypothesised that hoopoes should prefer for reproduction nest boxes previously used by conspecifics and thus predicted a higher usage of experimental nest boxes with old hoopoe material added. However, our experimental results do not agree with such prediction and hoopoes used at similar rates nest boxes with or without material taken from nests where hoopoes reproduced the previous season. This prediction was based on the possibility that old nest material was a reservoir of mutualistic bacteria for the uropygial gland of nesting hoopoes. Previous studies have pointed out that hoopoes commonly re-use cavities for reproduction (Cramp 1998, Martín-Vivaldi et al. 1999, van

Wijk et al. 2017). Our results suggest that this preference for particular nest sites is not mediated by particularities of materials from previous hoopoe reproduction, i.e. by the presence of symbiotic bacteria, because hoopoes reproduced at similar rates in nest-boxes of different experimental treatment. Nest box choice could also depend on the availability of appropriate nest sites (Stanback and Rockwell 2003) and the characteristics of the territory (Tschumi et al. 2014). This possibility would unlikely explain our results since experimental nest boxes were new (never used before) and installed in locations in a pairwise experimental design. Age and previous breeding experience could affect nest-box choice by hoopoes. We do not have a reliable criterion for estimating age in the females of this breeding season so we cannot control for it in the analyses. However, we have very few recruits (11.36% in 2015; 4 of 44 females) due to high mortality and high dispersion of the species (van Wijk et al. 2018). Thus, most breeders were new individuals arrived from other places to our study area. Moreover, the new experimental nest boxes were new for all hoopoes that arrived to the study area regardless of their age (recruits or not recruits) and both, old and new individuals, used new (experimental) and old nest boxes. Another possibility could be that nest box choice was affected by the bacterial community of the individual hoopoes. Thus, although we think this alternative explanation is improbable, we cannot rule it out.

Despite not showing preference for nests with old hoopoe nest material, we found support to the expected effect of the experimental nest material on the composition of females' uropygial secretion, and on the bacterial load of the eggshells. Whittaker et al. 2016 found in dark-eyed junco that shared environments had an influence in shaping bacterial and volatile profiles of the uropygial gland skin and secretion. In our case, the bacteria were only stemmed from the uropygial secretion. This result shows, for the first

time in hoopoes, that the nest material could be a source of strains for their incorporation to the uropygial gland that can interact with other components of the community.

Results from the two statistical approaches used for the comparison of bacterial communities of experimental groups agreed in the identity of the two OTUs whose rarefied abundances were more strongly affected by the experiment. LEfSe analysis is more sensitive and detected additional biased taxa that deserve to be investigated as possible biomarkers of these environmental conditions. Our relative low sample size and the relatively high inter-individual variation detected, impede to point out statistical support when considering averaged repeated t-tests on random repetitions of rarefactions.

There were two particular OTUs of glands that were greatly affected by the presence of hoopoe old nest material, but in opposed directions. OTU29 was only present in nests with hoopoe old nest material, while OTU12 was much more prevalent in nests without such old material. This suggests that OTU29 will only be incorporated to the secretion bacterial community when hoopoes use cavities where conspecifics previously reproduced. We do not know to what extent the incorporation of these bacteria in the secretion is beneficial or not to the host, or even if any of them is potentially pathogenic, since we have not found effects of the experiment on breeding success or antimicrobial activity of secretions. Nevertheless, since both are correlated with the abundance of other components of the community, they may have a role in the uropygial gland microbiota dynamics.

Moreover, our findings show that old nest material could affect the microbial community established in female glands. In this way, females could be able to acquire bacteria from the old nest material. This could also explain the differences found in the bacterial community of uropygial secretion of females and nestlings since the latter had six OTUs not present in females. Another non-exclusive possibility is that nestlings could

acquire it from the nest after female leaves it, but not from sources present in the old material. Females stay inside the nest until the first nestlings are 8 days old and nestlings leave the nest after 24-30 days (Martín-Vivaldi et al. 2014b). In this way, these OTUs present only in nestlings glands could be obtained from the diverse kind of food remains, faeces, dead nestlings, etc. accumulated in the nest along the nestling period. Alternatively, nestlings´ glands may be less selective in the acquisition of strains from the environment than females. The association of age with the maturity of the immune system might determine or control the microbiota of the uropygial gland. This possibility is also consistent with the idea that young animals host more rare and transient symbionts than adults (e.g. Palmer et al. 2007). Whatever the reason, some other studies also found a greater number of total bacterial OTUs in nestlings than in adults (van Dongen et al. 2013, Whittaker et al. 2016) and, thus, our result might adjust to a general trend in birds. However, the microbiota of the nest materials has not been analyzed. Therefore, it is possible that the results found are due to indirect effects of the nest material or environmental conditions. Many studies have found that there is an important role of environmental conditions for bacterial load that could influence the final microbiome (Palmer et al. 2007, Peralta-Sánchez et al. 2012, Brandl et al. 2014, D'Alba and Shawkey 2015, Ruiz-Castellano et al. 2016, Whittaker et al. 2016, Martín-Vivaldi et al. 2018).

Regarding bacterial load of the eggshell, previous experimental studies detected the influence of nest materials on eggshell microbiota in several bird species (Brandl et al. 2014, Grizard et al. 2014, Martínez-García et al. 2016a, Ruiz-Castellano et al. 2016, van Veelen et al. 2018). These bacteria are mostly derived from faeces, digestive tract and bare skin of the female, feathers and nest material itself (Peralta-Sanchez et al. 2010, van Veelen et al. 2017, 2018). Moreover, uropygial secretion might reach eggshells and, thus, because of its antimicrobial properties, determine at least partially their microbiota

(Peralta-Sánchez et al. 2012). In the case of hoopoes, uropygial secretion also contains bacteriocin-producing bacteria, and females actively smear eggshells with their uropygial gland secretion (Soler et al. 2014) and fill up special structures (i.e. crypts) that enhance the adhesion of the secretion full of symbiotic bacteria (Martín-Vivaldi et al., 2014b). Therefore, since we have found an effect of experimental nest material on the bacterial community of uropygial secretion of female hoopoes, the detected effects on the bacterial load of the eggshell might not only be directly determined by the microbiota of nest material, but indirectly by its effect on the bacterial community of female secretion.

The absence of effects of the type of nest material on breeding success suggests that the bacterial strains acquired from the nest by hoopoes are not especially important for them or that these bacteria are functionally redundant. The core components of the usual microbiome might be acquired vertically, probably maintained from one to another breeding season on reservoirs on the body, or can be obtained horizontally from environmental sources others than nest materials (Ruiz-Rodríguez et al. 2014, Martínez-García et al. 2016b, Martín-Vivaldi et al. 2018, Rodríguez-Ruano et al. 2018). This result may further explain why hoopoes have not evolved preference for nests with old hoopoe nest material. The effects of nest re-use on nestling fitness and reproductive success are still unclear. Some studies found negative effects (Tomás et al. 2007; González-Braojos et al., 2012). However, most studies agree with our results, and did not find that nest re-use affects hatching or breeding success (review on Mazgajski, 2007; Martínez-García et al., 2016a; Podofillini et al., 2018). Even though the experiment did not affect breeding success of pairs, we have found that several OTUs from female uropygial secretions were positively associated with hatching success. These OTUs belong to the phylum *Firmicutes*, as *enterococci*, that produce bacteriocins defending from feather degrading bacteria and trans-shell infection of embryos (Martín-Platero et al., 2006; Soler et al.,

2008; Martín-Vivaldi et al., 2014b). The experiment did not affect these bacteria and, thus, it is unlikely that they came from the nest material, so it is worthwhile to continue exploring their origin. Another explanation for the absence of a significant effect on breeding success could be the small sample size obtained, being insufficient for detecting statistically significant effects.

In summary, the existence of nest material seems more important for hoopoes´nest-site selection than the possibility of obtaining a reservoir of beneficial bacteria from it. Nevertheless, the experiment confirms an important effect of such re-used nest material on the bacterial loads of the eggshells, and this is the first time that it is shown that it affects also the composition of the uropygial gland secretion bacterial community. More importantly, several particular OTUs resulted related to hatching success. This result highlights the possibility that a main function of the uropygial secretion of female hoopoes is to protect the eggs from infection, using a variety of cultivated bacterial strains, as suggested by previous results mainly for *enterococci* (Ruiz-Rodríguez et al. 2013, Martín-Vivaldi et al. 2014a, Peralta-Sánchez et al. 2014). The information provided by the description of the whole microbial community inhabiting these glands (Rodríguez-Ruano et al. 2018 and the present study), will lead to the next interesting step of studying the complete set of interactions among them and their effects on bird health and breeding success using network analyses. In addition, it would be worth to analyze the microbiota of the nest material itself, to understand how it interacts with the bacterial composition of the uropygial secretion and to reinforce the results and conclusions derived from this experiment.

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Permits – The study was conducted according to relevant Spanish national (Decreto 105/2011, 19 de abril) and regional guidelines. The protocol was approved by the ethics committee of the Univ. of Granada (Comité de Ética en Experimentación Animal, CEEA, Ref.: 785), and all necessary permits for hoopoe's manipulations were provided by Consejería de Medio Ambiente de la Junta de Andalucía, Spain (Ref: SGYB/FOA/AFR/CFS and SGMN/GyB/ JMIF). Our study area is not protected, but privately owned, and the owners allowed us to work in their properties. The time spent in each hoopoe nest was the minimum necessary for the experiment.

REFERENCES

Alberdi, A., Aizpurua, O., Bohmann, K., Zepeda-Mendoza, M. L. and Gilbert, M. T. P. 2016. Do Vertebrate Gut Metagenomes Confer Rapid Ecological Adaptation? - Trends Ecol. Evol. 31: 689–699.

Alexander, G. D., Houston, D. C. and Campbell, M. 1994. A possible acoustic function for the casque structure in hornbills (Aves: Bucerotidae). - J. Zool. 233: 57–67.

Amat, J. A., Rendón, M. A., Garrido-Fernández, J., Garrido, A., Rendón-martos, M. and Pérez-gálvez, A. 2011. Greater flamingos Phoenicopterus roseus use uropygial secretions as make-up. - Behav. Ecol. 65: 665–673.

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Zech Xu, Z., Kightley, E. P., Thompson, L. R., Hyde, E. R., Gonzalez, A. and Knight, R. 2017. Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. - mSystems 2: e00191-16.

Amundsen, T. 2000. Why are female birds ornamented? - Trends Ecol. Evol. 15: 149–155.

Amundsen, T. 2018. Sex roles and sexual selection: Lessons from a dynamic model system. - Curr. Zool. 64: 363–392.

Andersson, M. 1994. Sexual selection (Princeton Univ. Press, Ed.).

Andersson, M. and Simmons, L. W. 2006. Sexual selection and mate choice. - Trends Ecol. Evol. 21: 296–302.

Archie, E. A. and Theis, K. R. 2011. Animal behaviour meets microbial ecology. - Anim. Behav. 82: 425–436.

Arlettaz, R., Schaub, M., Fournier, J., Reichlin, T. S., Sierro, A., Watson, J. E. M. and Braunisch, V. 2010. From Publications to Public Actions: When Conservation Biologists Bridge the Gap between Research and Implementation. - Bioscience 60: 835–842.

Aronesty, E. 2011. ea-utils: Command-line tools for processing biological sequencing data.: https://github.com/ExpressionAnalysis/ea-utils.

Avilés, J. M. and Soler, J. J. 2009. Nestling colouration is adjusted to parent visual performance in altricial birds. - J. Evol. Biol. 22: 376–386.

Avilés, J. M., Soler, J. J. and Pérez-Contreras, T. 2006. Dark nests and egg colour in birds: a possible functional role of ultraviolet reflectance in egg detectability. - Proc. Biol. Sci. 273: 2821–2829.

Avilés, J. M., Pérez-Contreras, T., Navarro, C. and Soler, J. J. 2009. Male spotless starlings adjust feeding effort based on egg spots revealing ectoparasite load. - Anim. Behav. 78: 993–999.

Avilés, J. M., Vikan, J. R., Fossøy, F., Antonov, A., Moksnes, A., Røskaft, E. and Stokke, B. G. 2010. Avian colour perception predicts behavioural responses to experimental

brood parasitism in chaffinches. - J Evol Biol 23: 293–301.

Avilés, J. M., Soler, J. J. and Hart, N. S. 2011. Sexual selection based on egg colour: Physiological models and egg discrimination experiments in a cavity-nesting bird. - Behav. Ecol. Sociobiol. 65: 1721–1730.

Azcárate-García, M., González-Braojos, S., Díaz-Lora, S., Ruiz-Rodríguez, M., Martin-Vivaldi, M., Martínez-Bueno, M., Moreno, J. and Soler, J. J. 2020. Interspecific variation in deterioration and degradability of avian feathers: The evolutionary role of the microbiome. - J. Avian Biol.: in press.

Baker, L. J., Freed, L. L., Easson, C. G., Lopez, J. V, Fenolio, D., Sutton, T. T., Nyholm, S. V and Hendry, T. A. 2019. Diverse deep-sea anglerfishes share a genetically reduced luminous symbiont that is acquired from the environment. - Elife 8: e47606.

Bandyopadhyay, A. and Bhattacharyya, S. P. 1996. Influence of fowl uropygial gland and its secretory lipid components on growth of skin surface bacteria of fowl. - Indian J. Exp. Biol. 34: 48–52.

Banning, J. L., Weddle, A. L., Wahl III, G. W., Simon, M. A., Lauer, A., Walters, R. L. and Harris, R. N. 2008. Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, Hemidactylium scutatum. - Oecologia 156: 423–429.

Barr, J. J., Auro, R., Furlan, M., Whiteson, K. L., Erb, M. L., Pogliano, J., Stotland, A., Wolkowicz, R., Cutting, A. S., Doran, K. S., Salamon, P., Youle, M. and Rohwer, F. 2013. Bacteriophage adhering to mucus provide a non–host-derived immunity. - Proc. Natl. Acad. Sci. 110: 10771–10776.

Beltran-Bech, S. and Richard, F.-J. 2014. Impact of infection on mate choice. - Anim. Behav. 90: 159–170.

Bennett, G. M. and Moran, N. A. 2015. Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. - Proc. Natl. Acad. Sci. 112: 10169–10176.

Bentley, D. R., Balasubramanian, S., Swerdlow, H. P., Smith, G. P., Milton, J., Brown, C. G., Hall, K. P., Evers, D. J., Barnes, C. L., Bignell, H. R., Boutell, J. M., Bryant, J., Carter, R. J., Keira Cheetham, R., Cox, A. J., Ellis, D. J., Flatbush, M. R., Gormley, N. A., Humphray, S. J., Irving, L. J., Karbelashvili, M. S., Kirk, S. M., Li, H., Liu, X., Maisinger, K. S., Murray, L. J., Obradovic, B., Ost, T., Parkinson, M. L., Pratt, M. R., Rasolonjatovo, I. M. J., Reed, M. T., Rigatti, R., Rodighiero, C., Ross, M. T., Sabot, A., Sankar, S. V., Scally, A., Schroth, G. P., Smith, M. E., Smith, V. P.,

Spiridou, A., Torrance, P. E., Tzonev, S. S., Vermaas, E. H., Walter, K., Wu, X., Zhang, L., Alam, M. D., Anastasi, C., Aniebo, I. C., Bailey, D. M. D., Bancarz, I. R., Banerjee, S., Barbour, S. G., Baybayan, P. A., Benoit, V. A., Benson, K. F., Bevis, C., Black, P. J., Boodhun, A., Brennan, J. S., Bridgham, J. A., Brown, R. C., Brown, A. A., Buermann, D. H., Bundu, A. A., Burrows, J. C., Carter, N. P., Castillo, N., Catenazzi, M. C. E., Chang, S., Neil Cooley, R., Crake, N. R., Dada, O. O., Diakoumakos, K. D., Dominguez-Fernandez, B., Earnshaw, D. J., Egbujor, U. C., Elmore, D. W., Etchin, S. S., Ewan, M. R., Fedurco, M., Fraser, L. J., Fuentes Fajardo, K. V., Scott Furey, W., George, D., Gietzen, K. J., Goddard, C. P., Golda, G. S., Granieri, P. A., Green, D. E., Gustafson, D. L., Hansen, N. F., Harnish, K., Haudenschild, C. D., Heyer, N. I., Hims, M. M., et al. 2008. Accurate whole human genome sequencing using reversible terminator chemistry. - Nature 456: 53–59.

Boisbunon, A., Canu, S., Fourdrinier, D., Strawderman, W. and Wells, M. T. 2013. AIC , Cp and estimators of loss for elliptically symmetric distributions. - hal-00851206

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., Mills, D. A. and Caporaso, J. G. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. - Nat. Methods 10: 57–59.

Brandl, H. B., van Dongen, W. F. D., Darolová, A., Kristofík, J., Majtan, J. and Hoi, H. 2014. Composition of Bacterial Assemblages in Different Components of Reed Warbler Nests and a Possible Role of Egg Incubation in Pathogen Regulation. - PLoS One 9(12): 1–19.

Braun, M. S., Zimmermann, S., Danner, M., Rashid, H. and Wink, M. 2016. Corynebacterium uropygiale sp. nov., isolated from the preen gland of Turkeys (Meleagris gallopavo). - Syst. Appl. Microbiol. 39: 88–92.

Braun, M. S., Wang, E., Zimmermann, S. and Wink, M. 2018a. Corynebacterium heidelbergense sp. nov., isolated from the preen glands of Egyptian geese (Alopochen aegyptiacus). - Syst. Appl. Microbiol. 41: 564–569.

Braun, M. S., Wang, E., Zimmermann, S., Boutin, S. and Wink, M. 2018b. Kocuria uropygioeca sp. nov. and Kocuria uropygialis sp. nov., isolated from the preen glands of Great Spotted Woodpeckers (Dendrocopos major). - Syst. Appl. Microbiol. 41: 38–43.

Braun, M. S., Sporer, F., Zimmermann, S. and Wink, M. 2018c. Birds, feather-degrading bacteria and preen glands: The antimicrobial activity of preen gland secretions from

turkeys (Meleagris gallopavo) is amplified by keratinase. - FEMS Microbiol. Ecol. 94: fiy117.

Braun, M. S., Wang, E., Zimmermann, S., Boutin, S., Wagner, H. and Wink, M. 2019a. Kocuria tytonicola, new bacteria from the preen glands of American barn owls (Tyto furcata). - Syst. Appl. Microbiol. 42: 198–204.

Braun, M. S., Wang, E., Zimmermann, S., Wagner, H. and Wink, M. 2019b. Kocuria tytonis sp. nov., isolated from the uropygial gland of an American barn owl (Tyto furcata). - Int. J. Syst. Evol. Microbiol. 69: 447–451.

Bright, M. and Bulgheresi, S. 2010. A complex journey: transmission of microbial symbionts. - Nat. Rev. Microbiol. 8: 218–230.

Brulez, K., Pike, T. W. and Reynolds, S. J. 2015. Egg signalling: The use of visual, auditory, and chemical stimuli. - In: Deeming, D. C. and Reynolds, S. J. (eds), Nest, Egg, and Incubation. New ideas about avian reproduction. Oxford University Press, pp. 127–141.

Brunetti, A. E., Lyra, M. L., Melo, W. G. P., Andrade, L. E., Palacios-Rodríguez, P., Prado, B. M., Haddad, C. F. B., Pupo, M. T. and Lopes, N. P. 2019. Symbiotic skin bacteria as a source for sex-specific scents in frogs. - Proc. Natl. Acad. Sci. 116: 2124–2129.

Bulla, M., Šálek, M. and Gosler, A. G. 2012. Eggshell spotting does not predict male incubation but marks thinner areas of a shorebird's shells. - Auk 129: 26–35.

Burger, B. V, Reiter, B., Borzyk, O. and du Plessis, M. A. 2004. Avian Exocrine Secretions. I. Chemical Characterization of the Volatile Fraction of the Uropygial Secretion of the Green Woodhoopoe, Phoeniculus purpureus. - J. Chem. Ecol. 30: 1603–1611.

Burley, N. 1986. Sexual Selection for Aesthetic Traits in Species with Biparental Care. - Am. Nat. 127: 415–445.

Burley, N. 1988. The Differential Allocation hypothesis. An experimental test. - Am. Nat. 132: 611–628.

Callahan, B. J., McMurdie, P. J. and Holmes, S. P. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. - ISME J. 11: 2639–2643.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., González Peña, A., Goodrich, J. K., Gordon, J. I., Huttley, G. A.,

Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., Mcdonald, D., Muegge, B., Pirrung, M., Reeder, J., Sevinsky, J., Turnbaugh, P., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J. and Knight, R. 2010. QIIME allows analysis of high-throughput community sequencing data. - Nat Methods. 7: 335–336.

Caruso, V., Song, X., Asquith, M. and Karstens, L. 2019. Performance of Microbiome Sequence Inference Methods in Environments with Varying Biomass (SM Gibbons, Ed.). - mSystems 4: e00163-18.

Chapman, T., Arnqvist, G., Bangham, J. and Rowe, L. 2003. Sexual conflict. - Trends Ecol. Evol. 18: 41–47.

Cherry, M. I. and Gosler, A. G. 2010. Avian eggshell coloration: New perspectives on adaptive explanations. - Biol. J. Linn. Soc. 100: 753–762.

Chiarello, M., Villéger, S., Bouvier, C., Auguet, J. C. and Bouvier, T. 2017. Captive bottlenose dolphins and killer whales harbor a species-specific skin microbiota that varies among individuals. - Sci. Rep. 7: 15269.

Clayton, D. H. 1991. The influence of parasites on host sexual selection. - Parasitol. Today 7: 329–334.

Clutton-Brock, T. H. 1991. The evolution of parental care (Princeton University Press, Ed.).

Clutton-Brock, T. 2007. Sexual Selection in Males and Females. - Science (80-.). 318: 1882–1885.

Clutton-Brock, T. 2009. Sexual selection in females. - Anim. Behav. 77: 3–11.

Cook, M. I., Beissinger, S. R., Toranzos, G. A. and Arendt, W. J. 2005. Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection. - Ecol. Lett. 8: 532–537.

Corti, M., Romano, A., Costanzo, A., Bentz, A. B., Navara, K. J., Parolini, M., Saino, N. and Rubolini, D. 2018. Protoporphyrin-based eggshell pigmentation predicts hatching success and offspring sex ratio in the barn swallow. - J. Avian Biol. 49: 1–17.

Cramp, S. 1998. The complete birds of the western Palearctic on CD-ROM. - Oxford University Press.

Cunningham, E. J. A. and Russell, A. F. 2000. Egg investment is influenced by male attractiveness in the mallard. - Nature 404: 74–77.

Currie, C. R., Scott, J. A., Summerbell, R. C. and Malloch, D. 1999. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. - Nature 398: 701–704.

D'Alba, L. and Shawkey, M. D. 2015. Mechanisms of antimicrobial defense in avian eggs. - J. Ornithol. 156: S399–S408.

Darby, A. C. and Douglas, A. E. 2003. Elucidation of the Transmission Patterns of an Insect-Borne Bacterium. - Appl. Environ. Microbiol. 69: 4403 LP – 4407.

Darwin, C. 1871. The descent of man and selection in relation to sex (J Murray, Ed.).

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A. and Callahan, B. J. 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. - Microbiome 6: 226.

de Goffau, M. C., Lager, S., Salter, S. J., Wagner, J., Kronbichler, A., Charnock-Jones, D. S., Peacock, S. J., Smith, G. C. S. and Parkhill, J. 2018. Recognizing the reagent microbiome. - Nat. Microbiol. 3: 851–853.

Delhey, K., Peters, A. and Kempenaers, B. 2007. Cosmetic Coloration in Birds: Occurrence, Function, and Evolution. - Am. Nat. 169: S145–S158.

Dequiedt, S., Thioulouse, J., Jolivet, C., Saby, N. P. A., Lelievre, M., Maron, P.-A., Martin, M. P., Prévost-Bouré, N. C., Toutain, B., Arrouays, D., Lemanceau, P. and Ranjard, L. 2009. Biogeographical patterns of soil bacterial communities. - Environ. Microbiol. Rep. 1: 251–255.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P. and Andersen, G. L. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. - Appl. Environ. Microbiol. 72: 5069–5072.

Devaynes, A., Antunes, A., Bedford, A. and Ashton, P. 2018. Progression in the bacterial load during the breeding season in nest boxes occupied by the Blue Tit and its potential impact on hatching or fledging success. - J. Ornithol. 159: 1009–1017.

Díaz-Lora, S., Pérez-Contreras, T., Azcárate-García, M., Peralta-Sánchez, J. M., Martínez-Bueno, M., Soler, J. J. and Martín-Vivaldi, M. Cosmetic coloration of cross-fostered eggs affects male parental investment in the Hoopoe (Upupa epops). - Under Rev. in press.

Díaz-Lora, S., Pérez-Contreras, T., Azcárate-García, M., Martínez-Bueno, M., Soler, J. J. and Martín-Vivaldi, M. Hoopoe (Upupa epops) male feeding effort is related to female cosmetic egg colouration. - Under Rev. in press.

Díaz-Lora, S., Martín-Vivaldi, M., Juárez-García-Pelayo, N., Azcárate-García, M., Rodríguez-Ruano, S., M., Martínez-Bueno, M. and Soler, J. J. 2019. Data from:

experimental old nest material predicts hoopoe Upupa epops eggshell and uropygial gland microbiota. − Dryad Digital Repository, <http://dx.doi.org/10.5061/dryad.8574ft0>.

Dillon, R. J., Vennard, C. T., Buckling, A. and Charnley, A. K. 2005. Diversity of locust gut bacteria protects against pathogen invasion. - Ecol. Lett. 8: 1291–1298.

Douglas, A. E. 1998. Nutritional Interactions in Insect-Microbial Symbioses: Aphids and Their Symbiotic Bacteria Buchnera. - Annu. Rev. Entomol. 43: 17–37.

Dumbrell, A. J., Ferguson, R. M. W. and Clark, D. R. 2017. Microbial Community Analysis by Single-Amplicon High-Throughput Next Generation Sequencing: Data Analysis – From Raw Output to Ecology. - In: McGenity, T. J. et al. (eds), Springer. Hydrocarbon and Lipid Microbiology Protocols: Microbial Quantitation, Community Profiling and Array Approaches, pp. 155–206.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. - Bioinformatics 26: 2460–2461.

Edgar, R. C. 2017. UNCROSS: Filtering of high-frequency cross-talk in 16S amplicon reads. - PeerJ 5: e3889.

Edward, D. A. and Chapman, T. 2011. The evolution and significance of male mate choice. - Trends Ecol. Evol. 26: 647–654.

Eisenhofer, R., Minich, J. J., Marotz, C., Cooper, A., Knight, R. and Weyrich, L. S. 2019. Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. - Trends Microbiol. 27: 105–117.

Ellegaard, K. M. and Engel, P. 2016. Beyond 16S rRNA Community Profiling: Intra-Species Diversity in the Gut Microbiota . - Front. Microbiol. 7: 1475.

Endler, J. A. 1990. On the measurement and classification of color in studies of animal color patterns. - Biol. J. Linn. Soc. 41: 315–352.

Endler, J. A. and Mielke, P. W. 2005. Comparing color patterns as birds see them. - Biol. J. Linn. Soc. 86: 405–431.

English, P. A. and Montgomerie, R. 2011. Robin's egg blue: Does egg color influence male parental care? - Behav. Ecol. Sociobiol. 65: 1029–1036.

Erckmann, W., Beletsky, L., Orians, G., Johnsen, T., Sharbaugh, S. and D´Antonio, C. 1990. Old nests as cues for nest-site selection: an experimental test with Red-winged Blackbirds. - Condor 92: 113–117.

Ezenwa, V. O. and Williams, A. E. 2014. Microbes and animal olfactory communication:

Where do we go from here? - BioEssays 36: 847–854.

Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M. and Xavier, J. B. 2012. Animal Behavior and the Microbiome. - Science (80-.). 338: 198–199.

Fitzpatrick, C. L. and Servedio, M. R. 2018. The evolution of male mate choice and female ornamentation: A review of mathematical models. - Curr. Zool. 64: 323–333.

Flórez, L. V. and Kaltenpoth, M. 2017. Symbiont dynamics and strain diversity in the defensive mutualism between *Lagria* beetles and *Burkholderia*. - Environ. Microbiol. 19: 3674–3688.

Flórez, L. V, Biedermann, P. H. W., Engl, T. and Kaltenpoth, M. 2015. Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. - Nat. Prod. Rep. 32: 904–936.

Folstad, I. and Karter, A. J. 1992. Parasites, bright males, and the immunocompetence handicap. - Am. Nat. 139: 603–622.

Fons, M., Gomez, A. and Karjalainen, T. 2000. Mechanisms of Colonisation and Colonisation Resistance of the Digestive Tract Part 2: Bacteria/Bacteria Interactions. - Microb. Ecol. Health Dis. 12: 240–246.

Fontaine, J. J. and Martin, T. E. 2006. Parent birds assess nest predation risk and adjust their reproductive strategies. - Ecol. Lett. 9: 428–434.

Fox, H. M. and Vevers, G. 1960. The nature of animal colors.

Friedman, J. and Alm, E. J. 2012. Inferring Correlation Networks from Genomic Survey Data. - PLoS Comput. Biol. 8: e1002687.

Fronstin, R. B., Doucet, S. M. and Christians, J. K. 2016. Haematocrit, eggshell colouration and sexual signaling in the European starling (Sturnus vulgaris). - BMC Ecol. in press.

Gamble, K. C. 2007. Internal Anatomy of the Hornbill Casque Described by Radiography , Contrast Radiography , and Computed Tomography. - J. Avian Med. Surg. 21: 38–49.

Gamble, K. C. 2012. Chapter 37. Squamous Cell Carcinoma in Buceros Hornbills. - In: Miller, R. E. and Fowler, M. (eds), Fowler's Zoo and Wild Animal Medicine. pp. 281–285.

Garamszegi, Z. and Møller, A. P. 2010. Effects of sample size and intraspecific variation in phylogenetic comparative studies : a meta-analytic review. - Biol. Rev. 85: 797–805.

Garamszegi, L. Z. and Møller, A. P. 2011. Nonrandom Variation in Within-Species Sample Size and Missing Data in Phylogenetic Comparative Studies. - Syst. Biol. 60: 876–880.

Gaston, A. J., De Forest, L. N. and G.Noble, D. 1993. Egg Recognition and Egg Stealing in Murres (Uria spp.). - Anim. Behav. 45: 301–306.

Gawryszewski, F. M. 2018. Color vision models: Some simulations, a general n-dimensional model, and the colourvision R package. - Ecol. Evol.: 8159–8170.

Giard, J.-C., Laplace, J.-M., Rincé, A., Pichereau, V., Benachour, A., Leboeuf, C., Flahaut, S., Auffray, Y. and Hartke, A. 2001. The stress proteome of *Enterococcus faecalis*. - Electrophoresis 22: 2947–2954.

Gil-Turnes, M. S. and Fenical, W. 1992. Embryos of Homarus americanus are Protected by Epibiotic Bacteria. - Biol. Bull. 182: 105–108.

Giordano, M., Costantini, D., Pick, J. L. and Tschirren, B. 2015. Female oxidative status, egg antioxidant protection and eggshell pigmentation: a supplemental feeding experiment in great tits. - Behav. Ecol. Sociobiol. 69: 777–785.

Goldstein, G., Flory, K. R., Browne, B. A., Majid, S., Ichida, J. M. and Burtt Jr., E. H. 2004. Bacterial Degradation of Black and White Feathers. - Auk 121: 656–659.

Golob, J. L., Margolis, E., Hoffman, N. G. and Fredricks, D. N. 2017. Evaluating the accuracy of amplicon-based microbiome computational pipelines on simulated human gut microbial communities. - BMC Bioinformatics 18: 283.

Gómez, D. 2006. AVICOL a program to analyse spectrometric data. Last update october 2011. Free executable available at http://sites.google.com/site/avicolprogram/ or from the author at dodogomez@yahoo.fr. in press.

González-Braojos, S., Vela, A. I., Ruiz-De-Castañeda, R., Briones, V., Cantarero, A. and Moreno, J. 2012. Is Nestling Growth Affected by Nest Reuse and Skin Bacteria in Pied Flycatchers *Ficedula hypoleuca?* - Acta Ornithol. 47: 119–127.

Gonzalez, J. C. T., Sheldon, B. C., Collar, N. J. and Tobias, J. A. 2013. A comprehensive molecular phylogeny for the hornbills (Aves: Bucerotidae). - Mol. Phylogenet. Evol. 67: 468–483.

Gosler, A. G., Higham, J. P. and Reynolds, S. J. 2005. Why are birds ' eggs speckled ? - Ecol. Lett. 8: 1105–1113.

Grether, G. F., Hudon, J. and Millie, D. F. 1999. Carotenoid limitation of sexual coloration along an environmental gradient in guppies. - Proc. R. Soc. London. Ser. B

Biol. Sci. 266: 1317–1322.

Griggio, M., Valera, F., Casas, A. and Pilastro, A. 2005. Males prefer ornamented females: A field experiment of male choice in the rock sparrow. - Anim. Behav. 69: 1243–1250.

Grizard, S., Dini-Andreote, F., Tieleman, B. I. and Salles, J. F. 2014. Dynamics of bacterial and fungal communities associated with eggshells during incubation. - Ecol. Evol. 4: 1140–1157.

Grizard, S., Versteegh, M. A., Ndithia, H. K., Salles, J. F. and Tieleman, B. I. 2015. Shifts in Bacterial Communities of Eggshells and Antimicrobial Activities in Eggs during Incubation in a Ground-Nesting Passerine. - PLoS One: 10(4).

Gunderson, A. R. 2008. Feather-Degrading Bacteria: A New Frontier in Avian and Host–Parasite Research? - Auk 125: 972–979.

Gwinner, H. and Berger, S. 2005. European starlings: Nestling condition, parasites and green nest material during the breeding season. - J. Ornithol. 146: 365–371.

Haine, E. R. 2008. Symbiont-mediated protection. - Proc. R. Soc. B Biol. Sci. 275: 353–361.

Hamilton, W. D. and Zuk, M. 1982. Heritable true fitness and bright birds: a role for parasites? - Science (80-.). 218: 384–387.

Hanley, D. 2012. The function and evolution of egg colour in birds. - Electron. Theses Diss.: 382.

Hanley, D. and Doucet, S. M. 2009. Egg coloration in ring-billed gulls (Larus delawarensis): A test of the sexual signaling hypothesis. - Behav. Ecol. Sociobiol. 63: 719–729.

Hanley, D., Heiber, G. and Dearborn, D. C. 2008. Testing an Assumption of the Sexual-Signaling Hypothesis: Does Blue-Green Egg Color Reflect Maternal Antioxidant Capacity? - Condor 110: 767–771.

Hanley, D., Doucet, S. M. and Dearborn, D. C. 2010. A Blackmail Hypothesis for the Evolution of Conspicuous Egg Coloration in Birds. - Auk 127: 453–459.

Hargitai, R., Boross, N., Nyiri, Z. and Eke, Z. 2018a. Effects of food limitation on the intensity of blue-green and brown eggshell coloration: an experimental study with the canary. - J. Avian Biol.: e01486.

Hargitai, R., Herényi, M., Nagy, G. and Török, J. 2018b. Eggshell spotting pattern is related to hatching asynchrony, hematocrit value and growth of nestling great tits

Parus major. - J. Avian Biol. 49: e01827.

Hart, N. S. 2001. The visual ecology of avian Photoreceptors. - Prog. Retin. Eye Res. 20, No. 5: 675 to 703.

Hart, N. S. 2002. Vision in the peafowl (Aves: Pavo cristatus). - J. Exp. Biol. 205: 3925–3935.

Harvey, P. H. and Pagel, M. D. 1991. The Comparative Method in Evolutionary Biology. - Oxford University Press.

He, Y., Caporaso, J. G., Jiang, X.-T., Sheng, H.-F., Huse, S. M., Rideout, J. R., Edgar, R. C., Kopylova, E., Walters, W. A., Knight, R. and Zhou, H.-W. 2015. Stability of operational taxonomic units: an important but neglected property for analyzing microbial diversity. - Microbiome 3: 20.

Heath-Heckman, E. A. C., Peyer, S. M., Whistler, C. A., Apicella, M. A., Goldman, W. E. and McFall-Ngai, M. J. 2013. Bacterial Bioluminescence Regulates Expression of a Host Cryptochrome Gene in the Squid-Vibrio Symbiosis (J Handelsman, Ed.). - MBio 4: e00167-13.

Heath, K. D. and Tiffin, P. 2007. Context dependence in the coevolution of plant and rhizobial mutualists. - Proc. R. Soc. B Biol. Sci. 274: 1905–1912.

Henry, L. P., Bruijning, M., Forsberg, S. K. G. and Ayroles, J. F. 2019. Can the microbiome influence host evolutionary trajectories? - bioRxiv: 700237.

Hill, M. J. 1997. Intestinal flora and endogenous vitamin synthesis. - Eur. J. Cancer Prev. 6 Suppl 1: S43-45.

Hill, G. E. 2000. Energetic constraints on expression of carotenoid-based plumage coloration. - J. Avian Biol. 31: 559–566.

Hill, G. E. 2002. A red bird in a brown bag: the function and evolution of ornamental plumage coloration in the house finch. - Oxford University Press.

Hill, G. E. 2006. Environmental Regulation of Ornamental Coloration. - In: Hill, G. E. and Mcgraw, K. J. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 507–560.

Hill, G. E., Farmer, K. L. and Beck, M. L. 2004. The effect of mycoplasmosis on carotenoid plumage coloration in male house finches. - J. Exp. Biol. 207: 2095–2099.

Hird, S. M. 2017. Evolutionary Biology Needs Wild Microbiomes. - Front. Microbiol. 8: 725.

Hoffmann, J., Postma, E. and Schaub, M. 2015. Factors influencing double brooding in

Eurasian Hoopoes Upupa epops. - Ibis (Lond. 1859). 157: 17–30.

Holveck, M.-J., Doutrelant, C., Guerreiro, R., Perret, P., Gomez, D. and Grégoire, A. 2010. Can eggs in a cavity be a female secondary sexual signal? Male nest visits and modelling of egg visual discrimination in blue tits. - Biol. Lett. 6: 453–457.

Holveck, M., Guerreiro, R., Perret, P., Doutrelant, C. and Grégoire, A. 2019. Eggshell coloration indicates female condition during egg-laying: a field experiment in blue tits. - Biol. J. Linn. Soc. XX: 1–20.

Honza, M., Požgayová, M., Procházka, P. and Cherry, M. I. 2011. Blue-green eggshell coloration is not a sexually selected signal of female quality in an open-nesting polygynous passerine. - Naturwissenschaften 98: 493–499.

Hornung, B. V. H., Zwittink, R. D. and Kuijper, E. J. 2019. Issues and current standards of controls in microbiome research. - FEMS Microbiol. Ecol. 95: fiz045.

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y. and Fukatsu, T. 2010. Wolbachia as a bacteriocyte-associated nutritional mutualist. - Proc. Natl. Acad. Sci. 107: 769–774.

Hoyt, D. F. 1979. Practical methods of estimating volume and fresh weight of bird eggs. - Auk 96: 73–77.

Jacob, J. and Ziswiler, V. 1982. The uropygial gland. - In: Farner, D. S. et al. (eds), Avian biology, vol VI. Academic Press, pp. 359–362.

Jacob, S., Parthuisot, N., Vallat, A., Ramon-Portugal, F., Helfenstein, F. and Heeb, P. 2015. Microbiome affects egg carotenoid investment, nestling development and adult oxidative costs of reproduction in Great tits. - Funct. Ecol. 29: 1048–1058.

Janzen, D. H. 1985. The natural history of mutualism. - In: Boucher, D. H. (ed), The Biology of Mutualism: Ecology and Evolution. Oxford University Press, pp. 40–99.

Javůrková, V. G., Kreisinger, J., Procházka, P., Požgayová, M., Ševčíková, K., Brlík, V., Adamík, P., Heneberg, P. and Porkert, J. 2019. Unveiled feather microcosm: feather microbiota of passerine birds is closely associated with host species identity and bacteriocin-producing bacteria. - ISME J.: 2363–2376.

Jervis-Bardy, J., Leong, L. E. X., Marri, S., Smith, R. J., Choo, J. M., Smith-Vaughan, H. C., Nosworthy, E., Morris, P. S., O'Leary, S., Rogers, G. B. and Marsh, R. L. 2015. Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. - Microbiome 3: 19.

Jetz, W., Thomas, G. H., Joy, J. B., Hartmann, K. and Mooers, A. O. 2012. The global diversity of birds in space and time. - Nature 491: 444–448.

Johnsen, A., Vesterkjær, K. and Slagsvold, T. 2011. Do Male Pied Flycatchers (Ficedula hypoleuca) Adjust Their Feeding Effort According to Egg Colour? - Ethology 117: 309–317.

Johnstone, R. A. 1995. Sexual selection, honest advertisement and the handicap principle: reviewing the evidence. - Biol. Rev. 70: 1–65.

Kaltenpoth, M., Göttler, W., Herzner, G. and Strohm, E. 2005. Symbiotic Bacteria Protect Wasp Larvae from Fungal Infestation. - Curr. Biol. 15: 475–479.

Kaltz, O. and Koella, J. C. 2003. Host growth conditions regulate the plasticity of horizontal and vertical transmission in holospora undulata, a bacterial parasite of the protozoan paramecium caudatum. - Evolution (N. Y). 57: 1535–1542.

Karstens, L., Asquith, M., Davin, S., Fair, D., Gregory, W. T., Wolfe, A. J., Braun, J. and McWeeney, S. 2019. Controlling for Contaminants in Low-Biomass 16S rRNA Gene Sequencing Experiments (JA Gilbert, Ed.). - mSystems 4: e00290-19.

Kaur, H., Hughes, M. N., Green, C. J., Naughton, P., Foresti, R. and Motterlini, R. 2003. Interaction of bilirubin and biliverdin with reactive nitrogen species. - FEBS Lett. 543: 113–119.

Keebaugh, E. S., Yamada, R., Obadia, B., Ludington, W. B. and Ja, W. W. 2018. Microbial Quantity Impacts Drosophila Nutrition, Development, and Lifespan. - iScience 4: 247–259.

Kemp, A. C. 2001. Family Bucerotidae (Hornbills). - In: Del Hoyo, J. et al. (eds), Handbook of the birds of the world, Piciformes to Bucerotiformes, vol 6. Lynx Editi. pp. 436–523.

Kemp, D. J., Herberstein, M. E., Fleishman, L. J., Endler, J. a., Bennett, A. T. D., Dyer, A. G., Hart, N. S., Marshall, J. and Whiting, M. J. 2015. An Integrative Framework for the Appraisal of Coloration in Nature. - Am. Nat. 185: 000–000.

Kikuchi, Y., Hayatsu, M., Hosokawa, T., Nagayama, A., Tago, K. and Fukatsu, T. 2012. Symbiont-mediated insecticide resistance. - Proc. Natl. Acad. Sci. 109: 8618–8622.

Kilner, R. M. 2006. The evolution of egg colour and patterning in birds. - Biol. Rev. Camb. Philos. Soc. 81: 383–406.

Kinnaird, M. F. and O'Brien, T. G. 2007. The Ecology and Conservation of Asian Hornbills: Farmers of the Forest.

Knight, R., Vrbanac, A., Taylor, B. C., Aksenov, A., Callewaert, C., Debelius, J., Gonzalez, A., Kosciolek, T., McCall, L. I., McDonald, D., Melnik, A. V., Morton, J.

T., Navas, J., Quinn, R. A., Sanders, J. G., Swafford, A. D., Thompson, L. R., Tripathi, A., Xu, Z. Z., Zaneveld, J. R., Zhu, Q., Caporaso, J. G. and Dorrestein, P. C. 2018. Best practices for analysing microbiomes. - Nat. Rev. Microbiol. 16: 410–422.

Kopylova, E., Navas-molina, J. A., Mercier, C., Xu, Z., Mahé, F., He, Y., Zhou, H.-W., Rognes, T., Caporaso, J. G. and Knight, R. 2016. Open-Source Sequence Clustering Methods Improve the State Of the Art. mSystems. 1: 1–16.

Kose, M. and Møller, A. P. 1999. Sexual selection, feather breakage and parasites: the importance of white spots in the tail of the barn swallow (Hirundo rustica). - Behav. Ecol. Sociobiol. 45: 430–436.

Kraaijeveld, K., Kraaijeveld-Smit, F. J. L. and Komdeur, J. 2007. The evolution of mutual ornamentation. - Anim. Behav. 74: 657–677.

Krist, M. and Grim, T. 2007. Are blue eggs a sexually selected signal of female collared flycatchers? A cross-fostering experiment. - Behav. Ecol. Sociobiol. 61: 863–876.

Kuczynski, J., Stombaugh, J., Walters, W. A., González, A., Caporaso, J. G. and Knight, R. 2012. Using QIIME to analyze 16s rRNA gene sequences from microbial communities. - Curr. Protoc. Microbiol. in press.

Labocha, M. K. and Hayes, J. P. 2012. Morphometric indices of body condition in birds: A review. - J. Ornithol. 153: 1–22.

Lahti, D. C. 2008. Population Differentiation and Rapid Evolution of Egg Color in Accordance With Solar Radiation. - Auk 125: 796–802.

Larsson, A. J. M., Stanley, G., Sinha, R., Weissman, I. L. and Sandberg, R. 2018. Computational correction of index switching in multiplexed sequencing libraries. - Nat. Methods 15: 305–307.

Law-Brown, J. and Meyers, P. R. 2003. *Enterococcus phoeniculicola sp . nov .*, a novel member of the enterococci isolated from the uropygial gland of the Red-billed Woodhoopoe , *Phoeniculus purpureus.* - Int. J. Syst. Evol. Microbiol. 53: 683–685.

Leclaire, S., Nielsen, J. F. and Drea, C. M. 2014. Bacterial communities in meerkat anal scent secretions vary with host sex, age, and group membership. - Behav. Ecol. 25: 996–1004.

Leclaire, S., Jacob, S., Greene, L. K., Dubay, G. R. and Drea, C. M. 2017. Social odours covary with bacterial community in the anal secretions of wild meerkats. - Sci. Rep. 7: 3240.

Leftwich, P. T., Hutchings, M. I. and Chapman, T. 2018. Diet, Gut Microbes and Host

Mate Choice. - BioEssays 40: 1800053.

Leitner, S., Marshall, R. C., Leisler, B. and Catchpole, C. K. 2006. Male song quality, egg size and offspring sex in captive canaries (Serinus canaria). - Ethology 112: 554–563.

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D. and Gordon, J. I. 2005. Obesity alters gut microbial ecology. - Proc. Natl. Acad. Sci. 102: 11070–11075.

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R. and Gordon, J. I. 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. - Nat. Rev. Microbiol. 6: 776–788.

Li, D., Chen, B., Zhang, L., Gaur, U., Ma, T., Jie, H., Zhao, G., Wu, N., Xu, Z., Xu, H., Yao, Y., Lian, T., Fan, X., Yang, D., Yang, M., Zhu, Q. and Satkoski Trask, J. 2016. The musk chemical composition and microbiota of Chinese forest musk deer males. - Sci. Rep. 6: 18975.

Lindquist, N., Barber, P. H. and Weisz, J. B. 2005. Episymbiotic microbes as food and defence for marine isopods: unique symbioses in a hostile environment. - Proc. R. Soc. B Biol. Sci. 272: 1209–1216.

Linville, S. U., Breitwisch, R. and Schilling, A. J. 1998. Plumage brightness as an indicator of parental care in northern cardinals. - Anim. Behav. 55: 119–127.

Lopetuso, L. R., Scaldaferri, F., Petito, V. and Gasbarrini, A. 2013. Commensal Clostridia: leading players in the maintenance of gut homeostasis. - Gut Pathog. 5: 23.

López-Rull, I., Gil, M. and Gil, D. 2007. Spots in starling Sturnus unicolor eggs are good indicators of ectoparasite load by Carnus hemapterus (Diptera: Carnidae). - Ardeola 54: 131–134.

López-Rull, I., Pagán, I. and Macías Garcia, C. 2010. Cosmetic enhancement of signal coloration: Experimental evidence in the house finch. - Behav. Ecol. 21: 781–787.

Lozupone, C. and Knight, R. 2005. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. - Appl. Environ. Microbiol. 71: 8228–8235.

Lyu, N., Servedio, M. R., Lloyd, H. and Sun, Y. 2017. The evolution of postpairing male mate choice. - Evolution (N. Y). 71: 1465–1477.

Macpherson, A. J. and Harris, N. L. 2004. Interactions between commensal intestinal bacteria and the immune system. - Nat. Rev. Immunol. 4: 478–485.

Maddison, W. . and Maddison, D. R. 2018. Mesquite: a modular system for evolutionary

analysis. Version 3.6.: http://www.mesquiteproject.org.

Maier, R. M., Pepper, I. L. and Gerba, C. P. 2000. Environmental Microbiology. - Academic Press, San Diego, California.

Mainwaring, M. C., Hartley, I. R., Lambrechts, M. M. and Deeming, D. C. 2014. The design and function of birds' nests. - Ecol. Evol. 4: 3909–3928.

Mallows, C. L. 1973. Some Comments on Cp. - Technometrics 15: 661–675.

Maraci, Ö., Engel, K. and Caspers, B. A. 2018. Olfactory communication via microbiota: what is known in birds? - Genes (Basel). in press.

Martín-Platero, A. M., Valdivia, E., Ruíz-Rodríguez, M., Soler, J. J., Martín-Vivaldi, M., Maqueda, M. and Martínez-Bueno, M. 2006. Characterization of antimicrobial substances produced by *Enterococcus faecalis* MRR 10-3, isolated from the uropygial gland of the hoopoe (*Upupa epops*). - Appl. Environ. Microbiol. 72: 4245–4249.

Martín-Vivaldi, M., Palomino, J. J., Soler, M. and Soler, J. J. 1999. Determinants of reproductive success in the Hoopoe Upupa epops, a hole-nesting non-passerine bird with asynchronous hatching. - Bird Study 46: 205–216.

Martín-Vivaldi, M., Ruiz-Rodríguez, M., Soler, J. J., Peralta-Sánchez, J. M., Méndez, M., Valdivia, E., Martín-Platero, A. M. and Martínez-Bueno, M. 2009. Seasonal, sexual and developmental differences in hoopoe Upupa epops preen gland morphology and secretions: Evidence for a role of bacteria. - J. Avian Biol. 40: 191–205.

Martín-Vivaldi, M., Peña, A., Peralta-Sánchez, J. M., Sánchez, L., Ananou, S., Ruiz-Rodríguez, M. and Soler, J. J. 2010. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. - Proc. Biol. Sci. 277: 123–130.

Martín-Vivaldi, M., Soler, J. J., Peralta-Sánchez, J. M., Arco, L., Martín-Platero, A. M., Martínez-Bueno, M., Ruiz-Rodríguez, M. and Valdivia, E. 2014a. Special structures of hoopoe eggshells enhance the adhesion of symbiont-carrying uropygial secretion that increase hatching success. - J. Anim. Ecol. 83: 1289–1301.

Martín-Vivaldi, M., Doña, J., Romero Masegosa, J. and Soto Cárdenas, M. 2014b. Abubilla - Upupa epops Linnaeus, 1758. - En Encicl. Virtual los Vertebr. Españoles. Salvador. A., Morales, M. B. (Eds.). Mus. Nac. Ciencias Nat. Madrid. http//www.vertebradosibericos.org/

Martín-Vivaldi, M., Soler, J. J., Martínez-García, Á., Arco, L., Juárez-García-Pelayo, N., Ruiz-Rodríguez, M. and Martínez-Bueno, M. 2018. Acquisition of Uropygial Gland

Microbiome by Hoopoe Nestlings. - Microb. Ecol. 76: 285–297.

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. - EMBnet.journal 17: 10–12.

Martin, T. E. and Badyaev, A. V 1996. Sexual dichromatism in birds: importance of nest predation and nest location for females versus males. - Evolution (N. Y). 50: 2454–2460.

Martínez-De La Puente, J., Merino, S., Moreno, J., Tomás, G., Morales, J., Lobato, E., García-Fraile, S. and Martínez, J. 2007. Are eggshell spottiness and colour indicators of health and condition in blue tits Cyanistes caeruleus? - J. Avian Biol. 38: 377–384.

Martínez-García, Á. 2015. Biodiversidad bacteriana de la secreción uropigial y la cáscara de los huevos de abubilla: estabilidad y adquisición.

Martínez-García, Á., Soler, J. J., Rodríguez-Ruano, S. M., Martínez-Bueno, M., Martín-Platero, A. M., Juárez-García, N. and Martín-Vivaldi, M. 2015. Preening as a Vehicle for Key Bacteria in Hoopoes. - Microb. Ecol. 70: 1024–1033.

Martínez-García, Á., Martín-Vivaldi, M., Rodríguez-Ruano, S. M., Peralta-Sánchez, J. M., Valdivia, E. and Soler, J. J. 2016a. Nest bacterial environment affects microbiome of hoopoe eggshells, but not that of the uropygial secretion. - PLoS One 11(7): e0158158.

Martínez-García, Á., Martín-Vivaldi, M., Ruiz-Rodríguez, M., Martínez-Bueno, M., Arco, L., Rodríguez-Ruano, S. M., Peralta-Sánchez, J. M. and Soler, J. J. 2016b. The Microbiome of the Uropygial Secretion in Hoopoes Is Shaped Along the Nesting Phase. - Microb. Ecol. 72: 252–261.

Massaro, M., Davis, L. S. and Darby, J. T. 2003. Carotenoid-derived ornaments reflect parental quality in male and female yellow-eyed penguins (Megadyptes antipodes). - Behav. Ecol. Sociobiol. 55: 169–175.

Mazgajski, T. D. 2007. Effect of old nest material on nest site selection and breeding parameters in secondary hole nesters — a review. - Acta Ornithol. 42: 1–14.

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., Desantis, T. Z., Probst, A., Andersen, G. L., Knight, R. and Hugenholtz, P. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. - ISME J. 6: 610–618.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U.,

King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E., Rawls, J. F., Reid, A., Ruby, E. G., Rumpho, M., Sanders, J. G., Tautz, D. and Wernegreen, J. J. 2013. Animals in a bacterial world, a new imperative for the life sciences. - Proc. Natl. Acad. Sci. U. S. A. 110: 3229–3236.

Mcgraw, K. J. and Hill, G. E. 2004. Plumage color as a dynamic trait : carotenoid pigmentation of male house finches (Carpodacus mexicanus) fades during the breeding season. - Can. J. Zool 82: 734–738.

McGraw, K. J. and Ardia, D. R. 2003. Carotenoids, Immunocompetence, and the Information Content of Sexual Colors: An Experimental Test. - Am. Nat. 162: 704–712.

McKnight, D. T., Huerlimann, R., Bower, D. S., Schwarzkopf, L., Alford, R. A. and Zenger, K. R. 2019. microDecon: A highly accurate read-subtraction tool for the post-sequencing removal of contamination in metabarcoding studies. - Environ. DNA 1: 14–25.

Mikšík, I., Holáň, V. and Deyl, Z. 1996. Avian eggshell pigments and their variability. - Comp. Biochem. Physiol. 113: 607–612.

Miller, R. E., Trampel, D. W., Boever, W. J. and Kling, M. A. 1985. Carcinoma in the Casque of a Greater Indian Hornbill (Buceros bicornis). - J. Zoo Anim. Med. 16: 131–136.

Minich, J. J., Zhu, Q., Janssen, S., Hendrickson, R., Amir, A., Vetter, R., Hyde, J., Doty, M. M., Stillwell, K., Benardini, J., Kim, J. H., Allen, E. E., Venkateswaran, K. and Knight, R. 2018. KatharoSeq Enables High-Throughput Microbiome Analysis from Low-Biomass Samples (MJ McFall-Ngai, Ed.). - mSystems 3: e00218-17.

Møller, A. P. 1993. Sexual Selection in the Barn Swallow Hirundo rustica. III. Female Tail Ornaments. - Evolution (N. Y). 47: 417–431.

Møller, A. P. and Erritzoe, J. 1996. Parasite Virulence and Host Immune Defense : Host Immune Response is Related to Nest Reuse in Birds. - Evolution (N. Y). 50: 2066–2072.

Møller, A. P., Arriero, E., Lobato, E. and Merino, S. 2009. A meta-analysis of parasite virulence in nestling birds. - Biol. Rev. 84: 567–588.

Montgomerie, R. 2006. Cosmetic and adventitious colors. - In: Hill, E. and McGraw, K. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 399–427.

Morales, J., Sanz, J. J. and Moreno, J. 2006. Egg colour reflects the amount of yolk maternal antibodies and fledging success in a songbird. - Biol. Lett. 2: 334–336.

Morales, J., Moreno, J., Merino, S., Sanz, J. J., Tomás, G., Arriero, E., Lobato, E. and Martínez-De La Puente, J. 2007. Female ornaments in the Pied Flycatcher Ficedula hypoleuca: Associations with age, health and reproductive success. - Ibis (Lond. 1859). 149: 245–254.

Morales, J., Velando, A. and Moreno, J. 2008. Pigment allocation to eggs decreases plasma antioxidants in a songbird. - Behav. Ecol. Sociobiol. 63: 227–233.

Moran, N. A. 2006. Symbiosis. - Curr. Biol. 16: R866–R871.

Moran, N. A., McCutcheon, J. P. and Nakabachi, A. 2008. Genomics and Evolution of Heritable Bacterial Symbionts. - Annu. Rev. Genet. 42: 165–190.

Moreau, R. E. 1934. Breeding Habits of Hornbills. - Nature 134: 899.

Moreno, J. and Osorno, J. L. 2003. Avian egg colour and sexual selection: Does eggshell pigmentation reflect female condition and genetic quality? - Ecol. Lett. 6: 803–806.

Moreno, J., Briones, V., Merino, S., Ballesteros, C., Sanz, J. J. and Tomás, G. 2003. Beneficial Effects of Cloacal Bacteria on Growth and Fledging Size in Nestling Pied Flycatchers (Ficedula Hypoleuca) in Spain. - Auk 120: 784–790.

Moreno, J., Osorno, J. L., Morales, J., Merino, S. and Tomás, G. 2004. Egg Colouration and Male Parental Effort in the Pied Flycatcher Ficedula Hypoleuca. - J. Avian Biol. 35: 300–304.

Moreno, J., Morales, J., Lobato, E., Merino, S., Tomás, G. and Martínez-De La Puente, J. 2005. Evidence for the signaling function of egg color in the pied flycatcher Ficedula hypoleuca. - Behav. Ecol. 16: 931–937.

Moreno, J., Morales, J., Lobato, E., Merino, S., Tomás, G. and Martínez-De La Puente, J. 2006a. More colourful eggs induce a higher relative paternal investment in the pied flycatcher Ficedula hypoleuca: A cross-fostering experiment. - J. Avian Biol. 37: 555–560.

Moreno, J., Lobato, E., Morales, J., Merino, S., Tomás, G., Martínez-De La Puente, J., Sanz, J. J., Mateo, R. and Soler, J. J. 2006b. Experimental evidence that egg color indicates female condition at laying in a songbird. - Behav. Ecol. 17: 651–655.

Moreno, J., Lobato, E., Merino, S. and Martínez-De La Puente, J. 2008. Blue-green eggs in pied flycatchers: An experimental demonstration that a supernormal stimulus elicits improved nestling condition. - Ethology 114: 1078–1083.

Morton, J. T., Sanders, J., Quinn, R. A., McDonald, D., Gonzalez, A., Vázquez-Baeza, Y., Navas-Molina, J. A., Song, S. J., Metcalf, J. L., Hyde, E. R., Lladser, M., Dorrestein, P. C. and Knight, R. 2017. Balance Trees Reveal Microbial Niche Differentiation (JK Jansson, Ed.). - mSystems 2: e00162-16.

Mushegian, A. A., Walser, J.-C., Sullam, K. E. and Ebert, D. 2018. The microbiota of diapause: How host–microbe associations are formed after dormancy in an aquatic crustacean. - J. Anim. Ecol. 87: 400–413.

Naish, D. 2015. The Ecology and Conservation of Asian Hornbills: Farmers of the Forest. - Hist. Biol. 27: 954–956.

Navas-Molina, J. A., Peralta-Sánchez, J. M., González, A., McMurdie, P. J., Vázquez-Baeza, Y., Xu, Z., Ursell, L. K., Lauber, C., Zhou, H., Song, S. J., Huntley, J., Ackermann, G. L., Berg-Lyons, D., Holmes, S., Caporaso, J. G. and Knight, R. 2013. Advancing our understanding of the human microbiome using QIIME. - Elsevier Inc.

Negro, J. J., Margalida, A., Hiraldo, F. and Heredia, R. 1999. The function of the cosmetic coloration of bearded vultures : when art imitates life. - Anim. Behav. S8: 14–17.

Nicholls, E. M. and Rienits, K. G. 1971. Tryptophan derivatives and pigment in the hair of some Australian marsupials. - Int. J. Biochem. 2: 593–603.

Ödeen, A. and Håstad, O. 2003. Complex distribution of avian color vision systems revealed by sequencing the SWS1 opsin from total DNA. - Mol. Biol. Evol. 20: 855–861.

Oliver, K. M., Russell, J. A., Moran, N. A. and Hunter, M. S. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. - Proc. Natl. Acad. Sci. 100: 1803 LP – 1807.

Olson, V. A. and Owens, I. P. F. 1998. Costly sexual signals: are carotenoids rare, risky or required? - Trends Ecol. Evol. 13: 510–514.

Olsson, K. and Allander, K. 1995. Do Fleas, and/or Old Nest Material, Influence Nest-site preference in Hole-nesting Passerines? - Ethology 101: 160–170.

Olsson, P., Lind, O. and Kelber, A. 2015. Bird colour vision: behavioural thresholds reveal receptor noise. - J. Exp. Biol. 218: 184–193.

Orell, M., Rytkönen, S. and Ilomäki, K. 1993. Do pied flycatchers prefer nest boxes with old nest material? - Ann. Zool. Fennici 30: 313–316.

Pagel, M. 1997. Inferring evolutionary processes from phylogenies. - Zool. Scr. 26: 331–348.

Pagel, M. 1999. Inferring the historical patterns of biological evolution. - Nature 401: 877–884.

Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A. and Brown, P. O. 2007. Development of the Human Infant Intestinal Microbiota. - PLoS Biol. 5: e177.

Paradis, E., Claude, J. and Strimmer, K. 2004. APE : Analyses of Phylogenetics and Evolution in R language. - Bioinformatics 20: 289–290.

Paz, L.-C., Schramm, A. and Lund, M. B. 2017. Biparental transmission of Verminephrobacter symbionts in the earthworm Aporrectodea tuberculata (Lumbricidae). - FEMS Microbiol. Ecol. 93: fix025.

Pearce, D. S., Hoover, B. A., Jennings, S., Nevitt, G. A. and Docherty, K. M. 2017. Morphological and genetic factors shape the microbiome of a seabird species (Oceanodroma leucorhoa) more than environmental and social factors. - Microbiome 5: 146.

Peig, J. and Green, A. J. 2010. The paradigm of body condition: A critical reappraisal of current methods based on mass and length. - Funct. Ecol. 24: 1323–1332.

Peralta-Sanchez, J. M., Møller, A. P., Martin-Platero, A. M. and Soler, J. J. 2010. Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: An experimental study. - Funct. Ecol. 24: 426–433.

Peralta-Sánchez, J. M., Martín-Vivaldi, M., Martín-Platero, A. M., Martínez-Bueno, M., Oñate, M., Ruiz-Rodríguez, M. and Soler, J. J. 2012. Avian life history traits influence eggshell bacterial loads: A comparative analysis. - Ibis (Lond. 1859). 154: 725–737.

Peralta-Sánchez, J. M., Soler, J. J., Martín-Platero, A. M., Knight, R., Martínez-Bueno, M. and Møller, A. P. 2014. Eggshell Bacterial Load Is Related to Antimicrobial Properties of Feathers Lining Barn Swallow Nests. - Microb. Ecol. 67: 480–487.

Pérez-Rodríguez, L., Mougeot, F. and Bortolotti, G. R. 2011. The effects of preen oils and soiling on the UV – visible reflectance of carotenoid-pigmented feathers. - Behav. Ecol. Sociobiol. 65: 1425–1435.

Piault, R., Gasparini, J., Bize, P., Paulet, M., McGraw, K. J. and Roulin, A. 2008. Experimental support for the makeup hypothesis in nestling tawny owls (Strix aluco). - Behav. Ecol. 19: 703–709.

Plard, F., Arlettaz, R. and Schaub, M. 2018. Hoopoe males experience intra-seasonal while females experience inter-seasonal reproductive costs. - Oecologia 186: 665–

675.

Podofillini, S., Cecere, J. G., Griggio, M., Curcio, A., De Capua, E. L., Fulco, E., Pirrello, S., Saino, N., Serra, L., Visceglia, M. and Rubolini, D. 2018. Home, dirty home: effect of old nest material on nest-site selection and breeding performance in a cavity-nesting raptor. - Curr. Zool.: 1–10.

Poláček, M., Griggio, M., Mikšík, I., Bartíková, M., Eckenfellner, M. and Hoi, H. 2017. Eggshell coloration and its importance in postmating sexual selection. - Ecol. Evol. 7: 941–949.

Poonswad, P., Kemp, A. and Strange, M. 2013. Hornbills of the world: A photographic guide.

Price, M. N., Dehal, P. S. and Arkin, A. P. 2009. Fasttree: Computing large minimum evolution trees with profiles instead of a distance matrix. - Mol. Biol. Evol. 26: 1641–1650.

Prum, R. O. 2006. Anatomy, Physics, and Evolution of Structural Colors. - In: Hill, G. E. and Mcgraw, K. J. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 295–353.

Refsnider, J. M. and Janzen, F. J. 2010. Putting Eggs in One Basket: Ecological and Evolutionary Hypotheses for Variation in Oviposition-Site Choice. - Annu. Rev. Ecol. Evol. Syst. 41: 39–57.

Reichlin, T. S., Hobson, K. A., Van Wilgenburg, S. L., Schaub, M., Wassenaar, L. I., Martín-Vivaldi, M., Arlettaz, R. and Jenni, L. 2013. Conservation through connectivity: Can isotopic gradients in Africa reveal winter quarters of a migratory bird? - Oecologia 171: 591–600.

Renoult, J. P., Kelber, A. and Schaefer, H. M. 2017. Colour spaces in ecology and evolutionary biology. - Biol. Rev. 92: 292–315.

Reynolds, S. J., Martin, G. R. and Cassey, P. 2009. Is sexual selection blurring the functional significance of eggshell coloration hypotheses? - Anim. Behav. 78: 209–215.

Richard, F.-J. 2017. Symbiotic Bacteria Influence the Odor and Mating Preference of Their Hosts. - Front. Ecol. Evol. 5: 1–9.

Rideout, J. R., He, Y., Navas-Molina, J. A., Walters, W. A., Ursell, L. K., Gibbons, S. M., Chase, J., McDonald, D., Gonzalez, A., Robbins-Pianka, A., Clemente, J. C., Gilbert, J. A., Huse, S. M., Zhou, H.-W., Knight, R. and Caporaso, J. G. 2014. Subsampled

open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. - PeerJ 2: e545.

Riehl, C. 2011. Paternal Investment and the "Sexually Selected Hypothesis" For the Evolution of Eggshell Coloration: Revisiting the Assumptions. - Auk 128: 175–179.

Riley, M. A. and Wertz, J. E. 2002. Bacteriocins: Evolution, Ecology, and Application. - Annu. Rev. Microbiol. 56: 117–137.

Rizzo, C. and Lo Giudice, A. 2018. Marine Invertebrates: Underexplored Sources of Bacteria Producing Biologically Active Molecules. - Diversity 10: 52.

Rodríguez-Ruano, S. M. 2015. Diversidad bacteriana en la glándula uropigial de la abubilla: dinámica estacional y beneficios asociados.

Rodríguez-Ruano, S. M., Martín-Vivaldi, M., Martín-Platero, A. M., López-López, J. P., Peralta-Sánchez, J. M., Ruiz-Rodríguez, M., Soler, J. J., Valdivia, E. and Martínez-Bueno, M. 2015. The hoopoe's uropygial gland hosts a bacterial community influenced by the living conditions of the bird. - PLoS One 10: 1–18.

Rodríguez-Ruano, S. M., Martín-Vivaldi, M., Peralta-Sánchez, J. M., García-Martín, A. B., Martínez-García, Á., Soler, J. J., Valdivia, E. and Martínez-Bueno, M. 2018. Seasonal and Sexual Differences in the Microbiota of the Hoopoe Uropygial Secretion. - Genes (Basel). 9: 407.

Rognes, T., Flouri, T., Nichols, B., Quince, C. and Mahé, F. 2016. VSEARCH: a versatile open source tool for metagenomics. - PeerJ 4: e2584.

Rolig, A. S., Parthasarathy, R., Burns, A. R., Bohannan, B. J. M. and Guillemin, K. 2015. Individual Members of the Microbiota Disproportionately Modulate Host Innate Immune Responses. - Cell Host Microbe 18: 613–620.

Rollins-Smith, L. A., Ramsey, J. P., Pask, J. D., Reinert, L. K. and Woodhams, D. C. 2011. Amphibian Immune Defenses against Chytridiomycosis: Impacts of Changing Environments. - Integr. Comp. Biol. 51: 552–562.

Rosvall, K. A. 2011. Intrasexual competition in females: evidence for sexual selection? - Behav. Ecol. 22: 1131–1140.

Rubolini, D., Liker, A., Garamszegi, L. Z., Møller, A. P. and Saino, N. 2015. Using the birdtree.org website to obtain robust phylogenies for avian comparative studies: A primer. - Curr. Zool. 61: 959–965.

Ruiz-Castellano, C., Tomás, G., Ruiz-Rodríguez, M., Martín-Gálvez, D. and Soler, J. J. 2016. Nest material shapes eggs bacterial environment. - PLoS One 11: e0148894.

Ruiz-de-Castañeda, R., Burtt Jr, E. H., González-Braojos, S. and Moreno, J. 2012. Bacterial degradability of an intrafeather unmelanized ornament: a role for feather-degrading bacteria in sexual selection? - Biol. J. Linn. Soc. 105: 409–419.

Ruiz-De-Castañeda, R., Burtt, E. H., González-Braojos, S. and Moreno, J. 2015. Bacterial degradability of white patches on primary feathers is associated with breeding date and parental effort in a migratory bird. - Ibis (Lond. 1859). 157: 871–876.

Ruiz-Rodríguez, M., Azcárate-García, M., Ruiz-Castellano, C., Tomás, G., Díaz-Lora, S., Pérez-Contreras, T. and Soler, J. J. Antimicrobial capacity is related to body colouration and reproductive success in female spotless starlings. - Under Rev. in press.

Ruiz-Rodríguez, M., Valdivia, E., Soler, J. J., Martín-Vivaldi, M., Martín-Platero, A. M. and Martínez-Bueno, M. 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. - J. Exp. Biol. 212: 3621–6.

Ruiz-Rodríguez, M., Valdivia, E., Martín-Vivaldi, M., Martín-Platero, A. M., Martínez-Bueno, M., Méndez, M., Peralta-Sánchez, J. M. and Soler, J. J. 2012. Antimicrobial activity and genetic profile of *enteroccoci* isolated from hoopoes uropygial gland. - PLoS One 7: 1–6.

Ruiz-Rodríguez, M., Martínez-Bueno, M., Martín-Vivaldi, M., Valdivia, E. and Soler, J. J. 2013. Bacteriocins with a broader antimicrobial spectrum prevail in *enterococcal* symbionts isolated from the hoopoe's uropygial gland. - FEMS Microbiol. Ecol. 85: 495–502.

Ruiz-Rodríguez, M., Soler, J. J., Martín-Vivaldi, M., Martín-Platero, A. M., Méndez, M., Peralta-Sánchez, J. M., Ananou, S., Valdivia, E. and Martínez-Buenob, M. 2014. Environmental factors shape the community of symbionts in the hoopoe uropygial gland more than genetic factors. - Appl. Environ. Microbiol. 80: 6714–6723.

Ruiz-Rodríguez, M., Tomás, G., Martín-Gálvez, D., Ruiz-Castellano, C. and Soler, J. J. 2015. Bacteria and the evolution of honest signals. The case of ornamental throat feathers in spotless starlings. - Funct. Ecol. 29: 701–709.

Ruiz-Rodríguez, M., Møller, A. P., Mousseau, T. A. and Soler, J. J. 2016. Defenses against keratinolytic bacteria in birds living in radioactively contaminated areas. - Sci. Nat. 103: 71.

Sachs, J. L., Skophammer, R. G. and Regus, J. U. 2011. Evolutionary transitions in bacterial symbiosis. - Proc. Natl. Acad. Sci. 108: 10800 LP – 10807.

Saino, N., Romano, M., Rubolini, D., Ambrosini, R., Caprioli, M., Milzani, A., Costanzo, A., Colombo, G., Canova, L. and Wakamatsu, K. 2013. Viability Is Associated with Melanin-Based Coloration in the Barn Swallow (Hirundo rustica). - PLoS One 8: e60426.

Sanz, J. J. and García-Navas, V. 2009. Eggshell pigmentation pattern in relation to breeding performance of blue tits Cyanistes caeruleus. - J. Anim. Ecol. 78: 31–41.

Scott, J. J., Oh, D.-C., Yuceer, M. C., Klepzig, K. D., Clardy, J. and Currie, C. R. 2008. Bacterial Protection of Beetle-Fungus Mutualism. - Science (80-.). 322: 63.

Searcy, W. A. and Nowicki, S. 2005. The evolution of animal communication. - Princeton Univ. Press, Princeton, NJ.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S. and Huttenhower, C. 2011. Metagenomic biomarker discovery and explanation. - Genome Biol. 12: R60.

Senar, J. C. and Pascual, J. 1997. Tarsus lenght predictor body size. - Ardea 85: 269–274.

Sender, R., Fuchs, S. and Milo, R. 2016. Revised Estimates for the Number of Human and Bacteria Cells in the Body. - PLOS Biol. 14: e1002533.

Sergio, F. and Newton, I. 2003. Occupancy as a measure of habitat quality. - J Anim Ecol. 72: 857–865.

Shapira, M. 2016. Gut Microbiotas and Host Evolution: Scaling Up Symbiosis. - Trends Ecol. Evol. 31: 539–549.

Sharon, G., Segal, D., M.Ringo, H., Hefetz, A., Zilber-Rosenberg, I. and Rosenberg, E. 2010. Commensal bacteria play a role in mating preference of Drosophila melanogaster. - PNAS 107: 20051–20056.

Shawkey, M. D., Pillai, S. R. and Hill, G. E. 2003. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. - J. Avian Biol. 34: 345–349.

Shawkey, M. D., Pillai, S. R. and Hill, G. E. 2009. Do feather-degrading bacteria affect sexually selected plumage color? - Naturwissenschaften 96: 123–128.

Sheldon, B. C. 2000. Differential allocation: Tests, mechanisms and implications. - Trends Ecol. Evol. 15: 397–402.

Shephard, K. L. 1994. Functions for fish mucus. - Rev. Fish Biol. Fish. 4: 401–429.

Sherwin, E., Bordenstein, S. R., Quinn, J. L., Dinan, T. G. and Cryan, J. F. 2019. Microbiota and the social brain. - Science (80-.). 366: eaar2016.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. and Ishikawa, H. 2000. Genome

sequence of the endocellular bacterial symbiont of aphids Buchnera sp. APS. - Nature 407: 81–86.

Siefferman, L., Navara, K. J. and Hill, G. E. 2006. Egg coloration is correlated with female condition in eastern bluebirds (Sialia sialis). - Behav. Ecol. Sociobiol. 59: 651–656.

Singleton, D. R. and Harper, R. G. 1996. Bacteria in Old House Wren nests. - J F. Ornithol 69: 71–74.

Smith, H. G. and Montgomerie, R. 1991. Nestling American robins compete with siblings by begging. - Behav. Ecol. Sociobiol. 29: 307–312.

Soini, H. A., Whittaker, D. J., Wiesler, D., Ketterson, E. D. and Novotny, M. V. 2013. Chemosignaling diversity in songbirds: Chromatographic profiling of preen oil volatiles in different species. - J. Chromatogr. A 1317: 186–192.

Soler, J. J. and Møller, A. P. 1996. A comparative analysis of the evolution of variation in appearance of eggs of European passerines in relation to brood parasitism. - Behav. Ecol. 7: 89–94.

Soler, J. J., Moreno, J., Aviles, J. M. and Møller, A. P. 2005. Blue and green egg-color intensity is associated with parental effort and mating system in passerines: support for the sexual selection hypothesis. - Evolution (N. Y). 59: 636–644.

Soler, J. J., Martín-Vivaldi, M., Ruiz-Rodríguez, M., Valdivia, E., Martín-Platero, A. M., Martínez-Bueno, M., Peralta-Sánchez, J. M. and Méndez, M. 2008a. Symbiotic association between hoopoes and antibiotic-producing bacteria that live in their uropygial gland. - Funct. Ecol. 22: 864–871.

Soler, J. J., Navarro, C., Contreras, T. P., Avilés, J. M. and Cuervo, J. J. 2008b. Sexually Selected Egg Coloration in Spotless Starlings. - Am. Nat. 171: 183–194.

Soler, J. J., Martín-vivaldi, M., Peralta-sánchez, J. M. and Ruiz-rodríguez, M. 2010. Antibiotic-Producing Bacteria as a Possible Defence of Birds against Pathogenic Microorganisms. - Open Ornithol. J. 3: 93–100.

Soler, J. J., Martín-Vivaldi, M., Peralta-Sánchez, J. M., Arco, L. and Juárez-García-Pelayo, N. 2014. Hoopoes color their eggs with antimicrobial uropygial secretions. - Naturwissenschaften 101: 697–705.

Soler, J. J., Martínez-García, Á., Rodríguez-Ruano, S. M., Martínez-Bueno, M., Martín-Platero, A. M., Peralta-Sánchez, J. M. and Martín-Vivaldi, M. 2016. Nestedness of hoopoes' bacterial communities: symbionts from the uropygial gland to the eggshell.

- Biol. J. Linn. Soc. 118: 763–773.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martín-Vivaldi, M., Martínez-de la Puente, J., Ruiz-Rodríguez, M. and Tomás, G. 2017. Telomere length and dynamics of spotless starling nestlings depend on nest-building materials used by parents. - Anim. Behav. 126: 89–100.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martínez-de la Puente, J., Ruiz-Rodríguez, M. and Tomás, G. 2018. Egg colouration predicts brood size, telomere length and body condition of spotless starling fledglings. - J. Avian Biol. 49: 1–12.

Soler, J. J., Morales, J., Cuervo, J. J. and Moreno, J. 2019. Conspicuousness of passerine females is associated with the nest-building behaviour of males. - Biol. J. Linn. Soc. 126: 824–835.

Solís, J. C. and de Lope, F. 1995. Nest and Egg Crypsis in the Ground-Nesting Stone Curlew Burhinus oedicnemus. - J. Avian Biol. 26: 135–138.

Spicer, M. E., Pruitt, J. N. and Keiser, C. N. 2019. Spiders, microbes and sex: Bacterial exposure on copulatory organs alters mating behaviour in funnel-web spiders. - Ethology 125: 677–685.

Spor, A., Koren, O. and Ley, R. 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. - Nat. Rev. Microbiol. 9: 279–290.

Stanback, M. T. and Rockwell, E. K. 2003. Nest-site fidelity in Eastern Bluebirds (Sialia sialis) depends on the quality of alternative cavities. - Auk 120: 1029–1032.

Statsoft, I. 2006. STATISTICA (data analysis software system), version 7. Available at www.statsoft.com.

Steinert, M., Hentschel, U. and Hacker, J. 2000. Symbiosis and pathogenesis: evolution of the microbe-host interaction. - Naturwissenschaften 87: 1—11.

Stoddard, M. C. and Prum, R. O. 2008. Evolution of avian plumage color in a tetrahedral colour space: a phylogenetic analysis of New World buntings. - Am. Nat. 171: 755–776.

Stoddard, M. C. and Osorio, D. 2019. Animal Coloration Patterns: Linking Spatial Vision to Quantitative Analysis. - Am. Nat. 193: 000–000.

Stoddard, M. C., Fayet, A. L., Kilner, R. M. and Hinde, C. A. 2012. Egg speckling patterns do not advertise offspring quality or influence male provisioning in great tits. - PLoS One 7: e40211.

Suedmeyer, W. K., McCaw, D. and Turnquist, S. 2001. Attempted Photodynamic Therapy

of Squamous Cell Carcinoma in the Casque of a Great Hornbill (Buceros bicornis). - J. Avian Med. Surg. 15: 44–49.

Sumasgutner, P., Vasko, V., Varjonen, R. and Korpimäki, E. 2014. Public information revealed by pellets in nest sites is more important than ecto-parasite avoidance in the settlement decisions of Eurasian kestrels. - Behav. Ecol. Sociobiol. 68: 2023–2034.

Surmacki, A. and Nowakowski, J. K. 2007. Soil and preen waxes influence the expression of carotenoid-based plumage coloration. - Naturwissenschaften 94: 829–835.

Suzuki, T. A. 2017. Links between Natural Variation in the Microbiome and Host Fitness in Wild Mammals. - Integr. Comp. Biol. 57: 756–769.

Swynnerton, C. F. M. 1916. On the coloration of the mouths and eggs of birds. II. On the coloration of eggs. - Ibis (Lond. 1859). 4: 529–606.

Swynnerton, C. F. M. 1918. Rejections by birds of eggs unlike their own: with remarks on some of the cuckoo problems. - Ibis (Lond. 1859).: 127–154.

Team, R. D. C. 2019. R: A Language and Environment for Statistical Computing (RDC Team, Ed.). - R Found. Stat. Comput. in press.

Theis, K. R., Venkataraman, A., Dycus, J. A., Koonter, K. D., Schmitt-Matzen, E. N., Wagner, A. P., Holekamp, K. E. and Schmidt, T. M. 2013. Symbiotic bacteria appear to mediate hyena social odors. - Proc. Natl. Acad. Sci. 110: 19832–19837.

Tomás, G., Merino, S., Moreno, J., Sanz, J. J., Morales, J. and García-Fraile, S. 2006. Nest Weight and Female Health in the Blue Tit (Cyanistes Caeruleus). - Auk 123: 1013–1021.

Tomás, G., Merino, S., Moreno, J. and Morales, J. 2007. Consequences of nest reuse for parasite burden and female health and condition in blue tits, Cyanistes caeruleus. - Anim. Behav. 73: 805–814.

Tomás, G., Martín-Gálvez, D., Ruiz-Castellano, C., Ruiz-Rodríguez, M., Peralta-Sánchez, J. M., Martín-Vivaldi, M. and Soler, J. J. 2018. Ectoparasite Activity During Incubation Increases Microbial Growth on Avian Eggs. - Microb. Ecol. 76: 555–564.

Trail, P. W. 2007. African hornbills: keystone species threatened by habitat loss, hunting and international trade. - Ostrich 78: 609–613.

Trivers, R. L. 1972. Parental investment and sexual selection. - In: Campbell, B. (ed), Sexual selection and the descent of man 1871-1971. pp. 136–179.

Tschumi, M., Schaub, M. and Arlettaz, R. 2014. Territory occupancy and parental quality as proxies for spatial prioritization of conservation areas. - PLoS One 9: e97679.

Uchida, Y. 1970. On the color change in Japanese crested ibis. A new type of cosmetic coloration in birds. - Misc Rep Yamashina Inst Ornithol 6: 54–72. In Japanese, with English summary.

Umesaki, Y., Setoyama, H., Matsumoto, S., Imaoka, A. and Itoh, K. 1999. Differential Roles of Segmented Filamentous Bacteria and Clostridia in Development of the Intestinal Immune System. - Infect. Immun. 67: 3504 LP – 3511.

Underwood, T. and Sealy, S. 2002. Adaptive significance of egg coloration. - In: Deeming DC (ed), Avian incubation: behaviour, environment, and evolution. Oxford Uni. pp. 280–298.

van Dongen, W. F., White, J., Brandl, H. B., Moodley, Y., Merkling, T., Leclaire, S., Blanchard, P., Danchin, É., Hatch, S. A. and Wagner, R. H. 2013. Age-related differences in the cloacal microbiota of a wild bird species. - BMC Ecol. 13: 11.

van Veelen, H. P. J., Falcao Salles, J. and Tieleman, B. I. 2017. Multi-level comparisons of cloacal, skin, feather and nest-associated microbiota suggest considerable influence of horizontal acquisition on the microbiota assembly of sympatric woodlarks and skylarks. - Microbiome 5: 156.

van Veelen, H. P. J., Salles, J. F. and Tieleman, B. I. 2018. Microbiome assembly of avian eggshells and their potential as transgenerational carriers of maternal microbiota. - ISME J. 12: 1375–1388.

van Wijk, R. E., Schaub, M. and Bauer, S. 2017. Dependencies in the timing of activities weaken over the annual cycle in a long-distance migratory bird. - Behav. Ecol. Sociobiol. 71: 73.

van Wijk, R. E., Schaub, M., Hahn, S., Juárez-García-Pelayo, N., Schäfer, B., Viktora, L., Martín-Vivaldi, M., Zischewski, M. and Bauer, S. 2018. Diverse migration strategies in hoopoes (Upupa epops) lead to weak spatial but strong temporal connectivity. - Sci. Nat. 105: 42.

Victoria, J. K. 1972. Clutch characteristics and egg discriminative ability of African village weaverbird Ploceus cucullatus. - Ibis (Lond. 1859). 114: 367–376.

Videler, H., Geertjes, G. J. and Videler, J. J. 1999. Biochemical characteristics and antibiotic properties of the mucous envelope of the queen parrotfish. - J. Fish Biol. 54: 1124–1127.

Viseshakul, N., Charoennitikul, W., Kitamura, S., Kemp, A., Thong-Aree, S., Surapunpitak, Y., Poonswad, P. and Ponglikitmongkol, M. 2011. A phylogeny of

frugivorous hornbills linked to the evolution of Indian plants within Asian rainforests. - J. Evol. Biol. 24: 1533–1545.

Wallace AR 1889. Darwinism: an exposition of the theory of natural selection, with some of its applications.

Walters, L. A. and Getty, T. 2010. Are brighter eggs better? Egg color and parental investment by House Wrens. - J. F. Ornithol. 81: 155–166.

Wang, B., Wan, L., Wang, A. and Li, L. M. 2017. An adaptive decorrelation method removes Illumina DNA base-calling errors caused by crosstalk between adjacent clusters. - Sci. Rep. 7: 41348.

Warnecke, L., Turner, J. M., Bollinger, T. K., Lorch, J. M., Misra, V., Cryan, P. M., Wibbelt, G., Blehert, D. S. and Willis, C. K. R. 2012. Inoculation of bats with European Geomyces destructans supports the novel pathogen hypothesis for the origin of white-nose syndrome. - Proc. Natl. Acad. Sci. 109: 6999–7003.

Weiß, B. M., Kücklich, M., Thomsen, R., Henkel, S., Jänig, S., Kulik, L., Birkemeyer, C. and Widdig, A. 2018. Chemical composition of axillary odorants reflects social and individual attributes in rhesus macaques. - Behav. Ecol. Sociobiol. 72: 65.

Werren, J. H. 2012. Symbionts provide pesticide detoxification. - Proc. Natl. Acad. Sci. 109: 8364–8365.

Werren, J. H., Baldo, L. and Clark, M. E. 2008. Wolbachia: master manipulators of invertebrate biology. - Nat. Rev. Microbiol. 6: 741–751.

Wesolowski, T. and Maziarz, M. 2012. Dark tree cavities – a challenge for hole nesting birds? - J. Avian Biol. 43: 454–460.

Westcott, S. L. and Schloss, P. D. 2015. De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. - PeerJ 3: e1487.

Whittaker, D. J. and Theis, K. R. 2016. Bacterial Communities Associated with Junco Preen Glands : Preliminary Ramifications for Chemical Signaling. - In: Schulte, B. A. et al. (ed), Chemical Signals in Vertebrates 13. Springer International Publishing, pp. 105–117.

Whittaker, D. J., Soini, H. A., Atwell, J. W., Hollars, C., Novotny, M. V and Ketterson, E. D. 2010. Songbird chemosignals: volatile compounds in preen gland secretions vary among individuals, sexes, and populations. - Behav. Ecol. 21: 608–614.

Whittaker, D. J., Richmond, K. M., Miller, A. K., Kiley, R., Bergeon Burns, C., Atwell, J.

W. and Ketterson, E. D. 2011. Intraspecific preen oil odor preferences in dark-eyed juncos (Junco hyemalis). - Behav. Ecol. 22: 1256–1263.

Whittaker, D. J., Gerlach, N. M., Slowinski, S. P., Corcoran, K. P., Winters, A. D., Soini, H. A., Novotny, M. V., Ketterson, E. D. and Theis, K. R. 2016. Social Environment Has a Primary Influence on the Microbial and Odor Profiles of a Chemically Signaling Songbird. - Front. Ecol. Evol. 4: 1–15.

Whittaker, D. J., Slowinski, S. P., Greenberg, J. M., Alian, O., Winters, A. D., Ahmad, M. M., Burrell, M. J. E., Soini, H. A., Novotny, M. V., Ketterson, E. D. and Theis, K. R. 2019. Experimental evidence that symbiotic bacteria produce chemical cues in a songbird. - J. Exp. Biol.: jeb.202978.

Wright, E. S. and Vetsigian, K. H. 2016. Quality filtering of Illumina index reads mitigates sample cross-talk. - BMC Genomics 17: 876.

Zahavi, A. 1975. Mate selection-A selection for a handicap. - J. Theor. Biol. 53: 205–214.

Zahavi, A. and Zahavi, A. 1997. The handicap principle; a missing piece of Darwin's puzzle.

Zamzow, J. P. and Losey, G. S. 2002. Ultraviolet radiation absorbance by coral reef fish mucus: Photo-protection and visual communication. - Environ. Biol. Fishes 63: 41–47.

Zinter, M. S., Mayday, M. Y., Ryckman, K. K., Jelliffe-Pawlowski, L. L. and DeRisi, J. L. 2019. Towards precision quantification of contamination in metagenomic sequencing experiments. - Microbiome 7: 62.

SUPPLEMENTARY MATERIAL

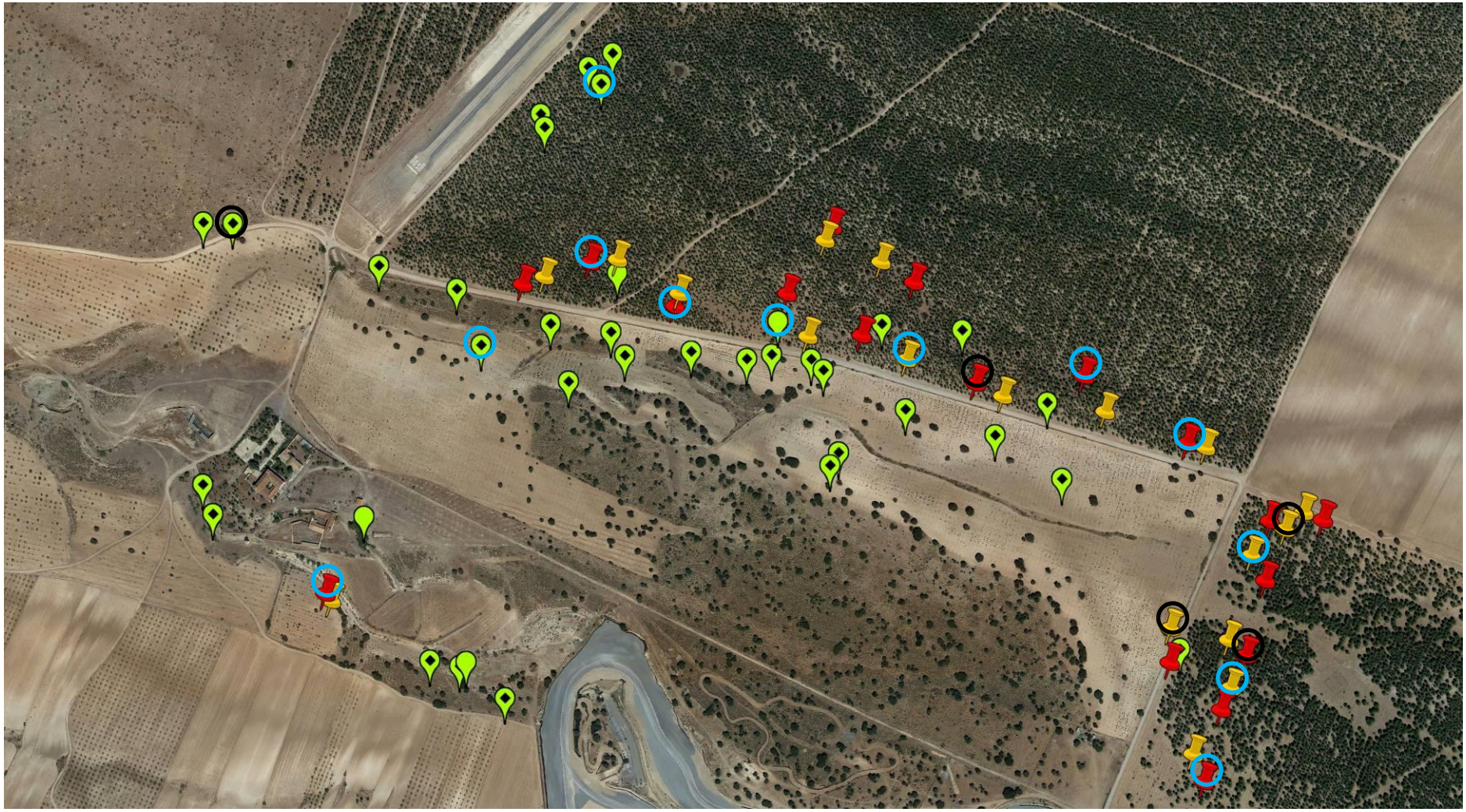

Fig S1. Map showing the disposition of the different types of nest boxes in one of our study areas in 2015 in addition to those that were already during 2014 that were cleaned at the beginning of the breeding season (green). The ones that are green with a black square inside indicate a pair of empty nest boxes placed in the same tree: new and old (3 nest boxes of this pair were used: two were old and 1 was new). The experimental nest boxes were placed in pairs: yellow (with sawdust) and red (sawdust and old hoopoe nest material). The nest boxes used that year are surrounded by a circle: blue ones are first clutches and black ones second clutches.

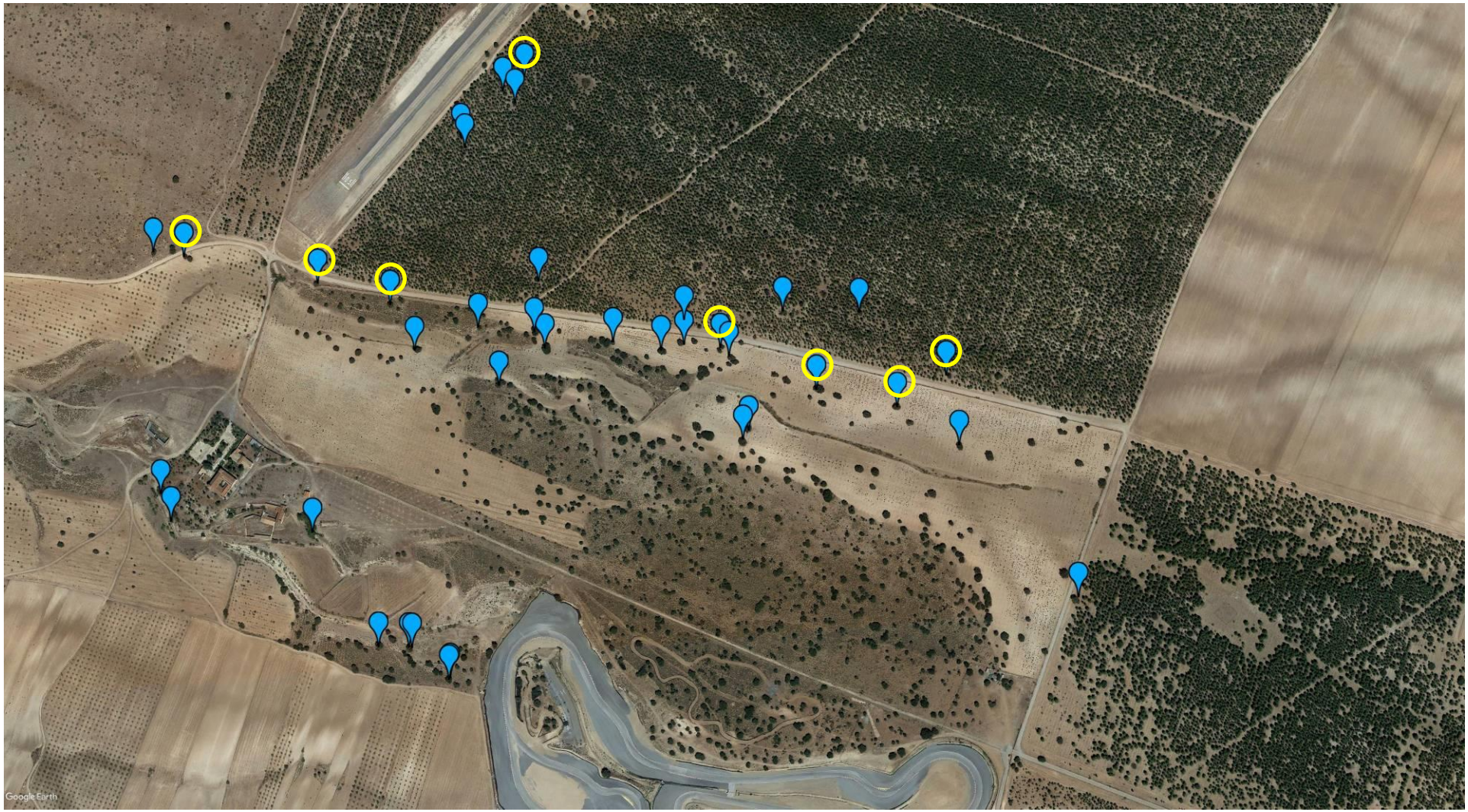

Fig S2. Map showing the disposition of the old nest boxes in one of our study areas in 2014. The nest boxes used that year are surrounded by a yellow circle.

CAPÍTULO II

CAPÍTULO II

Hoopoe (*Upupa epops*) male feeding effort is related to female cosmetic egg colouration

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Abstract

Avian eggshell colouration might function as a post-mating sexually selected signal of female quality influencing male parental investment and, hence, reproductive success. This hypothesis has been tested for intrinsic eggshell pigments as biliverdin (blue-green colouration) and/or protoporphyrin (brown coloured spots), but not for colourations due to post-laying events. Post-laying staining colouration due to, for instance, uropygial secretion of females, could reflect its phenotypic properties and, thus, might influence male investment in reproduction. In hoopoes, the uropygial gland of incubating females host symbiotic bacteria that are responsible of the brown colour of their uropygial secretion and of the eggshells, as they use their uropygial secretion to cover their bluish-grey eggshells. The secretion protects embryos from pathogenic trans-shell infections and, thus, egg colouration may function as a post-mating sexually selected signal of antimicrobial potential in hoopoes. Here, in a wild hoopoe population breeding in nest boxes in Spain, we test this hypothesis by exploring whether egg colour predicts male parental investment in reproduction. In accordance with the hypothesis, we found that the amount of food provided by males to incubating females was higher in nests with lower saturation of the cosmetic colouration of the eggshells. This relationship was not affected by female body condition nor clutch size, suggesting that it is not an indirect effect due to

other cues of female quality. Given that eggshell saturation is negatively related to density of bacterial symbionts in uropygial secretions, the results suggest that males may regulate their parental investment in accordance to the expected characteristics of mutualistic bacteria hosted in uropygial glands and deposited on eggshells. We discussed alternative explanations concluding that experimental modification of egg colour is needed to test this hypothesis further.

Keywords: Eggshell colour, Parental investment, Signalling, Symbiotic bacteria, *Upupa epops*, Uropygial secretion.

INTRODUCTION

Parental care, in its broad sense, is defined as any form of parental behaviour that increases the fitness of a parent's offspring at the cost of survival and future reproductive success of parents (Clutton-Brock 1991). While fitness benefits of parental care depend on the total care provided by males and females, sexual differences in reproductive investment are widespread in nature (Trivers 1972, Clutton-Brock 1991). Within this context, a sexual conflict of interests emerges where each of the parents tries to reduce their parental effort at the expense of the other sex (Trivers 1972). The optimal resolution of the conflict for each sex is the one that maximise reproductive success of the pair while minimising its own costs (i.e. parental care activity) (reviewed in Chapman et al. 2003). For males, reproductive success greatly depends on female investment in reproduction in terms of, for instance, clutch size and egg quality. Thus, males should differentially invest more in reproduction when paired with females that lay large clutches or eggs of better

quality. Conversely, everything equal, fitness benefits for females would depend on reproductive investment of their males and/or their genetic quality and, thus, females should differentially invest more in reproduction when paired with high quality males (Clutton-Brock 1991, Chapman et al. 2003). Interestingly, some phenotypic characteristics of males and of females reflect their phenotypic and or genetic quality, including reproductive abilities, which are related to mate choice preferences and, thus, are sexually selected (Andersson 1994). Moreover, these sexually selected traits are typically related to reproductive success, which is explained by both direct effect of the holders, and the differential investment in reproduction of the partner. Thus, differential investment in reproduction in relation to secondary sexual characteristics can be seen as a post-mating sexual selection process that contributes to the evolution of these traits (Burley 1986, Andersson 1994, Sheldon 2000). These processes have been mainly explored for secondary sexual traits of males, while those of females have only recently been considered (Soler et al. 2019).

Females may signal their phenotypic quality by means of their plumage (Møller 1993, Griggio et al. 2005, Morales et al. 2007), their nest building ability (Tomás et al. 2006, Soler et al. 2019), or the colour of their eggs (Moreno and Osorno 2003, Moreno et al. 2004, 2005, 2006a, Soler et al. 2005, 2018, Siefferman et al. 2006, Krist and Grim 2007, Giordano et al. 2015, Hargitai et al. 2018a) and, therefore, males should invest in reproduction in accordance with the expression of these secondary sexual traits of females. Egg colouration has only recently been considered as a possible sexually selected signal of females (Moreno and Osorno 2003) for which evidences are accumulating in the literature (e.g. Soler et al. 2005, Moreno et al. 2006a). Initially, the "sexually selected eggshell colouration (SSEC) hypothesis" was proposed for blue-green eggshell colouration (Moreno and Osorno 2003) because it depends on biliverdin (Mikšík et al. 1996), which is

a potent antioxidant (Kaur et al. 2003). Thus, blue-green colour intensity would signal the female's antioxidant capacity and thereby their immunocompetence (Morales et al. 2006, 2008, Moreno et al. 2006b, Hanley et al. 2008), and males would adjust their reproductive effort to eggshell colouration, favouring the evolution of such colours in a typical post-mating sexual selection process (Moreno and Osorno 2003). More recently, this hypothesis has been extended to red-brown eggshell colouration due to protoporphyrin (Martínez-De La Puente et al. 2007, Sanz and García-Navas 2009, Giordano et al. 2015, Poláček et al. 2017, Corti et al. 2018, Hargitai et al. 2018b). Thus, eggshell colouration can reflect phenotypic quality of females (Moreno et al. 2005, 2006b, Siefferman et al. 2006, Krist and Grim 2007, Hanley et al. 2008, Giordano et al. 2015); although it does not necessarily imply that it is a sexually selected trait (Cherry and Gosler 2010). Some studies, though not all, have detected that males invest more in reproduction when their nests harbour more intensely coloured eggs, as it would be expected from males maximising their fitness (Soler et al. 2005, 2008a, Moreno et al. 2006a, Hanley et al. 2008, English and Montgomerie 2011, but see Krist and Grim 2007, Hanley and Doucet 2009, Honza et al. 2011, Johnsen et al. 2011) and, thus, further research is necessary to reach firm conclusions.

The predicted associations between eggshell colouration, female condition and male feeding effort have been widely investigated regarding eggshell colouration produced by intrinsic pigments. However, cosmetic eggshell colouration might also indicate future reproductive success and/or female phenotypic condition and, therefore, affect male feeding effort. This is the case of extrinsic eggshell spots from ectoparasite activity (López-Rull et al. 2007, Avilés et al. 2009), or eggshell colouration due to females staining the eggshell with uropygial secretion (Soler et al. 2014). In the former case, the

amount of brown drops from ectoparasite activity will inform males on the parasitic environment where their offspring will develop and, thus, should influence their feeding effort as it was experimentally demonstrated in spotless starlings (*Sturnus unicolor*) (Avilés et al. 2009). In the latter case, which implies the use of uropygial secretion as cosmetics, eggshell colouration might inform males on characteristics of the uropygial secretion of their females that might have important consequences on reproductive success (i.e. hatching success, Martín-Vivaldi et al. 2014a). In this case, males should also adjust reproductive effort to eggshell colouration; a prediction that, for the first time, we are testing here in a European hoopoe (*Upupa epops*) population.

Female hoopoes actively use their uropygial secretion to cover their eggs after laying, protecting embryos from pathogenic infections (Martín-Vivaldi et al. 2014a). Thus, since uropygial secretion of laying and incubating females is dark (Martín-Vivaldi et al. 2009), the eggshell colouration change from the bluish-grey colouration of recently laid eggs to the greenish-brown colouration of eggs that females have repeatedly stained with secretion (Soler et al. 2014) (Fig. 1). Darkness of secretion is positively related to bacterial density in the secretion (Martín-Vivaldi et al. 2009) and, thus, the colour of the eggshells could be a possible indicator of the abundance of antibiotic producing bacteria in the secretion. Several studies have shown that some bacteria living in the uropygial gland of hoopoe females are producers of antimicrobial compounds (Martín-Platero et al. 2006, Soler et al. 2008a, Martín-Vivaldi et al. 2010, Ruiz-Rodríguez et al. 2013). Moreover, they are transferred by incubating females to the egg surface (Martín-Vivaldi et al. 2014a), determining the composition of the eggshell microbiota (Soler et al. 2016). Interestingly, it has been experimentally shown that the uropygial secretion with bacteria is darker (Martín-Vivaldi et al. 2009) and that the natural colouration of female secretions can reflect their antimicrobial capacity (Soler et al. 2014). Specifically, the saturation of

secretion colour was negatively related to the antimicrobial potential of the uropygial secretion (Soler et al. 2014). Thus, the colour of female secretion, and hence cosmetic colouration of the hoopoe eggshells, might act as a post-mating honest signal in this species informing of properties of the hosted symbiotic bacterial community (Fig 1). Since antimicrobial producing bacteria of the uropygial secretion protect embryos from trans-shell microbial infection (Martín-Vivaldi et al. 2014a), eggshell colouration might predict reproductive success. Moreover, since nestlings inherited from mothers the microbial community of their uropygial gland including those with antimicrobial potential (Ruiz-Rodríguez et al. 2014, Martínez-García et al. 2016b, Martín-Vivaldi et al. 2018), egg colouration would also reflect characteristics of the microbiota that nestlings will acquire, which might have fitness effects in terms of prevention of antimicrobial infection. Thus, investing differentially in clutches based on egg colouration may be a selective advantage for male hoopoes (Soler et al. 2014). Here, we tested this hypothesis by exploring the association between colouration of incubated hoopoe eggshells and male feeding effort. Specifically, we predict that males should invest more in clutches with lower saturation values (Fig. 1). We explored this hypothesis by using bird physiological visual models, and by recording male feeding visits at different nest stages: during the incubation, and after hatching; when first nestlings were 3 and 11 days old.

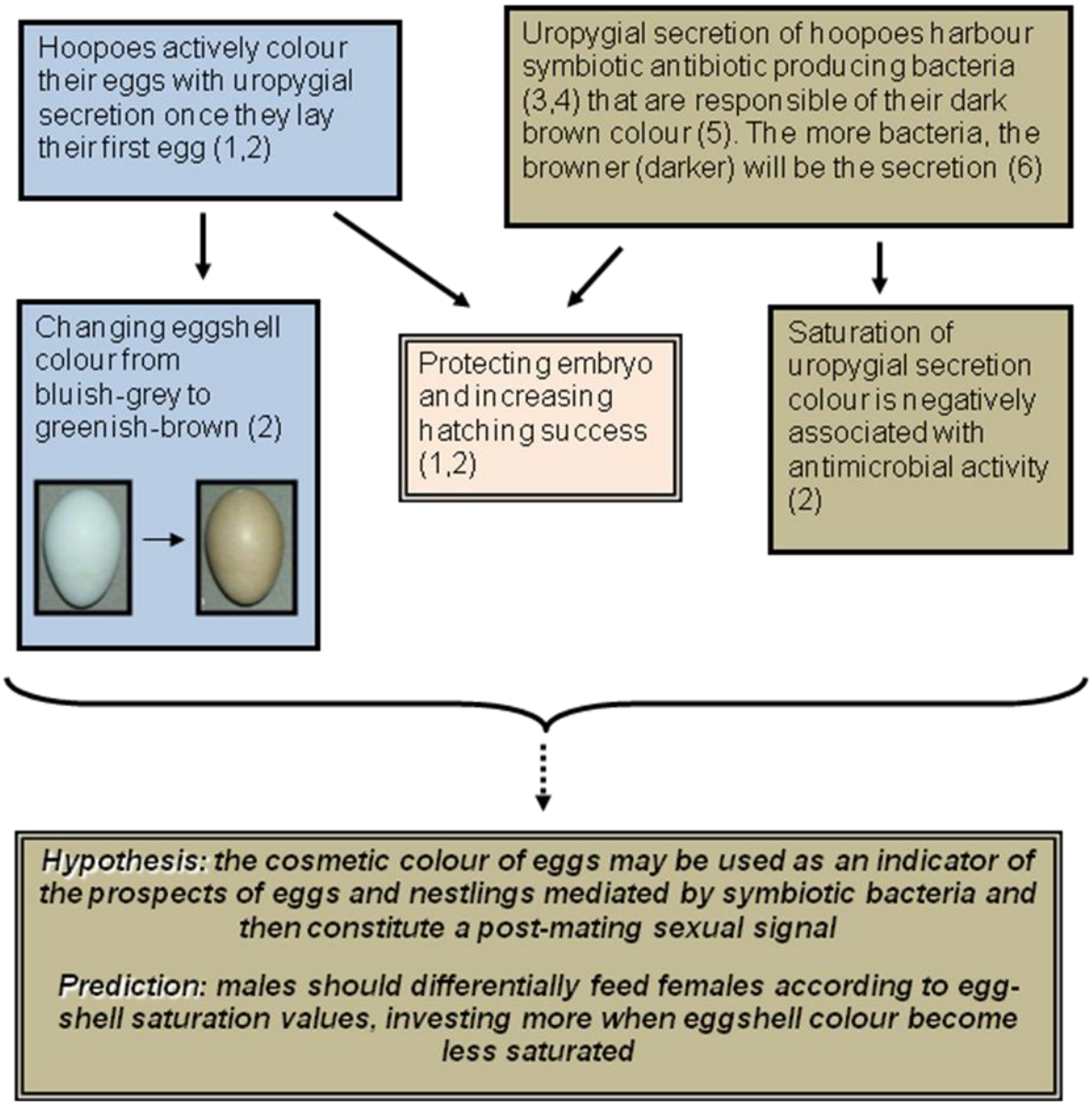


Fig. 1. Diagram showing premises supporting the hypothesis and prediction of the study. Numbers indicate references supporting premises ((1) Martín-Vivaldi, Soler, et al., 2014; (2) Soler et al., 2014; (3) Martín-Platero et al., 2006; (4) Ruiz-Rodríguez et al., 2013; (5) Martín-Vivaldi et al., 2010; (6) Martín-Vivaldi et al., 2009)).

MATERIAL AND METHODS

Study Species

The hoopoe is a migratory bird distributed throughout Europe, Asia and Africa. This species is a cavity nester that readily nests in nest boxes. Females lay usually between 6 and 8 eggs from February to July and one or two clutches (Martín-Vivaldi et al. 1999, Plard et al. 2018). They start to incubate, and paint eggshells with secretion, with the first or second egg in the laying sequence. The uropygial gland of all incubating females increases in size, producing a greater amount of secretion compared with non-breeding females (Martín-Vivaldi et al. 2009) allowing them to cover the big eggs surface of the entire clutch. In addition, the external structure of hoopoe eggshells is different from those of other birds (Martín-Vivaldi et al. 2014a), full of small crypts helping in the retention of the secretion. Hoopoes have asynchronous hatching since female incubates from the first or second egg, generating a size hierarchy within the brood (Cramp 1998). After the eighth day of the nestling phase, the size of the gland and the secretion returned to similar characteristic to those in the prelaying period (white and odorless secretions, without symbiotic bacteria) (Martín-Vivaldi et al. 2009). Thus, amounts of symbiotic bacteria seem to be consistent across the breeding season when the secretion is dark and after the eighth day of the nestling phase, they progressively decrease until disappearing. Only the female incubates, but both sexes care for offspring, and male feeds female while she is in the nest (from the start of incubation until the first nestling has 8 days). Then, both male and female provide food until fledging, 24-30 days after hatching (Martín-Vivaldi et al. 2014b). A variation in the colour of the uropygial secretion and eggshells is noticeable between individuals within our study population (Soler et al. 2014).

Study Area and General Procedures

The fieldwork was carried out in the Hoya de Guadix (37 ºC 18´N, 11´W), Granada (southern Spain) during the 2015 breeding season. This hoopoe population has been studied throughout the last 25 years. Hoopoes breed in this area in natural cavities and in nest boxes situated in trees. Nest boxes were made in cork with the following dimensions: 5.5 cm (entrance diameter), 24 cm (bottom-to-hole height), 35 × 18 × 21 cm (internal height × width × depth).

Nest boxes were visited from early March to the end of July every five days. Assuming that one egg was laid daily (Cramp 1998), laying date was considered the day when female laid her first egg, and hatching date as the day on which the first nestling hatched. Females were captured by hand inside the nest boxes twice, 15 days after laying date and 5 days after hatching of the first egg. Nestlings were sampled 19-20 days after hatching date. Bill and tarsus lengths were measured with a calliper (accuracy 1mm) and the body mass with a hanging scale (Pesola 0-100 g, accuracy 1 g). The uropygial gland secretion was extracted by means of automatic 1-10 μl micropipettes (see below for more details). Finally, all the individuals were marked with numbered aluminium rings (Spanish Institute for Nature Conservation, ICONA). Adult individuals were provided with unique colour ring combinations. After sampling, they were released into their nest box. To prevent contamination among nests and among nestlings, all the manipulations were made using disposable latex gloves previously cleaned with 96% ethanol.

Body condition of females was estimated as the residuals of body mass on tarsus length^3 (Senar and Pascual 1997, Peig and Green 2010, Labocha and Hayes 2012).

Parental Investment

The visits to the nest by parents were video-recorded at three different stages: incubation (10 days after laying the first egg) and days 3rd and 11th of the nestling period (nestlings 1 and 2, respectively). Digital video cameras (Sony Handycam DCR-SR55 and DCR SR190 models) were placed several metres away from the nest, camouflaged among vegetation, stones or trunks. Each recording started around 1600 hours and registered periods of approximately 3 hours. The period considered valid to estimate feeding effort started when males and females behaved normally. We identified individuals by their colour ring combinations and other characteristics of plumage or body cues. To estimate the amount of food provided by each individual, we counted the number of prey carried to the nest per hour (feeding rate) and average relative size of the prey carried. Relative size of prey was estimated in an ordinal scale. Value 1 was assigned to prey size that was less than a quarter of the beak length; value 2 when prey size was between a quarter and a half of the beak length; and value 3 when the prey were larger than a half beak length) (Martín-Vivaldi et al. 1999). Number of prey and prey size were multiplied to obtain an index of provisioning rate (feeding rate*prey average size). When it was not possible to estimate the size of the prey, we assigned the estimated average size for the recorded individual. We successfully recorded 39 nests that were visualized through VLC Media Player software (version 2.2.6). Only first clutches were considered in the analysis.

Egg Colour Measurements

Egg colour was measured on the 15th day of the incubation period, when all the eggs were completely covered by secretion, Hoopoes start to paint eggshells with secretion, with the first or second egg layed. Therefore, during laying, it is easily to notice a difference within

a single clutch between the first egg laid and the last one; simply because the former has been stained for longer period and, therefore is browner (similar to the colour of the secretion) (Soler et al. 2014). On the 15th day of the incubation period, all eggs are stained and the colouration is uniform in the entire clutch. The differences in colour among eggs along laying indicated above clearly suggest that females repeatedly re-stain their eggs after laying. Eggshell colouration was measured using an Ocean Optics S2000 spectrometer connected to a deuterium-halogen light (D2-W, Mini). A black bag that wrapped the tip of the optical fibre and the egg was used to standardise ambient light conditions. To prevent nest desertion by parents, all measures were performed close to the nest boxes as quick as possible (maximum of 15 minutes). The nest box entrance was blocked while the eggs and the female were measured.

Before the measurement of each clutch, the spectrometer was calibrated using a standard white and black reference. Reflectance spectra at 10nm intervals from 300-700nm was obtained for all eggs of the clutch. Eggshell colour was measured on five equidistant points on a random line along the long egg axis, from the apex to the base. To estimate repeatability, each zone was measured three times. Repeatability was calculated for each zone of the egg and among the eggs within the same clutch to verify that eggshell colouration was more variable among nests than within each of them. A principal components analysis (PCA) was run to summarise the colour information and calculated the repeatability with the three first PCA (Repeatability of each zone of the egg: GLM: PCA1: $r = 0.67$, $F_{380, 1524} = 11.38$, $P < 0.001$; PCA2: $r = 0.79$, $F_{380,1524} = 19.43$, $P < 0.001$, PCA3: $r = 0.71$, $F_{380, 1524} = 13.56$, $P < 0.001$. Repeatability among the eggs of each nest: GLM: PCA1: $r = 0.77$, $F_{51, 329} = 24.96$, $P < 0.0001$; PCA2: $r = 0.72$, $F_{51, 329} = 19.31$, $P < 0.001$, PCA3: $r = 0.62$, $F_{51,329} = 12.81$, $P < 0.0001$). Finally, average values of colour parameters for each egg and then for each nest were used.

In signalling contexts, it is desirable to analyse colour data in a way that is appropriate for the animal vision (Endler, 1990; Renoult, Kelber, & Schaefer, 2017). Moreover, birds have four types of cones in their retinas and, thus, we estimated the visual parameters of a physiological model for a tetrachromatic vision using Avicol V.6 software (Gómez 2006). Prior to all analysis, we applied the following corrections to our interpolated spectra data: negative values were settled to zero and reflectance curves were corrected for noise using triangular smoothing (Gómez 2006). Specifically, we used the model proposed by Endler and Mielke (2005) with some modifications proposed by Stoddard and Prum (2008), such as, the non-logarithmic transformation of the photoreceptor response values and the correction for dark colours. Among birds, hoopoes have a VS visual system, i.e. sensitive only to the violet range unlike other birds with an UV system, i.e. able to perceive wavelengths in the ultraviolet range (Hart 2001, Ödeen and Håstad 2003). Spectral sensitivity of the hoopoe is not available, and we used data from another non-passerine species, the peafowl (*Pavo cristatus*), as reference values of the VS system (Hart 2002). Moreover, we considered ambient light conditions inside a nest box to calculate the colour visualisation by hoopoes. Finally, we obtained qrQ, two variables with spherical coordinates tetha (θ) and phi (φ), and chroma (r). qrQ measure the quantum catch for the photoreceptor(s) responsible for brightness perception, adapted to the background (Gómez 2006). θ measure an angle (between -180º and 180º) in the red-green-blue plane and φ (between -90º and 90º) in the ultraviolet/violet sensitive (UV/V) range (Endler & Mielke, 2005). Both measures include information of hue, while r values inform on colour saturation (Stoddard and Prum 2008, Saino et al. 2013). Since the colour space is a tetrahedral and not a sphere, maximum potential chroma (r_{max}) depends on hue values. For this reason, we used achieved chroma (rA, hereafter saturation) in our

analyses, computed as: $rA=r/ r_{max}$ (Stoddard and Prum 2008). The data obtained take into account the sensitivity and the stimulation of each cone of the visual system of birds, allowing us to measure the colour in ways relevant to birds (Endler, 1990; Saino et al., 2013) (Figure S1. Supplementary Material). However little is known about neural processes, i.e. how the eyes and brain process colour patterns (Kemp et al. 2015, Renoult et al. 2017, Stoddard and Osorio 2019), therefore, even when using bird physiological visual models, we have to interpret these results with caution.

Uropygial Secretion Bacterial Loads

To collect the uropygial gland secretion, first, we cleaned the surroundings of the gland with a cotton swab soaked in 96% ethanol. Second, we gently introduced a previously autoclaved tip of an automatic 1-10 µl micropipette, into the opening of the papilla of the gland and directly pipetted the secretion. Finally, the secretion was introduced into a sterile microcentrifuge tube, repeating this procedure until the papilla emptied. To estimate bacterial loads, the samples were processed within the following 24h. Briefly, 5 µl of the secretion were homogenised with 45 µl of PBS in a sterile microcentrifuge tube. Then 5 µl of each tenfold dilutions to 10^{-4} of this mixture was spread onto the following medium (Scharlau Chemie S.A., Barcelona): Tryptone Soya Agar (TSA), a broadly used general medium to grow mesophilic bacteria. The plates were incubated aerobically at 37 ºC for 24h before colony counting. Estimates of bacterial loads were standardised to number of colony forming units (CFU) per millilitre of secretion (Nº colonies * $10^{\text{dilution factor}}$) / ml spread).

Statistical Analysis

To look for the best combination of eggshell colour variables of the physiological model that explained the bacterial load in the uropygial gland secretion of the female, we used a best subset General Regression Model (GRM) with qrQ, θ, φ and rA as predictors and CFU/ml as dependent variable. The best subset GRM model were analysed by means of Mallow´s CP (Mallows 1973), equivalent to Akaike´s Information Criterion (AIC) (Boisbunon et al. 2013). Since θ, φ values are located in the same quadrant (Range values: θ: from 4.45 to 90.42; φ: from -86.65 to -66.27), we can use these variables in linear scales in the sense that increasing values always imply changes in the same direction. Moreover, rA, the variable that explains bacterial load and feeding effort (see result), has a linear nature.

To explore the effect of eggshell saturation (rA) on male provisioning rates, we performed a multivariate General Lineal Model (GLM) that also included clutch size, laying date and female body condition as additional independent factors. To check the effect of those predictors in each phase separately, we used the corresponding Univariate GLM models.

All the variables followed approximately a normal distribution except for CFU/ml of uropygial secretion in TSA medium that was log10+ 0.1 transformed.

All statistical tests were performed with Statistica 7 software (Statsoft 2006).

RESULTS

In accordance with the assumption that eggshell colouration reflects the bacterial load of the uropygial secretion we found that saturation (rA) of the eggshell was negatively related to bacterial density of uropygial secretion as estimated in TSA medium (best subset GRM: rA: $F_{1,19}$ = 6.93, *Beta = -0.76, P* = 0.016, θ: $F_{1,19}$ = 3.99, *Beta = -1.69, P* = 0.060; φ: $F_{1,19}$ = 3.09, *Beta = 1.33, P* = 0.095, Fig. 2).

Egg colour (rA) was a significant predictor of male provisioning rate in hoopoes, as were laying date and female body condition (multivariate GLM. Table 1). In the case of eggshell colour, there was only a clear relationship during the incubation phase and no relationship in the other two phases with nestlings (univariate GLM. Table 1). Thus, the overall effect of the colour of the eggshells was mainly present during the incubation phase. Female body condition and laying date also predict male feeding effort, but mainly in the last phase with nestlings (univariate GLM. Table 1).

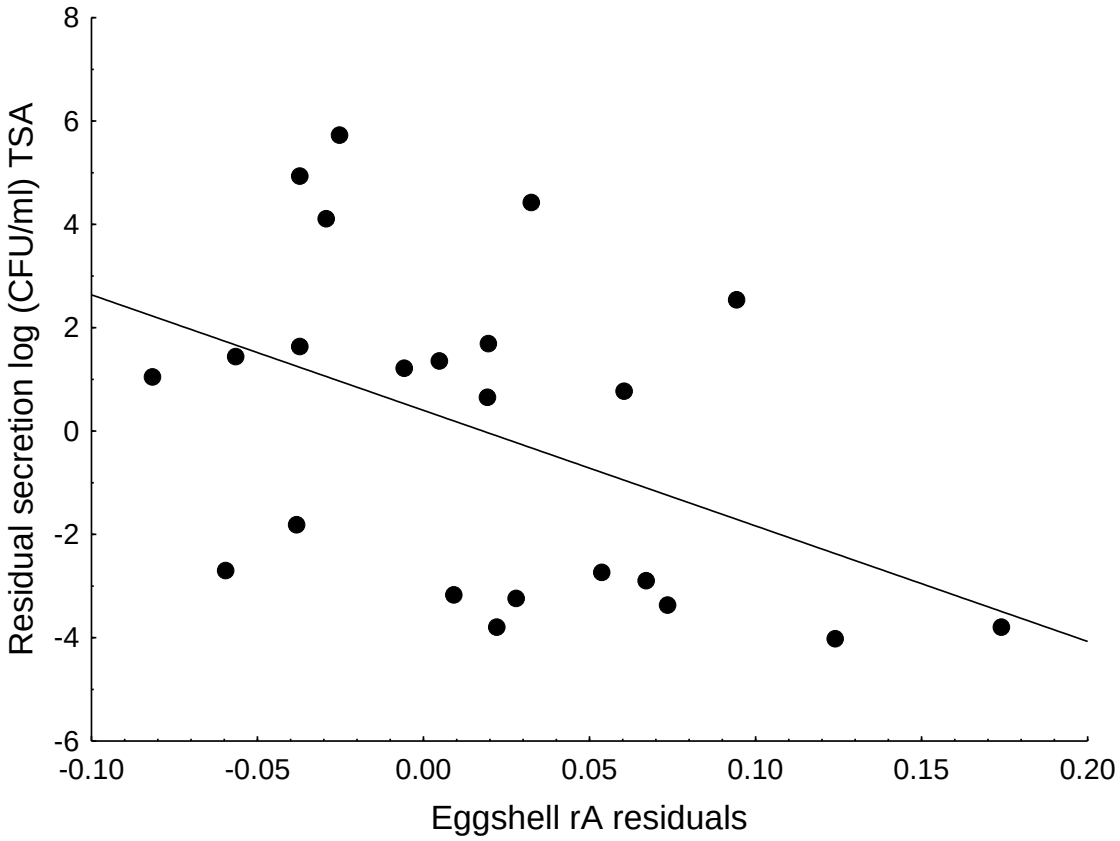


Fig. 2. Relationship between rA (saturation) of the eggshell and bacterial growth (log CFU/ml) in TSA medium of the uropygial secretion of females (mesophilic bacterial loads).

Eggshells with higher rA corresponded to lower male provisioning rates (Fig. 3). The best models for the nestling phases never included egg colour saturation (Table 1).

Table 1. Multivariate GLM testing the association between male provisioning rate in three different phases of the nesting period (incubation, nestlings 1 and nestlings 2) and eggshell colouration (rA) after controlling for the effects clutch size, laying date and body condition of females. Results from Univariate GLMs testing those predictors in each phase separately are also shown.

Multivariate GLM	***Wilks***	$F_{3, 21}$	***P***
Intercept	0.67	3.42	0.036
Clutch size	0.96	0.31	0.818
Laying date	0.68	3.31	**0.040**
rA	0.66	3.60	**0.030**
Female body condition	0.65	3.83	**0.025**
Univariate GLM: ***Incubation***		$F_{1,23}$	***P***
Clutch size		0.37	0.550
Laying date		2.82	0.106
rA		**9.85**	**0.004**
Female body condition		0.37	0.547
Univariate GLM: ***Nestlings 1***		$F_{1,23}$	***P***
Clutch size		0.01	0.915
Laying date		0.19	0.669
rA		<0.001	0.997
Female body condition		0.01	0.921
Univariate GLM: ***Nestling 2***		$F_{1,23}$	***P***
Clutch size		0.24	0.625
Laying date		4.04	0.056
rA		0.12	0.731
Female body condition		**6.12**	**0.021**

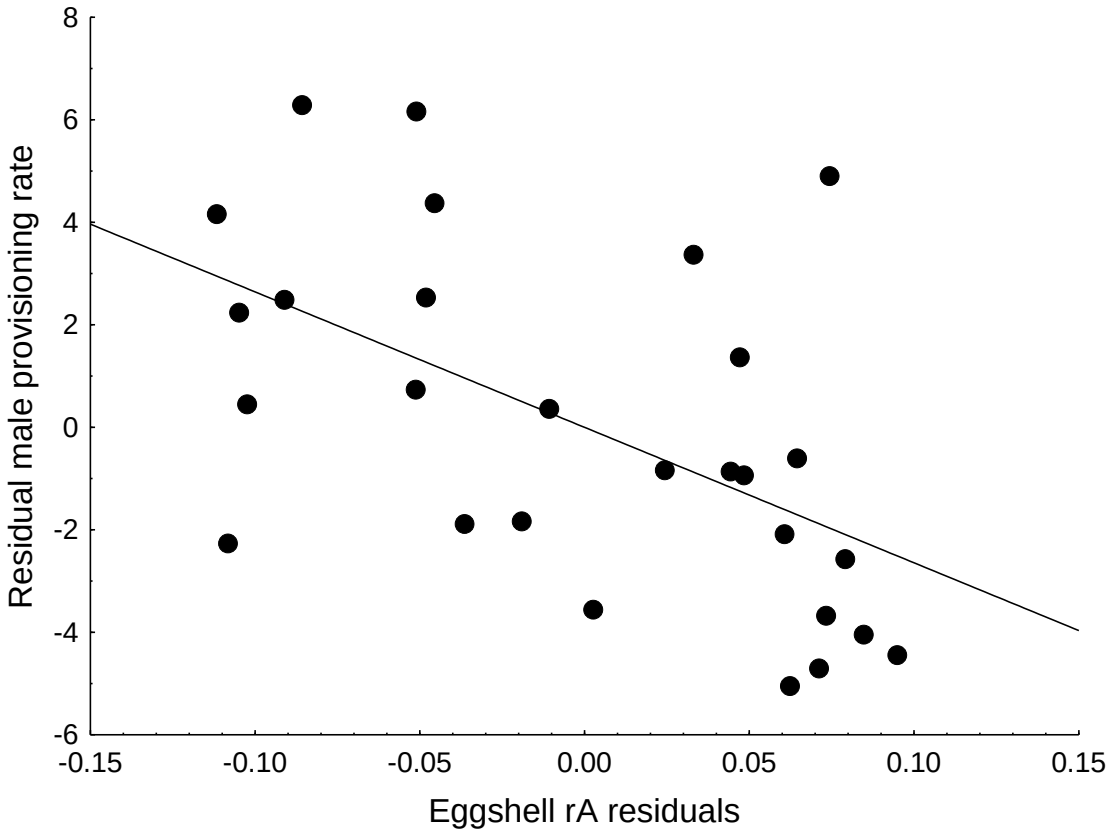


Fig. 3. Relationship between hoopoe male provisioning rate in the incubation period and eggshell saturation (rA). The residuals after correcting for laying date, clutch size and female body condition are presented.

DISCUSSION

Our main finding is that hoopoe males invested less in clutches of females with more saturated eggshell colours (higher rA values) and that secretion of females with more saturated eggshell colouration harboured a lower abundance of symbiotic bacteria during the incubation phase. These results are consistent with the hypothesis that the cosmetic colouration of the eggshell due to uropygial secretion could act as post-mating sexual signal of females. This signal would show antimicrobial potential of their uropygial

secretion that the males use to adjust their feeding effort accordingly. We discuss below the importance of these results under the hypothesis tested and possible alternative scenarios that could explain our correlative results.

Several previous studies have found a relationship between eggshell colouration and parental investment. However, most of them deal with intrinsic colouration due to biliverdin and/or protoporphyrin deposited within the oviduct during eggshell formation (Moreno et al. 2004, 2006a, Hanley et al. 2008, Soler et al. 2008a, English and Montgomerie 2011) and not with cosmetic colouration that are acquired after laying. Uropygial secretion of incubating individuals also reach the eggshells and reduced microbial growth or probability of trans-shell microbial infection (Cook et al. 2005, Martín-Vivaldi et al. 2014a), but might also change colouration of the eggshells (Soler et al. 2014). The use of uropygial secretion as cosmetic influencing avian colouration has mainly been studied in feathers (Montgomerie 2006, reviewed in Delhey et al. 2007, Piault et al. 2008) in scenarios of sexual selection (Negro et al. 1999, Delhey et al. 2007, López-Rull et al. 2010). Here, we find correlative evidence supporting the hypothesis that staining the eggshells with uropygial secretion of particular colour influences feeding effort of males during the incubation period.

Several adaptive functions have been proposed to explain intra and interspecific variation in eggshell colouration in scenarios of, for instance, predator-prey interactions, brood parasitism, thermoregulation, resistance to breakage or sexual selection (Soler and Møller 1996, Moreno and Osorno 2003, Gosler et al. 2005, Soler et al. 2005, 2018, Kilner 2006, Reynolds et al. 2009, Cherry and Gosler 2010). We know that female hoopoes actively cover their eggs with the secretion (Martín-Vivaldi et al. 2014a), which might imply several costs and benefits. Uropygial secretion protects feathers from feather degrading bacteria (Ruiz-Rodríguez et al. 2009) and using it to colour the eggshell implies

costs in terms of time and energy and in the amount of secretion that could have been invested in its entirely for their feathers. On the other hand, using the uropygial secretion to colour the eggs protect embryos and increase hatching success (Martín-Vivaldi et al. 2014a), which implies direct benefits of this females' behaviour. Interestingly, we know that antimicrobial potential of the uropygial secretion of females is related to its colouration (Martín-Vivaldi et al. 2009, Soler et al. 2014). Thus, males may infer properties of the uropygial secretion by evaluating eggshell colouration and adjust their feeding effort accordingly. Although this scenario is, in our opinion, the one which most likely explains our results, experimental demonstration is necessary to unambiguously support this conclusion.

Alternative explanations of the use of uropygial secretion to colour the eggs included that it could initially evolve to camouflage in the background since greenish-brown eggs are less conspicuous than the initial bluish grey ones, or for predator avoidance because of its malodorous volatiles (Martín-Vivaldi et al. 2010). However, females could have another anti-predatory strategy since it is common for them to defecate on their eggs when they are captured (Martín-Vivaldi et al. 2014b). A non-adaptive possibility is that females spread their eggs with their uropygial secretion to protect their ventral feathers, which are in close contact with eggs and with nest material, where prevalence of parasites and microbes is high. If this was the case, eggshells might become stained as a consequence of being in contact with smeared ventral feathers. However, video recordings have shown how females actively smear their uropygial secretion onto the eggshells (Martín-Vivaldi et al. 2014a). Therefore, even with other possible functions of the cosmetic colouration, the male would benefit from being able to interpret those clues, which would end up evolving to signals.

We have previously shown that, after elimination of the symbiotic bacteria from hoopoe uropygial gland with antibiotics, the secretions became less brown and more intensely red coloured (Martín-Vivaldi et al. 2010). Moreover, the natural variation in saturation of secretion colours was negatively related to the antimicrobial power of such secretions (Soler et al. 2014). In addition, we have found in this study that saturation of the eggshells correlates with the abundance of symbionts in the secretion. Given that almost every bacteria growing from secretions in TSA are *enterococci* producing bacteriocins (Soler et al 2008), these evidences together point to saturation of the secretion as a valid cue of its antimicrobial potential mediated by the symbionts of the gland. Males are not able to directly evaluate colouration of the uropygial secretion of their females, but can do it through evaluation of eggshell colouration. If that was the case, males should invest differentially in reproductions of higher fitness values and, thus, accordingly to the cosmetic egg-colour, likely reflecting not only abundance, but also composition of the community of bacteria hosted in female glands (Martínez-García et al. 2016b, Rodríguez-Ruano et al. 2018) transferred to eggs after laying (Soler et al. 2016).

The overall detected effect of the colour of the eggshell on male provisioning effort exclusively relies on the incubation phase, suggesting that effect stop when the eggshell colouration is not visible. In this species, females depend exclusively on the male to obtain food while they are incubating and until nestlings are about 8 days old (Martín-Vivaldi et al. 2014b). Therefore, an increased investment of males in this stage should be important for females and it would probably allow more efficient incubation of the eggs. Some other studies detected the expected association between eggshell colouration and feeding effort of males during early stages of the nestling period, which would later disappear (Soler et al. 2008b, English and Montgomerie 2011). We did not detect such an association during the nestlings phase and, thus, some other variables such as female provisioning rate,

number of nestlings, laying date and female body condition might have higher influence on male feeding effort. It is possible, however, that our index of male feeding effort during the nestling phase was underestimated. This is because males usually give the prey to the female when she is outside the nest instead of directly feeding nestlings (Martín-Vivaldi et al. 2014b). However, females usually stay within the nests during the first 8 days after hatching (Martín-Vivaldi et al. 2014b) and no effect of eggshell colouration was detected on feeding effort of males at this stage, i.e. when first nestling was 3 days old. Moreover, it is possible that behavioural signals like begging intensity become a more important predictor of offspring quality and needs for male feeding rate as nestling age (Smith and Montgomerie 1991, Moreno et al. 2006a).

Given the descriptive nature of our data, we cannot infer causality nor discard the possibility that the detected relationship was an indirect effect due to other variables that, such as nest predation risk (Fontaine and Martin 2006) or territory quality, covaried with colouration of eggshells or uropygial secretions. It is also possible that the quality of the secretion of the female was a consequence of the food received from the male and not the reverse. Males that invest more may be of higher quality or have a better territory, with more available food (Tschumi et al. 2014). Moreover, colouration of eggshells or uropygial secretions may covary with other characteristics that males could evaluate to decide differential investment in these nests. Plumage colouration of females (Morales et al. 2007, Soler et al. 2019), the length of their feathers (Møller 1993), the eggs size, or even other properties of the secretion, such as the odour, which also varies depending on the presence of symbiotic bacteria in the gland (Martín-Vivaldi et al. 2010), might in fact covary with eggshell colouration. Experimental manipulation of eggshell colouration is therefore necessary to reach firm conclusions. A method to do that may be a cross-

fostering experiment, swapping entire clutches of eggs between nests of approximately the same laying date, which would decouple possible indirect effects for eggshell colouration and male provisional rate (Riehl 2011, Stoddard et al. 2012). Moreover, the fact that males of hoopoes feed females during the incubation period (Martín-Vivaldi et al. 2014b) would allows us to directly test whether male provisioning rates to the female are caused by eggshell colour instead of nestling traits. We have found that female body condition during incubation was a predictor of male feeding effort, but it did not affect the effect of eggshell colour during incubation, and was mainly important in the nestling phases, specifically when first nestling was 11 days. This suggests that the differential investment of males detected in our analyses was not solely caused by their evaluation of the overall female quality.

In summary, our results suggest that the cosmetic colour of eggs caused by female uropygial secretion may be used by male hoopoes as a post-mating signal of female quality. Although descriptive, this study presents, for the first time, evidence that male feeding effort could be related to a cosmetic colouration produced by the uropygial secretion on eggshells. Experimental manipulations are needed to definitely support that the colour of the eggs is the variable causing such relationship.

Ethical Note

The study was conducted according to relevant Spanish national (Decreto 142/2013, 1 de octubre) and regional guidelines. The protocol was approved by the ethics committee of the University of Granada (Comité de Ética en Experimentación Animal, CEEA, Ref.:785), and all necessary permits for hoopoe manipulation were provided by the Consejería de Medio Ambiente de la Junta de Andalucía, Spain (Ref:SGYB/FOA/AFR/CFS and SGMN/GyB/JMIF). Our study area is not protected, but

privately owned, and the owners allowed us to work in their properties. The time spent in each hoopoe nest was the minimum necessary for sampling and for measuring the colouration of the eggshells. The protocols adhered to the ASAB/ABS Guidelines for the Use of Animals in Research.

REFERENCES

Alberdi, A., Aizpurua, O., Bohmann, K., Zepeda-Mendoza, M. L. and Gilbert, M. T. P. 2016. Do Vertebrate Gut Metagenomes Confer Rapid Ecological Adaptation? - Trends Ecol. Evol. 31: 689–699.

Alexander, G. D., Houston, D. C. and Campbell, M. 1994. A possible acoustic function for the casque structure in hornbills (Aves: Bucerotidae). - J. Zool. 233: 57–67.

Amat, J. A., Rendón, M. A., Garrido-Fernández, J., Garrido, A., Rendón-martos, M. and Pérez-gálvez, A. 2011. Greater flamingos Phoenicopterus roseus use uropygial secretions as make-up. - Behav. Ecol. 65: 665–673.

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Zech Xu, Z., Kightley, E. P., Thompson, L. R., Hyde, E. R., Gonzalez, A. and Knight, R. 2017. Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. - mSystems 2: e00191-16.

Amundsen, T. 2000. Why are female birds ornamented? - Trends Ecol. Evol. 15: 149–155.

Amundsen, T. 2018. Sex roles and sexual selection: Lessons from a dynamic model system. - Curr. Zool. 64: 363–392.

Andersson, M. 1994. Sexual selection (Princeton Univ. Press, Ed.).

Andersson, M. and Simmons, L. W. 2006. Sexual selection and mate choice. - Trends Ecol. Evol. 21: 296–302.

Archie, E. A. and Theis, K. R. 2011. Animal behaviour meets microbial ecology. - Anim. Behav. 82: 425–436.

Arlettaz, R., Schaub, M., Fournier, J., Reichlin, T. S., Sierro, A., Watson, J. E. M. and Braunisch, V. 2010. From Publications to Public Actions: When Conservation Biologists Bridge the Gap between Research and Implementation. - Bioscience 60:

835–842.

Aronesty, E. 2011. ea-utils: Command-line tools for processing biological sequencing data.: https://github.com/ExpressionAnalysis/ea-utils.

Avilés, J. M. and Soler, J. J. 2009. Nestling colouration is adjusted to parent visual performance in altricial birds. - J. Evol. Biol. 22: 376–386.

Avilés, J. M., Soler, J. J. and Pérez-Contreras, T. 2006. Dark nests and egg colour in birds: a possible functional role of ultraviolet reflectance in egg detectability. - Proc. Biol. Sci. 273: 2821–2829.

Avilés, J. M., Pérez-Contreras, T., Navarro, C. and Soler, J. J. 2009. Male spotless starlings adjust feeding effort based on egg spots revealing ectoparasite load. - Anim. Behav. 78: 993–999.

Avilés, J. M., Vikan, J. R., Fossøy, F., Antonov, A., Moksnes, A., Røskaft, E. and Stokke, B. G. 2010. Avian colour perception predicts behavioural responses to experimental brood parasitism in chaffinches. - J Evol Biol 23: 293–301.

Avilés, J. M., Soler, J. J. and Hart, N. S. 2011. Sexual selection based on egg colour: Physiological models and egg discrimination experiments in a cavity-nesting bird. - Behav. Ecol. Sociobiol. 65: 1721–1730.

Azcárate-García, M., González-Braojos, S., Díaz-Lora, S., Ruiz-Rodríguez, M., Martin-Vivaldi, M., Martínez-Bueno, M., Moreno, J. and Soler, J. J. 2020. Interspecific variation in deterioration and degradability of avian feathers: The evolutionary role of the microbiome. - J. Avian Biol.: in press.

Baker, L. J., Freed, L. L., Easson, C. G., Lopez, J. V, Fenolio, D., Sutton, T. T., Nyholm, S. V and Hendry, T. A. 2019. Diverse deep-sea anglerfishes share a genetically reduced luminous symbiont that is acquired from the environment. - Elife 8: e47606.

Bandyopadhyay, A. and Bhattacharyya, S. P. 1996. Influence of fowl uropygial gland and its secretory lipid components on growth of skin surface bacteria of fowl. - Indian J. Exp. Biol. 34: 48–52.

Banning, J. L., Weddle, A. L., Wahl III, G. W., Simon, M. A., Lauer, A., Walters, R. L. and Harris, R. N. 2008. Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, Hemidactylium scutatum. - Oecologia 156: 423–429.

Barr, J. J., Auro, R., Furlan, M., Whiteson, K. L., Erb, M. L., Pogliano, J., Stotland, A., Wolkowicz, R., Cutting, A. S., Doran, K. S., Salamon, P., Youle, M. and Rohwer, F.

2013. Bacteriophage adhering to mucus provide a non–host-derived immunity. - Proc. Natl. Acad. Sci. 110: 10771–10776.

Beltran-Bech, S. and Richard, F.-J. 2014. Impact of infection on mate choice. - Anim. Behav. 90: 159–170.

Bennett, G. M. and Moran, N. A. 2015. Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. - Proc. Natl. Acad. Sci. 112: 10169–10176.

Bentley, D. R., Balasubramanian, S., Swerdlow, H. P., Smith, G. P., Milton, J., Brown, C. G., Hall, K. P., Evers, D. J., Barnes, C. L., Bignell, H. R., Boutell, J. M., Bryant, J., Carter, R. J., Keira Cheetham, R., Cox, A. J., Ellis, D. J., Flatbush, M. R., Gormley, N. A., Humphray, S. J., Irving, L. J., Karbelashvili, M. S., Kirk, S. M., Li, H., Liu, X., Maisinger, K. S., Murray, L. J., Obradovic, B., Ost, T., Parkinson, M. L., Pratt, M. R., Rasolonjatovo, I. M. J., Reed, M. T., Rigatti, R., Rodighiero, C., Ross, M. T., Sabot, A., Sankar, S. V., Scally, A., Schroth, G. P., Smith, M. E., Smith, V. P., Spiridou, A., Torrance, P. E., Tzonev, S. S., Vermaas, E. H., Walter, K., Wu, X., Zhang, L., Alam, M. D., Anastasi, C., Aniebo, I. C., Bailey, D. M. D., Bancarz, I. R., Banerjee, S., Barbour, S. G., Baybayan, P. A., Benoit, V. A., Benson, K. F., Bevis, C., Black, P. J., Boodhun, A., Brennan, J. S., Bridgham, J. A., Brown, R. C., Brown, A. A., Buermann, D. H., Bundu, A. A., Burrows, J. C., Carter, N. P., Castillo, N., Catenazzi, M. C. E., Chang, S., Neil Cooley, R., Crake, N. R., Dada, O. O., Diakoumakos, K. D., Dominguez-Fernandez, B., Earnshaw, D. J., Egbujor, U. C., Elmore, D. W., Etchin, S. S., Ewan, M. R., Fedurco, M., Fraser, L. J., Fuentes Fajardo, K. V., Scott Furey, W., George, D., Gietzen, K. J., Goddard, C. P., Golda, G. S., Granieri, P. A., Green, D. E., Gustafson, D. L., Hansen, N. F., Harnish, K., Haudenschild, C. D., Heyer, N. I., Hims, M. M., et al. 2008. Accurate whole human genome sequencing using reversible terminator chemistry. - Nature 456: 53–59.

Boisbunon, A., Canu, S., Fourdrinier, D., Strawderman, W. and Wells, M. T. 2013. AIC , Cp and estimators of loss for elliptically symmetric distributions. - hal-00851206

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., Mills, D. A. and Caporaso, J. G. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. - Nat. Methods 10: 57–59.

Brandl, H. B., van Dongen, W. F. D., Darolová, A., Kristofík, J., Majtan, J. and Hoi, H. 2014. Composition of Bacterial Assemblages in Different Components of Reed

Warbler Nests and a Possible Role of Egg Incubation in Pathogen Regulation. - PLoS One 9(12): 1–19.

Braun, M. S., Zimmermann, S., Danner, M., Rashid, H. and Wink, M. 2016. Corynebacterium uropygiale sp. nov., isolated from the preen gland of Turkeys (Meleagris gallopavo). - Syst. Appl. Microbiol. 39: 88–92.

Braun, M. S., Wang, E., Zimmermann, S. and Wink, M. 2018a. Corynebacterium heidelbergense sp. nov., isolated from the preen glands of Egyptian geese (Alopochen aegyptiacus). - Syst. Appl. Microbiol. 41: 564–569.

Braun, M. S., Wang, E., Zimmermann, S., Boutin, S. and Wink, M. 2018b. Kocuria uropygioeca sp. nov. and Kocuria uropygialis sp. nov., isolated from the preen glands of Great Spotted Woodpeckers (Dendrocopos major). - Syst. Appl. Microbiol. 41: 38–43.

Braun, M. S., Sporer, F., Zimmermann, S. and Wink, M. 2018c. Birds, feather-degrading bacteria and preen glands: The antimicrobial activity of preen gland secretions from turkeys (Meleagris gallopavo) is amplified by keratinase. - FEMS Microbiol. Ecol. 94: fiy117.

Braun, M. S., Wang, E., Zimmermann, S., Boutin, S., Wagner, H. and Wink, M. 2019a. Kocuria tytonicola, new bacteria from the preen glands of American barn owls (Tyto furcata). - Syst. Appl. Microbiol. 42: 198–204.

Braun, M. S., Wang, E., Zimmermann, S., Wagner, H. and Wink, M. 2019b. Kocuria tytonis sp. nov., isolated from the uropygial gland of an American barn owl (Tyto furcata). - Int. J. Syst. Evol. Microbiol. 69: 447–451.

Bright, M. and Bulgheresi, S. 2010. A complex journey: transmission of microbial symbionts. - Nat. Rev. Microbiol. 8: 218–230.

Brulez, K., Pike, T. W. and Reynolds, S. J. 2015. Egg signalling: The use of visual, auditory, and chemical stimuli. - In: Deeming, D. C. and Reynolds, S. J. (eds), Nest, Egg, and Incubation. New ideas about avian reproduction. Oxford University Press, pp. 127–141.

Brunetti, A. E., Lyra, M. L., Melo, W. G. P., Andrade, L. E., Palacios-Rodríguez, P., Prado, B. M., Haddad, C. F. B., Pupo, M. T. and Lopes, N. P. 2019. Symbiotic skin bacteria as a source for sex-specific scents in frogs. - Proc. Natl. Acad. Sci. 116: 2124–2129.

Bulla, M., Šálek, M. and Gosler, A. G. 2012. Eggshell spotting does not predict male

incubation but marks thinner areas of a shorebird's shells. - Auk 129: 26–35.

Burger, B. V, Reiter, B., Borzyk, O. and du Plessis, M. A. 2004. Avian Exocrine Secretions. I. Chemical Characterization of the Volatile Fraction of the Uropygial Secretion of the Green Woodhoopoe, Phoeniculus purpureus. - J. Chem. Ecol. 30: 1603–1611.

Burley, N. 1986. Sexual Selection for Aesthetic Traits in Species with Biparental Care. - Am. Nat. 127: 415–445.

Burley, N. 1988. The Differential Allocation hypothesis. An experimental test. - Am. Nat. 132: 611–628.

Callahan, B. J., McMurdie, P. J. and Holmes, S. P. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. - ISME J. 11: 2639–2643.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., González Peña, A., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., Mcdonald, D., Muegge, B., Pirrung, M., Reeder, J., Sevinsky, J., Turnbaugh, P., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J. and Knight, R. 2010. QIIME allows analysis of high-throughput community sequencing data. - Nat Methods. 7: 335–336.

Caruso, V., Song, X., Asquith, M. and Karstens, L. 2019. Performance of Microbiome Sequence Inference Methods in Environments with Varying Biomass (SM Gibbons, Ed.). - mSystems 4: e00163-18.

Chapman, T., Arnqvist, G., Bangham, J. and Rowe, L. 2003. Sexual conflict. - Trends Ecol. Evol. 18: 41–47.

Cherry, M. I. and Gosler, A. G. 2010. Avian eggshell coloration: New perspectives on adaptive explanations. - Biol. J. Linn. Soc. 100: 753–762.

Chiarello, M., Villéger, S., Bouvier, C., Auguet, J. C. and Bouvier, T. 2017. Captive bottlenose dolphins and killer whales harbor a species-specific skin microbiota that varies among individuals. - Sci. Rep. 7: 15269.

Clayton, D. H. 1991. The influence of parasites on host sexual selection. - Parasitol. Today 7: 329–334.

Clutton-Brock, T. H. 1991. The evolution of parental care (Princeton University Press, Ed.).

Clutton-Brock, T. 2007. Sexual Selection in Males and Females. - Science (80-.). 318: 1882–1885.

Clutton-Brock, T. 2009. Sexual selection in females. - Anim. Behav. 77: 3–11.

Cook, M. I., Beissinger, S. R., Toranzos, G. A. and Arendt, W. J. 2005. Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection. - Ecol. Lett. 8: 532–537.

Corti, M., Romano, A., Costanzo, A., Bentz, A. B., Navara, K. J., Parolini, M., Saino, N. and Rubolini, D. 2018. Protoporphyrin-based eggshell pigmentation predicts hatching success and offspring sex ratio in the barn swallow. - J. Avian Biol. 49: 1–17.

Cramp, S. 1998. The complete birds of the western Palearctic on CD-ROM. - Oxford University Press.

Cunningham, E. J. A. and Russell, A. F. 2000. Egg investment is influenced by male attractiveness in the mallard. - Nature 404: 74–77.

Currie, C. R., Scott, J. A., Summerbell, R. C. and Malloch, D. 1999. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. - Nature 398: 701–704.

D'Alba, L. and Shawkey, M. D. 2015. Mechanisms of antimicrobial defense in avian eggs. - J. Ornithol. 156: S399–S408.

Darby, A. C. and Douglas, A. E. 2003. Elucidation of the Transmission Patterns of an Insect-Borne Bacterium. - Appl. Environ. Microbiol. 69: 4403 LP – 4407.

Darwin, C. 1871. The descent of man and selection in relation to sex (J Murray, Ed.).

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A. and Callahan, B. J. 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. - Microbiome 6: 226.

de Goffau, M. C., Lager, S., Salter, S. J., Wagner, J., Kronbichler, A., Charnock-Jones, D. S., Peacock, S. J., Smith, G. C. S. and Parkhill, J. 2018. Recognizing the reagent microbiome. - Nat. Microbiol. 3: 851–853.

Delhey, K., Peters, A. and Kempenaers, B. 2007. Cosmetic Coloration in Birds: Occurrence, Function, and Evolution. - Am. Nat. 169: S145–S158.

Dequiedt, S., Thioulouse, J., Jolivet, C., Saby, N. P. A., Lelievre, M., Maron, P.-A., Martin, M. P., Prévost-Bouré, N. C., Toutain, B., Arrouays, D., Lemanceau, P. and Ranjard, L. 2009. Biogeographical patterns of soil bacterial communities. - Environ. Microbiol. Rep. 1: 251–255.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber,

T., Dalevi, D., Hu, P. and Andersen, G. L. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. - Appl. Environ. Microbiol. 72: 5069–5072.

Devaynes, A., Antunes, A., Bedford, A. and Ashton, P. 2018. Progression in the bacterial load during the breeding season in nest boxes occupied by the Blue Tit and its potential impact on hatching or fledging success. - J. Ornithol. 159: 1009–1017.

Díaz-Lora, S., Pérez-Contreras, T., Azcárate-García, M., Peralta-Sánchez, J. M., Martínez-Bueno, M., Soler, J. J. and Martín-Vivaldi, M. Cosmetic coloration of cross-fostered eggs affects male parental investment in the Hoopoe (Upupa epops). - Under Rev. in press.

Díaz-Lora, S., Pérez-Contreras, T., Azcárate-García, M., Martínez-Bueno, M., Soler, J. J. and Martín-Vivaldi, M. Hoopoe (Upupa epops) male feeding effort is related to female cosmetic egg colouration. - Under Rev. in press.

Díaz-Lora, S., Martín-Vivaldi, M., Juárez-García-Pelayo, N., Azcárate-García, M., Rodríguez-Ruano, S., M., Martínez-Bueno, M. and Soler, J. J. 2019. Data from: experimental old nest material predicts hoopoe Upupa epops eggshell and uropygial gland microbiota. − Dryad Digital Repository, <http://dx.doi. org/10.5061/dryad.8574ft0>.

Dillon, R. J., Vennard, C. T., Buckling, A. and Charnley, A. K. 2005. Diversity of locust gut bacteria protects against pathogen invasion. - Ecol. Lett. 8: 1291–1298.

Douglas, A. E. 1998. Nutritional Interactions in Insect-Microbial Symbioses: Aphids and Their Symbiotic Bacteria Buchnera. - Annu. Rev. Entomol. 43: 17–37.

Dumbrell, A. J., Ferguson, R. M. W. and Clark, D. R. 2017. Microbial Community Analysis by Single-Amplicon High-Throughput Next Generation Sequencing: Data Analysis – From Raw Output to Ecology. - In: McGenity, T. J. et al. (eds), Springer. Hydrocarbon and Lipid Microbiology Protocols: Microbial Quantitation, Community Profiling and Array Approaches, pp. 155–206.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. - Bioinformatics 26: 2460–2461.

Edgar, R. C. 2017. UNCROSS: Filtering of high-frequency cross-talk in 16S amplicon reads. - PeerJ 5: e3889.

Edward, D. A. and Chapman, T. 2011. The evolution and significance of male mate

choice. - Trends Ecol. Evol. 26: 647–654.

Eisenhofer, R., Minich, J. J., Marotz, C., Cooper, A., Knight, R. and Weyrich, L. S. 2019. Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. - Trends Microbiol. 27: 105–117.

Ellegaard, K. M. and Engel, P. 2016. Beyond 16S rRNA Community Profiling: Intra-Species Diversity in the Gut Microbiota . - Front. Microbiol. 7: 1475.

Endler, J. A. 1990. On the measurement and classification of color in studies of animal color patterns. - Biol. J. Linn. Soc. 41: 315–352.

Endler, J. A. and Mielke, P. W. 2005. Comparing color patterns as birds see them. - Biol. J. Linn. Soc. 86: 405–431.

English, P. A. and Montgomerie, R. 2011. Robin's egg blue: Does egg color influence male parental care? - Behav. Ecol. Sociobiol. 65: 1029–1036.

Erckmann, W., Beletsky, L., Orians, G., Johnsen, T., Sharbaugh, S. and D´Antonio, C. 1990. Old nests as cues for nest-site selection: an experimental test with Red-winged Blackbirds. - Condor 92: 113–117.

Ezenwa, V. O. and Williams, A. E. 2014. Microbes and animal olfactory communication: Where do we go from here? - BioEssays 36: 847–854.

Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M. and Xavier, J. B. 2012. Animal Behavior and the Microbiome. - Science (80-.). 338: 198–199.

Fitzpatrick, C. L. and Servedio, M. R. 2018. The evolution of male mate choice and female ornamentation: A review of mathematical models. - Curr. Zool. 64: 323–333.

Flórez, L. V. and Kaltenpoth, M. 2017. Symbiont dynamics and strain diversity in the defensive mutualism between *Lagria* beetles and *Burkholderia*. - Environ. Microbiol. 19: 3674–3688.

Flórez, L. V, Biedermann, P. H. W., Engl, T. and Kaltenpoth, M. 2015. Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. - Nat. Prod. Rep. 32: 904–936.

Folstad, I. and Karter, A. J. 1992. Parasites, bright males, and the immunocompetence handicap. - Am. Nat. 139: 603–622.

Fons, M., Gomez, A. and Karjalainen, T. 2000. Mechanisms of Colonisation and Colonisation Resistance of the Digestive Tract Part 2: Bacteria/Bacteria Interactions. - Microb. Ecol. Health Dis. 12: 240–246.

Fontaine, J. J. and Martin, T. E. 2006. Parent birds assess nest predation risk and adjust

their reproductive strategies. - Ecol. Lett. 9: 428–434.

Fox, H. M. and Vevers, G. 1960. The nature of animal colors.

Friedman, J. and Alm, E. J. 2012. Inferring Correlation Networks from Genomic Survey Data. - PLoS Comput. Biol. 8: e1002687.

Fronstin, R. B., Doucet, S. M. and Christians, J. K. 2016. Haematocrit, eggshell colouration and sexual signaling in the European starling (Sturnus vulgaris). - BMC Ecol. in press.

Gamble, K. C. 2007. Internal Anatomy of the Hornbill Casque Described by Radiography , Contrast Radiography , and Computed Tomography. - J. Avian Med. Surg. 21: 38–49.

Gamble, K. C. 2012. Chapter 37. Squamous Cell Carcinoma in Buceros Hornbills. - In: Miller, R. E. and Fowler, M. (eds), Fowler's Zoo and Wild Animal Medicine. pp. 281–285.

Garamszegi, Z. and Møller, A. P. 2010. Effects of sample size and intraspecific variation in phylogenetic comparative studies : a meta-analytic review. - Biol. Rev. 85: 797–805.

Garamszegi, L. Z. and Møller, A. P. 2011. Nonrandom Variation in Within-Species Sample Size and Missing Data in Phylogenetic Comparative Studies. - Syst. Biol. 60: 876–880.

Gaston, A. J., De Forest, L. N. and G.Noble, D. 1993. Egg Recognition and Egg Stealing in Murres (Uria spp.). - Anim. Behav. 45: 301–306.

Gawryszewski, F. M. 2018. Color vision models: Some simulations, a general n-dimensional model, and the colourvision R package. - Ecol. Evol.: 8159–8170.

Giard, J.-C., Laplace, J.-M., Rincé, A., Pichereau, V., Benachour, A., Leboeuf, C., Flahaut, S., Auffray, Y. and Hartke, A. 2001. The stress proteome of *Enterococcus faecalis*. - Electrophoresis 22: 2947–2954.

Gil-Turnes, M. S. and Fenical, W. 1992. Embryos of Homarus americanus are Protected by Epibiotic Bacteria. - Biol. Bull. 182: 105–108.

Giordano, M., Costantini, D., Pick, J. L. and Tschirren, B. 2015. Female oxidative status, egg antioxidant protection and eggshell pigmentation: a supplemental feeding experiment in great tits. - Behav. Ecol. Sociobiol. 69: 777–785.

Goldstein, G., Flory, K. R., Browne, B. A., Majid, S., Ichida, J. M. and Burtt Jr., E. H.

2004. Bacterial Degradation of Black and White Feathers. - Auk 121: 656–659.

Golob, J. L., Margolis, E., Hoffman, N. G. and Fredricks, D. N. 2017. Evaluating the accuracy of amplicon-based microbiome computational pipelines on simulated human gut microbial communities. - BMC Bioinformatics 18: 283.

Gómez, D. 2006. AVICOL a program to analyse spectrometric data. Last update october 2011. Free executable available at http://sites.google.com/site/avicolprogram/ or from the author at dodogomez@yahoo.fr. in press.

González-Braojos, S., Vela, A. I., Ruiz-De-Castañeda, R., Briones, V., Cantarero, A. and Moreno, J. 2012. Is Nestling Growth Affected by Nest Reuse and Skin Bacteria in Pied Flycatchers *Ficedula hypoleuca?* - Acta Ornithol. 47: 119–127.

Gonzalez, J. C. T., Sheldon, B. C., Collar, N. J. and Tobias, J. A. 2013. A comprehensive molecular phylogeny for the hornbills (Aves: Bucerotidae). - Mol. Phylogenet. Evol. 67: 468–483.

Gosler, A. G., Higham, J. P. and Reynolds, S. J. 2005. Why are birds ' eggs speckled ? - Ecol. Lett. 8: 1105–1113.

Grether, G. F., Hudon, J. and Millie, D. F. 1999. Carotenoid limitation of sexual coloration along an environmental gradient in guppies. - Proc. R. Soc. London. Ser. B Biol. Sci. 266: 1317–1322.

Griggio, M., Valera, F., Casas, A. and Pilastro, A. 2005. Males prefer ornamented females: A field experiment of male choice in the rock sparrow. - Anim. Behav. 69: 1243–1250.

Grizard, S., Dini-Andreote, F., Tieleman, B. I. and Salles, J. F. 2014. Dynamics of bacterial and fungal communities associated with eggshells during incubation. - Ecol. Evol. 4: 1140–1157.

Grizard, S., Versteegh, M. A., Ndithia, H. K., Salles, J. F. and Tieleman, B. I. 2015. Shifts in Bacterial Communities of Eggshells and Antimicrobial Activities in Eggs during Incubation in a Ground-Nesting Passerine. - PLoS One: 10(4).

Gunderson, A. R. 2008. Feather-Degrading Bacteria: A New Frontier in Avian and Host–Parasite Research? - Auk 125: 972–979.

Gwinner, H. and Berger, S. 2005. European starlings: Nestling condition, parasites and green nest material during the breeding season. - J. Ornithol. 146: 365–371.

Haine, E. R. 2008. Symbiont-mediated protection. - Proc. R. Soc. B Biol. Sci. 275: 353–361.

Hamilton, W. D. and Zuk, M. 1982. Heritable true fitness and bright birds: a role for parasites? - Science (80-.). 218: 384–387.

Hanley, D. 2012. The function and evolution of egg colour in birds. - Electron. Theses Diss.: 382.

Hanley, D. and Doucet, S. M. 2009. Egg coloration in ring-billed gulls (Larus delawarensis): A test of the sexual signaling hypothesis. - Behav. Ecol. Sociobiol. 63: 719–729.

Hanley, D., Heiber, G. and Dearborn, D. C. 2008. Testing an Assumption of the Sexual-Signaling Hypothesis: Does Blue-Green Egg Color Reflect Maternal Antioxidant Capacity? - Condor 110: 767–771.

Hanley, D., Doucet, S. M. and Dearborn, D. C. 2010. A Blackmail Hypothesis for the Evolution of Conspicuous Egg Coloration in Birds. - Auk 127: 453–459.

Hargitai, R., Boross, N., Nyiri, Z. and Eke, Z. 2018a. Effects of food limitation on the intensity of blue-green and brown eggshell coloration: an experimental study with the canary. - J. Avian Biol.: e01486.

Hargitai, R., Herényi, M., Nagy, G. and Török, J. 2018b. Eggshell spotting pattern is related to hatching asynchrony, hematocrit value and growth of nestling great tits Parus major. - J. Avian Biol. 49: e01827.

Hart, N. S. 2001. The visual ecology of avian Photoreceptors. - Prog. Retin. Eye Res. 20, No. 5: 675 to 703.

Hart, N. S. 2002. Vision in the peafowl (Aves: Pavo cristatus). - J. Exp. Biol. 205: 3925–3935.

Harvey, P. H. and Pagel, M. D. 1991. The Comparative Method in Evolutionary Biology. - Oxford University Press.

He, Y., Caporaso, J. G., Jiang, X.-T., Sheng, H.-F., Huse, S. M., Rideout, J. R., Edgar, R. C., Kopylova, E., Walters, W. A., Knight, R. and Zhou, H.-W. 2015. Stability of operational taxonomic units: an important but neglected property for analyzing microbial diversity. - Microbiome 3: 20.

Heath-Heckman, E. A. C., Peyer, S. M., Whistler, C. A., Apicella, M. A., Goldman, W. E. and McFall-Ngai, M. J. 2013. Bacterial Bioluminescence Regulates Expression of a Host Cryptochrome Gene in the Squid-Vibrio Symbiosis (J Handelsman, Ed.). - MBio 4: e00167-13.

Heath, K. D. and Tiffin, P. 2007. Context dependence in the coevolution of plant and rhizobial mutualists. - Proc. R. Soc. B Biol. Sci. 274: 1905–1912.

Henry, L. P., Bruijning, M., Forsberg, S. K. G. and Ayroles, J. F. 2019. Can the microbiome influence host evolutionary trajectories? - bioRxiv: 700237.

Hill, M. J. 1997. Intestinal flora and endogenous vitamin synthesis. - Eur. J. Cancer Prev. 6 Suppl 1: S43-45.

Hill, G. E. 2000. Energetic constraints on expression of carotenoid-based plumage coloration. - J. Avian Biol. 31: 559–566.

Hill, G. E. 2002. A red bird in a brown bag: the function and evolution of ornamental plumage coloration in the house finch. - Oxford University Press.

Hill, G. E. 2006. Environmental Regulation of Ornamental Coloration. - In: Hill, G. E. and Mcgraw, K. J. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 507–560.

Hill, G. E., Farmer, K. L. and Beck, M. L. 2004. The effect of mycoplasmosis on carotenoid plumage coloration in male house finches. - J. Exp. Biol. 207: 2095–2099.

Hird, S. M. 2017. Evolutionary Biology Needs Wild Microbiomes. - Front. Microbiol. 8: 725.

Hoffmann, J., Postma, E. and Schaub, M. 2015. Factors influencing double brooding in Eurasian Hoopoes Upupa epops. - Ibis (Lond. 1859). 157: 17–30.

Holveck, M.-J., Doutrelant, C., Guerreiro, R., Perret, P., Gomez, D. and Grégoire, A. 2010. Can eggs in a cavity be a female secondary sexual signal? Male nest visits and modelling of egg visual discrimination in blue tits. - Biol. Lett. 6: 453–457.

Holveck, M., Guerreiro, R., Perret, P., Doutrelant, C. and Grégoire, A. 2019. Eggshell coloration indicates female condition during egg-laying: a field experiment in blue tits. - Biol. J. Linn. Soc. XX: 1–20.

Honza, M., Požgayová, M., Procházka, P. and Cherry, M. I. 2011. Blue-green eggshell coloration is not a sexually selected signal of female quality in an open-nesting polygynous passerine. - Naturwissenschaften 98: 493–499.

Hornung, B. V. H., Zwittink, R. D. and Kuijper, E. J. 2019. Issues and current standards of controls in microbiome research. - FEMS Microbiol. Ecol. 95: fiz045.

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y. and Fukatsu, T. 2010. Wolbachia as a bacteriocyte-associated nutritional mutualist. - Proc. Natl. Acad. Sci. 107: 769–774.

Hoyt, D. F. 1979. Practical methods of estimating volume and fresh weight of bird eggs. -

Auk 96: 73–77.

Jacob, J. and Ziswiler, V. 1982. The uropygial gland. - In: Farner, D. S. et al. (eds), Avian biology, vol VI. Academic Press, pp. 359–362.

Jacob, S., Parthuisot, N., Vallat, A., Ramon-Portugal, F., Helfenstein, F. and Heeb, P. 2015. Microbiome affects egg carotenoid investment, nestling development and adult oxidative costs of reproduction in Great tits. - Funct. Ecol. 29: 1048–1058.

Janzen, D. H. 1985. The natural history of mutualism. - In: Boucher, D. H. (ed), The Biology of Mutualism: Ecology and Evolution. Oxford University Press, pp. 40–99.

Javůrková, V. G., Kreisinger, J., Procházka, P., Požgayová, M., Ševčíková, K., Brlík, V., Adamík, P., Heneberg, P. and Porkert, J. 2019. Unveiled feather microcosm: feather microbiota of passerine birds is closely associated with host species identity and bacteriocin-producing bacteria. - ISME J.: 2363–2376.

Jervis-Bardy, J., Leong, L. E. X., Marri, S., Smith, R. J., Choo, J. M., Smith-Vaughan, H. C., Nosworthy, E., Morris, P. S., O'Leary, S., Rogers, G. B. and Marsh, R. L. 2015. Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. - Microbiome 3: 19.

Jetz, W., Thomas, G. H., Joy, J. B., Hartmann, K. and Mooers, A. O. 2012. The global diversity of birds in space and time. - Nature 491: 444–448.

Johnsen, A., Vesterkjær, K. and Slagsvold, T. 2011. Do Male Pied Flycatchers (Ficedula hypoleuca) Adjust Their Feeding Effort According to Egg Colour? - Ethology 117: 309–317.

Johnstone, R. A. 1995. Sexual selection, honest advertisement and the handicap principle: reviewing the evidence. - Biol. Rev. 70: 1–65.

Kaltenpoth, M., Göttler, W., Herzner, G. and Strohm, E. 2005. Symbiotic Bacteria Protect Wasp Larvae from Fungal Infestation. - Curr. Biol. 15: 475–479.

Kaltz, O. and Koella, J. C. 2003. Host growth conditions regulate the plasticity of horizontal and vertical transmission in holospora undulata, a bacterial parasite of the protozoan paramecium caudatum. - Evolution (N. Y). 57: 1535–1542.

Karstens, L., Asquith, M., Davin, S., Fair, D., Gregory, W. T., Wolfe, A. J., Braun, J. and McWeeney, S. 2019. Controlling for Contaminants in Low-Biomass 16S rRNA Gene Sequencing Experiments (JA Gilbert, Ed.). - mSystems 4: e00290-19.

Kaur, H., Hughes, M. N., Green, C. J., Naughton, P., Foresti, R. and Motterlini, R. 2003.

Interaction of bilirubin and biliverdin with reactive nitrogen species. - FEBS Lett. 543: 113–119.

Keebaugh, E. S., Yamada, R., Obadia, B., Ludington, W. B. and Ja, W. W. 2018. Microbial Quantity Impacts Drosophila Nutrition, Development, and Lifespan. - iScience 4: 247–259.

Kemp, A. C. 2001. Family Bucerotidae (Hornbills). - In: Del Hoyo, J. et al. (eds), Handbook of the birds of the world, Piciformes to Bucerotiformes, vol 6. Lynx Editi. pp. 436–523.

Kemp, D. J., Herberstein, M. E., Fleishman, L. J., Endler, J. a., Bennett, A. T. D., Dyer, A. G., Hart, N. S., Marshall, J. and Whiting, M. J. 2015. An Integrative Framework for the Appraisal of Coloration in Nature. - Am. Nat. 185: 000–000.

Kikuchi, Y., Hayatsu, M., Hosokawa, T., Nagayama, A., Tago, K. and Fukatsu, T. 2012. Symbiont-mediated insecticide resistance. - Proc. Natl. Acad. Sci. 109: 8618–8622.

Kilner, R. M. 2006. The evolution of egg colour and patterning in birds. - Biol. Rev. Camb. Philos. Soc. 81: 383–406.

Kinnaird, M. F. and O'Brien, T. G. 2007. The Ecology and Conservation of Asian Hornbills: Farmers of the Forest.

Knight, R., Vrbanac, A., Taylor, B. C., Aksenov, A., Callewaert, C., Debelius, J., Gonzalez, A., Kosciolek, T., McCall, L. I., McDonald, D., Melnik, A. V., Morton, J. T., Navas, J., Quinn, R. A., Sanders, J. G., Swafford, A. D., Thompson, L. R., Tripathi, A., Xu, Z. Z., Zaneveld, J. R., Zhu, Q., Caporaso, J. G. and Dorrestein, P. C. 2018. Best practices for analysing microbiomes. - Nat. Rev. Microbiol. 16: 410–422.

Kopylova, E., Navas-molina, J. A., Mercier, C., Xu, Z., Mahé, F., He, Y., Zhou, H.-W., Rognes, T., Caporaso, J. G. and Knight, R. 2016. Open-Source Sequence Clustering Methods Improve the State Of the Art. mSystems. 1: 1–16.

Kose, M. and Møller, A. P. 1999. Sexual selection, feather breakage and parasites: the importance of white spots in the tail of the barn swallow (Hirundo rustica). - Behav. Ecol. Sociobiol. 45: 430–436.

Kraaijeveld, K., Kraaijeveld-Smit, F. J. L. and Komdeur, J. 2007. The evolution of mutual ornamentation. - Anim. Behav. 74: 657–677.

Krist, M. and Grim, T. 2007. Are blue eggs a sexually selected signal of female collared flycatchers? A cross-fostering experiment. - Behav. Ecol. Sociobiol. 61: 863–876.

Kuczynski, J., Stombaugh, J., Walters, W. A., González, A., Caporaso, J. G. and Knight,

R. 2012. Using QIIME to analyze 16s rRNA gene sequences from microbial communities. - Curr. Protoc. Microbiol. in press.

Labocha, M. K. and Hayes, J. P. 2012. Morphometric indices of body condition in birds: A review. - J. Ornithol. 153: 1–22.

Lahti, D. C. 2008. Population Differentiation and Rapid Evolution of Egg Color in Accordance With Solar Radiation. - Auk 125: 796–802.

Larsson, A. J. M., Stanley, G., Sinha, R., Weissman, I. L. and Sandberg, R. 2018. Computational correction of index switching in multiplexed sequencing libraries. - Nat. Methods 15: 305–307.

Law-Brown, J. and Meyers, P. R. 2003. *Enterococcus phoeniculicola sp . nov* ., a novel member of the enterococci isolated from the uropygial gland of the Red-billed Woodhoopoe , *Phoeniculus purpureus.* - Int. J. Syst. Evol. Microbiol. 53: 683–685.

Leclaire, S., Nielsen, J. F. and Drea, C. M. 2014. Bacterial communities in meerkat anal scent secretions vary with host sex, age, and group membership. - Behav. Ecol. 25: 996–1004.

Leclaire, S., Jacob, S., Greene, L. K., Dubay, G. R. and Drea, C. M. 2017. Social odours covary with bacterial community in the anal secretions of wild meerkats. - Sci. Rep. 7: 3240.

Leftwich, P. T., Hutchings, M. I. and Chapman, T. 2018. Diet, Gut Microbes and Host Mate Choice. - BioEssays 40: 1800053.

Leitner, S., Marshall, R. C., Leisler, B. and Catchpole, C. K. 2006. Male song quality, egg size and offspring sex in captive canaries (Serinus canaria). - Ethology 112: 554–563.

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D. and Gordon, J. I. 2005. Obesity alters gut microbial ecology. - Proc. Natl. Acad. Sci. 102: 11070–11075.

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R. and Gordon, J. I. 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. - Nat. Rev. Microbiol. 6: 776–788.

Li, D., Chen, B., Zhang, L., Gaur, U., Ma, T., Jie, H., Zhao, G., Wu, N., Xu, Z., Xu, H., Yao, Y., Lian, T., Fan, X., Yang, D., Yang, M., Zhu, Q. and Satkoski Trask, J. 2016. The musk chemical composition and microbiota of Chinese forest musk deer males. - Sci. Rep. 6: 18975.

Lindquist, N., Barber, P. H. and Weisz, J. B. 2005. Episymbiotic microbes as food and defence for marine isopods: unique symbioses in a hostile environment. - Proc. R. Soc. B Biol. Sci. 272: 1209–1216.

Linville, S. U., Breitwisch, R. and Schilling, A. J. 1998. Plumage brightness as an indicator of parental care in northern cardinals. - Anim. Behav. 55: 119–127.

Lopetuso, L. R., Scaldaferri, F., Petito, V. and Gasbarrini, A. 2013. Commensal Clostridia: leading players in the maintenance of gut homeostasis. - Gut Pathog. 5: 23.

López-Rull, I., Gil, M. and Gil, D. 2007. Spots in starling Sturnus unicolor eggs are good indicators of ectoparasite load by Carnus hemapterus (Diptera: Carnidae). - Ardeola 54: 131–134.

López-Rull, I., Pagán, I. and Macías Garcia, C. 2010. Cosmetic enhancement of signal coloration: Experimental evidence in the house finch. - Behav. Ecol. 21: 781–787.

Lozupone, C. and Knight, R. 2005. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. - Appl. Environ. Microbiol. 71: 8228–8235.

Lyu, N., Servedio, M. R., Lloyd, H. and Sun, Y. 2017. The evolution of postpairing male mate choice. - Evolution (N. Y). 71: 1465–1477.

Macpherson, A. J. and Harris, N. L. 2004. Interactions between commensal intestinal bacteria and the immune system. - Nat. Rev. Immunol. 4: 478–485.

Maddison, W. . and Maddison, D. R. 2018. Mesquite: a modular system for evolutionary analysis. Version 3.6.: http://www.mesquiteproject.org.

Maier, R. M., Pepper, I. L. and Gerba, C. P. 2000. Environmental Microbiology. - Academic Press, San Diego, California.

Mainwaring, M. C., Hartley, I. R., Lambrechts, M. M. and Deeming, D. C. 2014. The design and function of birds' nests. - Ecol. Evol. 4: 3909–3928.

Mallows, C. L. 1973. Some Comments on Cp. - Technometrics 15: 661–675.

Maraci, Ö., Engel, K. and Caspers, B. A. 2018. Olfactory communication via microbiota: what is known in birds? - Genes (Basel). in press.

Martín-Platero, A. M., Valdivia, E., Ruíz-Rodríguez, M., Soler, J. J., Martín-Vivaldi, M., Maqueda, M. and Martínez-Bueno, M. 2006. Characterization of antimicrobial substances produced by *Enterococcus faecalis* MRR 10-3, isolated from the uropygial gland of the hoopoe (*Upupa epops*). - Appl. Environ. Microbiol. 72: 4245–4249.

Martín-Vivaldi, M., Palomino, J. J., Soler, M. and Soler, J. J. 1999. Determinants of reproductive success in the Hoopoe Upupa epops, a hole-nesting non-passerine bird with asynchronous hatching. - Bird Study 46: 205–216.

Martín-Vivaldi, M., Ruiz-Rodríguez, M., Soler, J. J., Peralta-Sánchez, J. M., Méndez, M., Valdivia, E., Martín-Platero, A. M. and Martínez-Bueno, M. 2009. Seasonal, sexual and developmental differences in hoopoe Upupa epops preen gland morphology and secretions: Evidence for a role of bacteria. - J. Avian Biol. 40: 191–205.

Martín-Vivaldi, M., Peña, A., Peralta-Sánchez, J. M., Sánchez, L., Ananou, S., Ruiz-Rodríguez, M. and Soler, J. J. 2010. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. - Proc. Biol. Sci. 277: 123–130.

Martín-Vivaldi, M., Soler, J. J., Peralta-Sánchez, J. M., Arco, L., Martín-Platero, A. M., Martínez-Bueno, M., Ruiz-Rodríguez, M. and Valdivia, E. 2014a. Special structures of hoopoe eggshells enhance the adhesion of symbiont-carrying uropygial secretion that increase hatching success. - J. Anim. Ecol. 83: 1289–1301.

Martín-Vivaldi, M., Doña, J., Romero Masegosa, J. and Soto Cárdenas, M. 2014b. Abubilla - Upupa epops Linnaeus, 1758. - En Encicl. Virtual los Vertebr. Españoles. Salvador. A., Morales, M. B. (Eds.). Mus. Nac. Ciencias Nat. Madrid. http//www.vertebradosibericos.org/

Martín-Vivaldi, M., Soler, J. J., Martínez-García, Á., Arco, L., Juárez-García-Pelayo, N., Ruiz-Rodríguez, M. and Martínez-Bueno, M. 2018. Acquisition of Uropygial Gland Microbiome by Hoopoe Nestlings. - Microb. Ecol. 76: 285–297.

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. - EMBnet.journal 17: 10–12.

Martin, T. E. and Badyaev, A. V 1996. Sexual dichromatism in birds: importance of nest predation and nest location for females versus males. - Evolution (N. Y). 50: 2454–2460.

Martínez-De La Puente, J., Merino, S., Moreno, J., Tomás, G., Morales, J., Lobato, E., García-Fraile, S. and Martínez, J. 2007. Are eggshell spottiness and colour indicators of health and condition in blue tits Cyanistes caeruleus? - J. Avian Biol. 38: 377–384.

Martínez-García, Á. 2015. Biodiversidad bacteriana de la secreción uropigial y la cáscara de los huevos de abubilla: estabilidad y adquisición.

Martínez-García, Á., Soler, J. J., Rodríguez-Ruano, S. M., Martínez-Bueno, M., Martín-

Platero, A. M., Juárez-García, N. and Martín-Vivaldi, M. 2015. Preening as a Vehicle for Key Bacteria in Hoopoes. - Microb. Ecol. 70: 1024–1033.

Martínez-García, Á., Martín-Vivaldi, M., Rodríguez-Ruano, S. M., Peralta-Sánchez, J. M., Valdivia, E. and Soler, J. J. 2016a. Nest bacterial environment affects microbiome of hoopoe eggshells, but not that of the uropygial secretion. - PLoS One 11(7): e0158158.

Martínez-García, Á., Martín-Vivaldi, M., Ruiz-Rodríguez, M., Martínez-Bueno, M., Arco, L., Rodríguez-Ruano, S. M., Peralta-Sánchez, J. M. and Soler, J. J. 2016b. The Microbiome of the Uropygial Secretion in Hoopoes Is Shaped Along the Nesting Phase. - Microb. Ecol. 72: 252–261.

Massaro, M., Davis, L. S. and Darby, J. T. 2003. Carotenoid-derived ornaments reflect parental quality in male and female yellow-eyed penguins (Megadyptes antipodes). - Behav. Ecol. Sociobiol. 55: 169–175.

Mazgajski, T. D. 2007. Effect of old nest material on nest site selection and breeding parameters in secondary hole nesters — a review. - Acta Ornithol. 42: 1–14.

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., Desantis, T. Z., Probst, A., Andersen, G. L., Knight, R. and Hugenholtz, P. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. - ISME J. 6: 610–618.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E., Rawls, J. F., Reid, A., Ruby, E. G., Rumpho, M., Sanders, J. G., Tautz, D. and Wernegreen, J. J. 2013. Animals in a bacterial world, a new imperative for the life sciences. - Proc. Natl. Acad. Sci. U. S. A. 110: 3229–3236.

Mcgraw, K. J. and Hill, G. E. 2004. Plumage color as a dynamic trait : carotenoid pigmentation of male house finches (Carpodacus mexicanus) fades during the breeding season. - Can. J. Zool 82: 734–738.

McGraw, K. J. and Ardia, D. R. 2003. Carotenoids, Immunocompetence, and the Information Content of Sexual Colors: An Experimental Test. - Am. Nat. 162: 704–712.

McKnight, D. T., Huerlimann, R., Bower, D. S., Schwarzkopf, L., Alford, R. A. and Zenger, K. R. 2019. microDecon: A highly accurate read-subtraction tool for the

post-sequencing removal of contamination in metabarcoding studies. - Environ. DNA 1: 14–25.

Mikšík, I., Holáň, V. and Deyl, Z. 1996. Avian eggshell pigments and their variability. - Comp. Biochem. Physiol. 113: 607–612.

Miller, R. E., Trampel, D. W., Boever, W. J. and Kling, M. A. 1985. Carcinoma in the Casque of a Greater Indian Hornbill (Buceros bicornis). - J. Zoo Anim. Med. 16: 131–136.

Minich, J. J., Zhu, Q., Janssen, S., Hendrickson, R., Amir, A., Vetter, R., Hyde, J., Doty, M. M., Stillwell, K., Benardini, J., Kim, J. H., Allen, E. E., Venkateswaran, K. and Knight, R. 2018. KatharoSeq Enables High-Throughput Microbiome Analysis from Low-Biomass Samples (MJ McFall-Ngai, Ed.). - mSystems 3: e00218-17.

Møller, A. P. 1993. Sexual Selection in the Barn Swallow Hirundo rustica. III. Female Tail Ornaments. - Evolution (N. Y). 47: 417–431.

Møller, A. P. and Erritzoe, J. 1996. Parasite Virulence and Host Immune Defense : Host Immune Response is Related to Nest Reuse in Birds. - Evolution (N. Y). 50: 2066–2072.

Møller, A. P., Arriero, E., Lobato, E. and Merino, S. 2009. A meta-analysis of parasite virulence in nestling birds. - Biol. Rev. 84: 567–588.

Montgomerie, R. 2006. Cosmetic and adventitious colors. - In: Hill, E. and McGraw, K. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 399–427.

Morales, J., Sanz, J. J. and Moreno, J. 2006. Egg colour reflects the amount of yolk maternal antibodies and fledging success in a songbird. - Biol. Lett. 2: 334–336.

Morales, J., Moreno, J., Merino, S., Sanz, J. J., Tomás, G., Arriero, E., Lobato, E. and Martínez-De La Puente, J. 2007. Female ornaments in the Pied Flycatcher Ficedula hypoleuca: Associations with age, health and reproductive success. - Ibis (Lond. 1859). 149: 245–254.

Morales, J., Velando, A. and Moreno, J. 2008. Pigment allocation to eggs decreases plasma antioxidants in a songbird. - Behav. Ecol. Sociobiol. 63: 227–233.

Moran, N. A. 2006. Symbiosis. - Curr. Biol. 16: R866–R871.

Moran, N. A., McCutcheon, J. P. and Nakabachi, A. 2008. Genomics and Evolution of Heritable Bacterial Symbionts. - Annu. Rev. Genet. 42: 165–190.

Moreau, R. E. 1934. Breeding Habits of Hornbills. - Nature 134: 899.

Moreno, J. and Osorno, J. L. 2003. Avian egg colour and sexual selection: Does eggshell pigmentation reflect female condition and genetic quality? - Ecol. Lett. 6: 803–806.

Moreno, J., Briones, V., Merino, S., Ballesteros, C., Sanz, J. J. and Tomás, G. 2003. Beneficial Effects of Cloacal Bacteria on Growth and Fledging Size in Nestling Pied Flycatchers (Ficedula Hypoleuca) in Spain. - Auk 120: 784–790.

Moreno, J., Osorno, J. L., Morales, J., Merino, S. and Tomás, G. 2004. Egg Colouration and Male Parental Effort in the Pied Flycatcher Ficedula Hypoleuca. - J. Avian Biol. 35: 300–304.

Moreno, J., Morales, J., Lobato, E., Merino, S., Tomás, G. and Martínez-De La Puente, J. 2005. Evidence for the signaling function of egg color in the pied flycatcher Ficedula hypoleuca. - Behav. Ecol. 16: 931–937.

Moreno, J., Morales, J., Lobato, E., Merino, S., Tomás, G. and Martínez-De La Puente, J. 2006a. More colourful eggs induce a higher relative paternal investment in the pied flycatcher Ficedula hypoleuca: A cross-fostering experiment. - J. Avian Biol. 37: 555–560.

Moreno, J., Lobato, E., Morales, J., Merino, S., Tomás, G., Martínez-De La Puente, J., Sanz, J. J., Mateo, R. and Soler, J. J. 2006b. Experimental evidence that egg color indicates female condition at laying in a songbird. - Behav. Ecol. 17: 651–655.

Moreno, J., Lobato, E., Merino, S. and Martínez-De La Puente, J. 2008. Blue-green eggs in pied flycatchers: An experimental demonstration that a supernormal stimulus elicits improved nestling condition. - Ethology 114: 1078–1083.

Morton, J. T., Sanders, J., Quinn, R. A., McDonald, D., Gonzalez, A., Vázquez-Baeza, Y., Navas-Molina, J. A., Song, S. J., Metcalf, J. L., Hyde, E. R., Lladser, M., Dorrestein, P. C. and Knight, R. 2017. Balance Trees Reveal Microbial Niche Differentiation (JK Jansson, Ed.). - mSystems 2: e00162-16.

Mushegian, A. A., Walser, J.-C., Sullam, K. E. and Ebert, D. 2018. The microbiota of diapause: How host–microbe associations are formed after dormancy in an aquatic crustacean. - J. Anim. Ecol. 87: 400–413.

Naish, D. 2015. The Ecology and Conservation of Asian Hornbills: Farmers of the Forest. - Hist. Biol. 27: 954–956.

Navas-Molina, J. A., Peralta-Sánchez, J. M., González, A., McMurdie, P. J., Vázquez-Baeza, Y., Xu, Z., Ursell, L. K., Lauber, C., Zhou, H., Song, S. J., Huntley, J.,

Ackermann, G. L., Berg-Lyons, D., Holmes, S., Caporaso, J. G. and Knight, R. 2013. Advancing our understanding of the human microbiome using QIIME. - Elsevier Inc.

Negro, J. J., Margalida, A., Hiraldo, F. and Heredia, R. 1999. The function of the cosmetic coloration of bearded vultures : when art imitates life. - Anim. Behav. S8: 14–17.

Nicholls, E. M. and Rienits, K. G. 1971. Tryptophan derivatives and pigment in the hair of some Australian marsupials. - Int. J. Biochem. 2: 593–603.

Ödeen, A. and Håstad, O. 2003. Complex distribution of avian color vision systems revealed by sequencing the SWS1 opsin from total DNA. - Mol. Biol. Evol. 20: 855–861.

Oliver, K. M., Russell, J. A., Moran, N. A. and Hunter, M. S. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. - Proc. Natl. Acad. Sci. 100: 1803 LP – 1807.

Olson, V. A. and Owens, I. P. F. 1998. Costly sexual signals: are carotenoids rare, risky or required? - Trends Ecol. Evol. 13: 510–514.

Olsson, K. and Allander, K. 1995. Do Fleas, and/or Old Nest Material, Influence Nest-site preference in Hole-nesting Passerines? - Ethology 101: 160–170.

Olsson, P., Lind, O. and Kelber, A. 2015. Bird colour vision: behavioural thresholds reveal receptor noise. - J. Exp. Biol. 218: 184–193.

Orell, M., Rytkönen, S. and Ilomäki, K. 1993. Do pied flycatchers prefer nest boxes with old nest material? - Ann. Zool. Fennici 30: 313–316.

Pagel, M. 1997. Inferring evolutionary processes from phylogenies. - Zool. Scr. 26: 331–348.

Pagel, M. 1999. Inferring the historical patterns of biological evolution. - Nature 401: 877–884.

Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A. and Brown, P. O. 2007. Development of the Human Infant Intestinal Microbiota. - PLoS Biol. 5: e177.

Paradis, E., Claude, J. and Strimmer, K. 2004. APE : Analyses of Phylogenetics and Evolution in R language. - Bioinformatics 20: 289–290.

Paz, L.-C., Schramm, A. and Lund, M. B. 2017. Biparental transmission of Verminephrobacter symbionts in the earthworm Aporrectodea tuberculata (Lumbricidae). - FEMS Microbiol. Ecol. 93: fix025.

Pearce, D. S., Hoover, B. A., Jennings, S., Nevitt, G. A. and Docherty, K. M. 2017.

Morphological and genetic factors shape the microbiome of a seabird species (Oceanodroma leucorhoa) more than environmental and social factors. - Microbiome 5: 146.

Peig, J. and Green, A. J. 2010. The paradigm of body condition: A critical reappraisal of current methods based on mass and length. - Funct. Ecol. 24: 1323–1332.

Peralta-Sanchez, J. M., Møller, A. P., Martin-Platero, A. M. and Soler, J. J. 2010. Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: An experimental study. - Funct. Ecol. 24: 426–433.

Peralta-Sánchez, J. M., Martín-Vivaldi, M., Martín-Platero, A. M., Martínez-Bueno, M., Oñate, M., Ruiz-Rodríguez, M. and Soler, J. J. 2012. Avian life history traits influence eggshell bacterial loads: A comparative analysis. - Ibis (Lond. 1859). 154: 725–737.

Peralta-Sánchez, J. M., Soler, J. J., Martín-Platero, A. M., Knight, R., Martínez-Bueno, M. and Møller, A. P. 2014. Eggshell Bacterial Load Is Related to Antimicrobial Properties of Feathers Lining Barn Swallow Nests. - Microb. Ecol. 67: 480–487.

Pérez-Rodríguez, L., Mougeot, F. and Bortolotti, G. R. 2011. The effects of preen oils and soiling on the UV – visible reflectance of carotenoid-pigmented feathers. - Behav. Ecol. Sociobiol. 65: 1425–1435.

Piault, R., Gasparini, J., Bize, P., Paulet, M., McGraw, K. J. and Roulin, A. 2008. Experimental support for the makeup hypothesis in nestling tawny owls (Strix aluco). - Behav. Ecol. 19: 703–709.

Plard, F., Arlettaz, R. and Schaub, M. 2018. Hoopoe males experience intra-seasonal while females experience inter-seasonal reproductive costs. - Oecologia 186: 665–675.

Podofillini, S., Cecere, J. G., Griggio, M., Curcio, A., De Capua, E. L., Fulco, E., Pirrello, S., Saino, N., Serra, L., Visceglia, M. and Rubolini, D. 2018. Home, dirty home: effect of old nest material on nest-site selection and breeding performance in a cavity-nesting raptor. - Curr. Zool.: 1–10.

Poláček, M., Griggio, M., Mikšík, I., Bartíková, M., Eckenfellner, M. and Hoi, H. 2017. Eggshell coloration and its importance in postmating sexual selection. - Ecol. Evol. 7: 941–949.

Poonswad, P., Kemp, A. and Strange, M. 2013. Hornbills of the world: A photographic guide.

Price, M. N., Dehal, P. S. and Arkin, A. P. 2009. Fasttree: Computing large minimum evolution trees with profiles instead of a distance matrix. - Mol. Biol. Evol. 26: 1641–1650.

Prum, R. O. 2006. Anatomy, Physics, and Evolution of Structural Colors. - In: Hill, G. E. and Mcgraw, K. J. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 295–353.

Refsnider, J. M. and Janzen, F. J. 2010. Putting Eggs in One Basket: Ecological and Evolutionary Hypotheses for Variation in Oviposition-Site Choice. - Annu. Rev. Ecol. Evol. Syst. 41: 39–57.

Reichlin, T. S., Hobson, K. A., Van Wilgenburg, S. L., Schaub, M., Wassenaar, L. I., Martín-Vivaldi, M., Arlettaz, R. and Jenni, L. 2013. Conservation through connectivity: Can isotopic gradients in Africa reveal winter quarters of a migratory bird? - Oecologia 171: 591–600.

Renoult, J. P., Kelber, A. and Schaefer, H. M. 2017. Colour spaces in ecology and evolutionary biology. - Biol. Rev. 92: 292–315.

Reynolds, S. J., Martin, G. R. and Cassey, P. 2009. Is sexual selection blurring the functional significance of eggshell coloration hypotheses? - Anim. Behav. 78: 209–215.

Richard, F.-J. 2017. Symbiotic Bacteria Influence the Odor and Mating Preference of Their Hosts. - Front. Ecol. Evol. 5: 1–9.

Rideout, J. R., He, Y., Navas-Molina, J. A., Walters, W. A., Ursell, L. K., Gibbons, S. M., Chase, J., McDonald, D., Gonzalez, A., Robbins-Pianka, A., Clemente, J. C., Gilbert, J. A., Huse, S. M., Zhou, H.-W., Knight, R. and Caporaso, J. G. 2014. Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. - PeerJ 2: e545.

Riehl, C. 2011. Paternal Investment and the "Sexually Selected Hypothesis" For the Evolution of Eggshell Coloration: Revisiting the Assumptions. - Auk 128: 175–179.

Riley, M. A. and Wertz, J. E. 2002. Bacteriocins: Evolution, Ecology, and Application. - Annu. Rev. Microbiol. 56: 117–137.

Rizzo, C. and Lo Giudice, A. 2018. Marine Invertebrates: Underexplored Sources of Bacteria Producing Biologically Active Molecules. - Diversity 10: 52.

Rodríguez-Ruano, S. M. 2015. Diversidad bacteriana en la glándula uropigial de la

abubilla: dinámica estacional y beneficios asociados.

Rodríguez-Ruano, S. M., Martín-Vivaldi, M., Martín-Platero, A. M., López-López, J. P., Peralta-Sánchez, J. M., Ruiz-Rodríguez, M., Soler, J. J., Valdivia, E. and Martínez-Bueno, M. 2015. The hoopoe's uropygial gland hosts a bacterial community influenced by the living conditions of the bird. - PLoS One 10: 1–18.

Rodríguez-Ruano, S. M., Martín-Vivaldi, M., Peralta-Sánchez, J. M., García-Martín, A. B., Martínez-García, Á., Soler, J. J., Valdivia, E. and Martínez-Bueno, M. 2018. Seasonal and Sexual Differences in the Microbiota of the Hoopoe Uropygial Secretion. - Genes (Basel). 9: 407.

Rognes, T., Flouri, T., Nichols, B., Quince, C. and Mahé, F. 2016. VSEARCH: a versatile open source tool for metagenomics. - PeerJ 4: e2584.

Rolig, A. S., Parthasarathy, R., Burns, A. R., Bohannan, B. J. M. and Guillemin, K. 2015. Individual Members of the Microbiota Disproportionately Modulate Host Innate Immune Responses. - Cell Host Microbe 18: 613–620.

Rollins-Smith, L. A., Ramsey, J. P., Pask, J. D., Reinert, L. K. and Woodhams, D. C. 2011. Amphibian Immune Defenses against Chytridiomycosis: Impacts of Changing Environments. - Integr. Comp. Biol. 51: 552–562.

Rosvall, K. A. 2011. Intrasexual competition in females: evidence for sexual selection? - Behav. Ecol. 22: 1131–1140.

Rubolini, D., Liker, A., Garamszegi, L. Z., Møller, A. P. and Saino, N. 2015. Using the birdtree.org website to obtain robust phylogenies for avian comparative studies: A primer. - Curr. Zool. 61: 959–965.

Ruiz-Castellano, C., Tomás, G., Ruiz-Rodríguez, M., Martín-Gálvez, D. and Soler, J. J. 2016. Nest material shapes eggs bacterial environment. - PLoS One 11: e0148894.

Ruiz-de-Castañeda, R., Burtt Jr, E. H., González-Braojos, S. and Moreno, J. 2012. Bacterial degradability of an intrafeather unmelanized ornament: a role for feather-degrading bacteria in sexual selection? - Biol. J. Linn. Soc. 105: 409–419.

Ruiz-De-Castañeda, R., Burtt, E. H., González-Braojos, S. and Moreno, J. 2015. Bacterial degradability of white patches on primary feathers is associated with breeding date and parental effort in a migratory bird. - Ibis (Lond. 1859). 157: 871–876.

Ruiz-Rodríguez, M., Azcárate-García, M., Ruiz-Castellano, C., Tomás, G., Díaz-Lora, S., Pérez-Contreras, T. and Soler, J. J. Antimicrobial capacity is related to body colouration and reproductive success in female spotless starlings. - Under Rev. in

press.

Ruiz-Rodríguez, M., Valdivia, E., Soler, J. J., Martín-Vivaldi, M., Martín-Platero, A. M. and Martínez-Bueno, M. 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. - J. Exp. Biol. 212: 3621–6.

Ruiz-Rodríguez, M., Valdivia, E., Martín-Vivaldi, M., Martín-Platero, A. M., Martínez-Bueno, M., Méndez, M., Peralta-Sánchez, J. M. and Soler, J. J. 2012. Antimicrobial activity and genetic profile of *enteroccoci* isolated from hoopoes uropygial gland. - PLoS One 7: 1–6.

Ruiz-Rodríguez, M., Martínez-Bueno, M., Martín-Vivaldi, M., Valdivia, E. and Soler, J. J. 2013. Bacteriocins with a broader antimicrobial spectrum prevail in enterococcal symbionts isolated from the hoopoe's uropygial gland. - FEMS Microbiol. Ecol. 85: 495–502.

Ruiz-Rodríguez, M., Soler, J. J., Martín-Vivaldi, M., Martín-Platero, A. M., Méndez, M., Peralta-Sánchez, J. M., Ananou, S., Valdivia, E. and Martínez-Buenob, M. 2014. Environmental factors shape the community of symbionts in the hoopoe uropygial gland more than genetic factors. - Appl. Environ. Microbiol. 80: 6714–6723.

Ruiz-Rodríguez, M., Tomás, G., Martín-Gálvez, D., Ruiz-Castellano, C. and Soler, J. J. 2015. Bacteria and the evolution of honest signals. The case of ornamental throat feathers in spotless starlings. - Funct. Ecol. 29: 701–709.

Ruiz-Rodríguez, M., Møller, A. P., Mousseau, T. A. and Soler, J. J. 2016. Defenses against keratinolytic bacteria in birds living in radioactively contaminated areas. - Sci. Nat. 103: 71.

Sachs, J. L., Skophammer, R. G. and Regus, J. U. 2011. Evolutionary transitions in bacterial symbiosis. - Proc. Natl. Acad. Sci. 108: 10800 LP – 10807.

Saino, N., Romano, M., Rubolini, D., Ambrosini, R., Caprioli, M., Milzani, A., Costanzo, A., Colombo, G., Canova, L. and Wakamatsu, K. 2013. Viability Is Associated with Melanin-Based Coloration in the Barn Swallow (Hirundo rustica). - PLoS One 8: e60426.

Sanz, J. J. and García-Navas, V. 2009. Eggshell pigmentation pattern in relation to breeding performance of blue tits Cyanistes caeruleus. - J. Anim. Ecol. 78: 31–41.

Scott, J. J., Oh, D.-C., Yuceer, M. C., Klepzig, K. D., Clardy, J. and Currie, C. R. 2008. Bacterial Protection of Beetle-Fungus Mutualism. - Science (80-.). 322: 63.

Searcy, W. A. and Nowicki, S. 2005. The evolution of animal communication. - Princeton Univ. Press, Princeton, NJ.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S. and Huttenhower, C. 2011. Metagenomic biomarker discovery and explanation. - Genome Biol. 12: R60.

Senar, J. C. and Pascual, J. 1997. Tarsus lenght predictor body size. - Ardea 85: 269–274.

Sender, R., Fuchs, S. and Milo, R. 2016. Revised Estimates for the Number of Human and Bacteria Cells in the Body. - PLOS Biol. 14: e1002533.

Sergio, F. and Newton, I. 2003. Occupancy as a measure of habitat quality. - J Anim Ecol. 72: 857–865.

Shapira, M. 2016. Gut Microbiotas and Host Evolution: Scaling Up Symbiosis. - Trends Ecol. Evol. 31: 539–549.

Sharon, G., Segal, D., M.Ringo, H., Hefetz, A., Zilber-Rosenberg, I. and Rosenberg, E. 2010. Commensal bacteria play a role in mating preference of Drosophila melanogaster. - PNAS 107: 20051–20056.

Shawkey, M. D., Pillai, S. R. and Hill, G. E. 2003. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. - J. Avian Biol. 34: 345–349.

Shawkey, M. D., Pillai, S. R. and Hill, G. E. 2009. Do feather-degrading bacteria affect sexually selected plumage color? - Naturwissenschaften 96: 123–128.

Sheldon, B. C. 2000. Differential allocation: Tests, mechanisms and implications. - Trends Ecol. Evol. 15: 397–402.

Shephard, K. L. 1994. Functions for fish mucus. - Rev. Fish Biol. Fish. 4: 401–429.

Sherwin, E., Bordenstein, S. R., Quinn, J. L., Dinan, T. G. and Cryan, J. F. 2019. Microbiota and the social brain. - Science (80-.). 366: eaar2016.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. and Ishikawa, H. 2000. Genome sequence of the endocellular bacterial symbiont of aphids Buchnera sp. APS. - Nature 407: 81–86.

Siefferman, L., Navara, K. J. and Hill, G. E. 2006. Egg coloration is correlated with female condition in eastern bluebirds (Sialia sialis). - Behav. Ecol. Sociobiol. 59: 651–656.

Singleton, D. R. and Harper, R. G. 1996. Bacteria in Old House Wren nests. - J F. Ornithol 69: 71–74.

Smith, H. G. and Montgomerie, R. 1991. Nestling American robins compete with siblings

by begging. - Behav. Ecol. Sociobiol. 29: 307–312.

Soini, H. A., Whittaker, D. J., Wiesler, D., Ketterson, E. D. and Novotny, M. V. 2013. Chemosignaling diversity in songbirds: Chromatographic profiling of preen oil volatiles in different species. - J. Chromatogr. A 1317: 186–192.

Soler, J. J. and Møller, A. P. 1996. A comparative analysis of the evolution of variation in appearance of eggs of European passerines in relation to brood parasitism. - Behav. Ecol. 7: 89–94.

Soler, J. J., Moreno, J., Aviles, J. M. and Møller, A. P. 2005. Blue and green egg-color intensity is associated with parental effort and mating system in passerines: support for the sexual selection hypothesis. - Evolution (N. Y). 59: 636–644.

Soler, J. J., Martín-Vivaldi, M., Ruiz-Rodríguez, M., Valdivia, E., Martín-Platero, A. M., Martínez-Bueno, M., Peralta-Sánchez, J. M. and Méndez, M. 2008a. Symbiotic association between hoopoes and antibiotic-producing bacteria that live in their uropygial gland. - Funct. Ecol. 22: 864–871.

Soler, J. J., Navarro, C., Contreras, T. P., Avilés, J. M. and Cuervo, J. J. 2008b. Sexually Selected Egg Coloration in Spotless Starlings. - Am. Nat. 171: 183–194.

Soler, J. J., Martín-vivaldi, M., Peralta-sánchez, J. M. and Ruiz-rodríguez, M. 2010. Antibiotic-Producing Bacteria as a Possible Defence of Birds against Pathogenic Microorganisms. - Open Ornithol. J. 3: 93–100.

Soler, J. J., Martín-Vivaldi, M., Peralta-Sánchez, J. M., Arco, L. and Juárez-García-Pelayo, N. 2014. Hoopoes color their eggs with antimicrobial uropygial secretions. - Naturwissenschaften 101: 697–705.

Soler, J. J., Martínez-García, Á., Rodríguez-Ruano, S. M., Martínez-Bueno, M., Martín-Platero, A. M., Peralta-Sánchez, J. M. and Martín-Vivaldi, M. 2016. Nestedness of hoopoes' bacterial communities: symbionts from the uropygial gland to the eggshell. - Biol. J. Linn. Soc. 118: 763–773.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martín-Vivaldi, M., Martínez-de la Puente, J., Ruiz-Rodríguez, M. and Tomás, G. 2017. Telomere length and dynamics of spotless starling nestlings depend on nest-building materials used by parents. - Anim. Behav. 126: 89–100.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martínez-de la Puente, J., Ruiz-Rodríguez, M. and Tomás, G. 2018. Egg colouration predicts brood size, telomere length and

body condition of spotless starling fledglings. - J. Avian Biol. 49: 1–12.

Soler, J. J., Morales, J., Cuervo, J. J. and Moreno, J. 2019. Conspicuousness of passerine females is associated with the nest-building behaviour of males. - Biol. J. Linn. Soc. 126: 824–835.

Solís, J. C. and de Lope, F. 1995. Nest and Egg Crypsis in the Ground-Nesting Stone Curlew Burhinus oedicnemus. - J. Avian Biol. 26: 135–138.

Spicer, M. E., Pruitt, J. N. and Keiser, C. N. 2019. Spiders, microbes and sex: Bacterial exposure on copulatory organs alters mating behaviour in funnel-web spiders. - Ethology 125: 677–685.

Spor, A., Koren, O. and Ley, R. 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. - Nat. Rev. Microbiol. 9: 279–290.

Stanback, M. T. and Rockwell, E. K. 2003. Nest-site fidelity in Eastern Bluebirds (Sialia sialis) depends on the quality of alternative cavities. - Auk 120: 1029–1032.

Statsoft, I. 2006. STATISTICA (data analysis software system), version 7. Available at www.statsoft.com.

Steinert, M., Hentschel, U. and Hacker, J. 2000. Symbiosis and pathogenesis: evolution of the microbe-host interaction. - Naturwissenschaften 87: 1—11.

Stoddard, M. C. and Prum, R. O. 2008. Evolution of avian plumage color in a tetrahedral colour space: a phylogenetic analysis of New World buntings. - Am. Nat. 171: 755–776.

Stoddard, M. C. and Osorio, D. 2019. Animal Coloration Patterns: Linking Spatial Vision to Quantitative Analysis. - Am. Nat. 193: 000–000.

Stoddard, M. C., Fayet, A. L., Kilner, R. M. and Hinde, C. A. 2012. Egg speckling patterns do not advertise offspring quality or influence male provisioning in great tits. - PLoS One 7: e40211.

Suedmeyer, W. K., McCaw, D. and Turnquist, S. 2001. Attempted Photodynamic Therapy of Squamous Cell Carcinoma in the Casque of a Great Hornbill (Buceros bicornis). - J. Avian Med. Surg. 15: 44–49.

Sumasgutner, P., Vasko, V., Varjonen, R. and Korpimäki, E. 2014. Public information revealed by pellets in nest sites is more important than ecto-parasite avoidance in the settlement decisions of Eurasian kestrels. - Behav. Ecol. Sociobiol. 68: 2023–2034.

Surmacki, A. and Nowakowski, J. K. 2007. Soil and preen waxes influence the expression of carotenoid-based plumage coloration. - Naturwissenschaften 94: 829–835.

Suzuki, T. A. 2017. Links between Natural Variation in the Microbiome and Host Fitness in Wild Mammals. - Integr. Comp. Biol. 57: 756–769.

Swynnerton, C. F. M. 1916. On the coloration of the mouths and eggs of birds. II. On the coloration of eggs. - Ibis (Lond. 1859). 4: 529–606.

Swynnerton, C. F. M. 1918. Rejections by birds of eggs unlike their own: with remarks on some of the cuckoo problems. - Ibis (Lond. 1859).: 127–154.

Team, R. D. C. 2019. R: A Language and Environment for Statistical Computing (RDC Team, Ed.). - R Found. Stat. Comput. in press.

Theis, K. R., Venkataraman, A., Dycus, J. A., Koonter, K. D., Schmitt-Matzen, E. N., Wagner, A. P., Holekamp, K. E. and Schmidt, T. M. 2013. Symbiotic bacteria appear to mediate hyena social odors. - Proc. Natl. Acad. Sci. 110: 19832–19837.

Tomás, G., Merino, S., Moreno, J., Sanz, J. J., Morales, J. and García-Fraile, S. 2006. Nest Weight and Female Health in the Blue Tit (Cyanistes Caeruleus). - Auk 123: 1013–1021.

Tomás, G., Merino, S., Moreno, J. and Morales, J. 2007. Consequences of nest reuse for parasite burden and female health and condition in blue tits, Cyanistes caeruleus. - Anim. Behav. 73: 805–814.

Tomás, G., Martín-Gálvez, D., Ruiz-Castellano, C., Ruiz-Rodríguez, M., Peralta-Sánchez, J. M., Martín-Vivaldi, M. and Soler, J. J. 2018. Ectoparasite Activity During Incubation Increases Microbial Growth on Avian Eggs. - Microb. Ecol. 76: 555–564.

Trail, P. W. 2007. African hornbills: keystone species threatened by habitat loss, hunting and international trade. - Ostrich 78: 609–613.

Trivers, R. L. 1972. Parental investment and sexual selection. - In: Campbell, B. (ed), Sexual selection and the descent of man 1871-1971. pp. 136–179.

Tschumi, M., Schaub, M. and Arlettaz, R. 2014. Territory occupancy and parental quality as proxies for spatial prioritization of conservation areas. - PLoS One 9: e97679.

Uchida, Y. 1970. On the color change in Japanese crested ibis. A new type of cosmetic coloration in birds. - Misc Rep Yamashina Inst Ornithol 6: 54–72. In Japanese, with English summary.

Umesaki, Y., Setoyama, H., Matsumoto, S., Imaoka, A. and Itoh, K. 1999. Differential Roles of Segmented Filamentous Bacteria and Clostridia in Development of the Intestinal Immune System. - Infect. Immun. 67: 3504 LP – 3511.

Underwood, T. and Sealy, S. 2002. Adaptive significance of egg coloration. - In: Deeming DC (ed), Avian incubation: behaviour, environment, and evolution. Oxford Uni. pp. 280–298.

van Dongen, W. F., White, J., Brandl, H. B., Moodley, Y., Merkling, T., Leclaire, S., Blanchard, P., Danchin, É., Hatch, S. A. and Wagner, R. H. 2013. Age-related differences in the cloacal microbiota of a wild bird species. - BMC Ecol. 13: 11.

van Veelen, H. P. J., Falcao Salles, J. and Tieleman, B. I. 2017. Multi-level comparisons of cloacal, skin, feather and nest-associated microbiota suggest considerable influence of horizontal acquisition on the microbiota assembly of sympatric woodlarks and skylarks. - Microbiome 5: 156.

van Veelen, H. P. J., Salles, J. F. and Tieleman, B. I. 2018. Microbiome assembly of avian eggshells and their potential as transgenerational carriers of maternal microbiota. - ISME J. 12: 1375–1388.

van Wijk, R. E., Schaub, M. and Bauer, S. 2017. Dependencies in the timing of activities weaken over the annual cycle in a long-distance migratory bird. - Behav. Ecol. Sociobiol. 71: 73.

van Wijk, R. E., Schaub, M., Hahn, S., Juárez-García-Pelayo, N., Schäfer, B., Viktora, L., Martín-Vivaldi, M., Zischewski, M. and Bauer, S. 2018. Diverse migration strategies in hoopoes (Upupa epops) lead to weak spatial but strong temporal connectivity. - Sci. Nat. 105: 42.

Victoria, J. K. 1972. Clutch characteristics and egg discriminative ability of African village weaverbird Ploceus cucullatus. - Ibis (Lond. 1859). 114: 367–376.

Videler, H., Geertjes, G. J. and Videler, J. J. 1999. Biochemical characteristics and antibiotic properties of the mucous envelope of the queen parrotfish. - J. Fish Biol. 54: 1124–1127.

Viseshakul, N., Charoennitikul, W., Kitamura, S., Kemp, A., Thong-Aree, S., Surapunpitak, Y., Poonswad, P. and Ponglikitmongkol, M. 2011. A phylogeny of frugivorous hornbills linked to the evolution of Indian plants within Asian rainforests. - J. Evol. Biol. 24: 1533–1545.

Wallace AR 1889. Darwinism: an exposition of the theory of natural selection, with some of its applications.

Walters, L. A. and Getty, T. 2010. Are brighter eggs better? Egg color and parental investment by House Wrens. - J. F. Ornithol. 81: 155–166.

Wang, B., Wan, L., Wang, A. and Li, L. M. 2017. An adaptive decorrelation method removes Illumina DNA base-calling errors caused by crosstalk between adjacent clusters. - Sci. Rep. 7: 41348.

Warnecke, L., Turner, J. M., Bollinger, T. K., Lorch, J. M., Misra, V., Cryan, P. M., Wibbelt, G., Blehert, D. S. and Willis, C. K. R. 2012. Inoculation of bats with European Geomyces destructans supports the novel pathogen hypothesis for the origin of white-nose syndrome. - Proc. Natl. Acad. Sci. 109: 6999–7003.

Weiß, B. M., Kücklich, M., Thomsen, R., Henkel, S., Jänig, S., Kulik, L., Birkemeyer, C. and Widdig, A. 2018. Chemical composition of axillary odorants reflects social and individual attributes in rhesus macaques. - Behav. Ecol. Sociobiol. 72: 65.

Werren, J. H. 2012. Symbionts provide pesticide detoxification. - Proc. Natl. Acad. Sci. 109: 8364–8365.

Werren, J. H., Baldo, L. and Clark, M. E. 2008. Wolbachia: master manipulators of invertebrate biology. - Nat. Rev. Microbiol. 6: 741–751.

Wesolowski, T. and Maziarz, M. 2012. Dark tree cavities – a challenge for hole nesting birds? - J. Avian Biol. 43: 454–460.

Westcott, S. L. and Schloss, P. D. 2015. De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. - PeerJ 3: e1487.

Whittaker, D. J. and Theis, K. R. 2016. Bacterial Communities Associated with Junco Preen Glands : Preliminary Ramifications for Chemical Signaling. - In: Schulte, B. A. et al. (ed), Chemical Signals in Vertebrates 13. Springer International Publishing, pp. 105–117.

Whittaker, D. J., Soini, H. A., Atwell, J. W., Hollars, C., Novotny, M. V and Ketterson, E. D. 2010. Songbird chemosignals: volatile compounds in preen gland secretions vary among individuals, sexes, and populations. - Behav. Ecol. 21: 608–614.

Whittaker, D. J., Richmond, K. M., Miller, A. K., Kiley, R., Bergeon Burns, C., Atwell, J. W. and Ketterson, E. D. 2011. Intraspecific preen oil odor preferences in dark-eyed juncos (Junco hyemalis). - Behav. Ecol. 22: 1256–1263.

Whittaker, D. J., Gerlach, N. M., Slowinski, S. P., Corcoran, K. P., Winters, A. D., Soini, H. A., Novotny, M. V., Ketterson, E. D. and Theis, K. R. 2016. Social Environment Has a Primary Influence on the Microbial and Odor Profiles of a Chemically

Signaling Songbird. - Front. Ecol. Evol. 4: 1–15.

Whittaker, D. J., Slowinski, S. P., Greenberg, J. M., Alian, O., Winters, A. D., Ahmad, M. M., Burrell, M. J. E., Soini, H. A., Novotny, M. V., Ketterson, E. D. and Theis, K. R. 2019. Experimental evidence that symbiotic bacteria produce chemical cues in a songbird. - J. Exp. Biol.: jeb.202978.

Wright, E. S. and Vetsigian, K. H. 2016. Quality filtering of Illumina index reads mitigates sample cross-talk. - BMC Genomics 17: 876.

Zahavi, A. 1975. Mate selection-A selection for a handicap. - J. Theor. Biol. 53: 205–214.

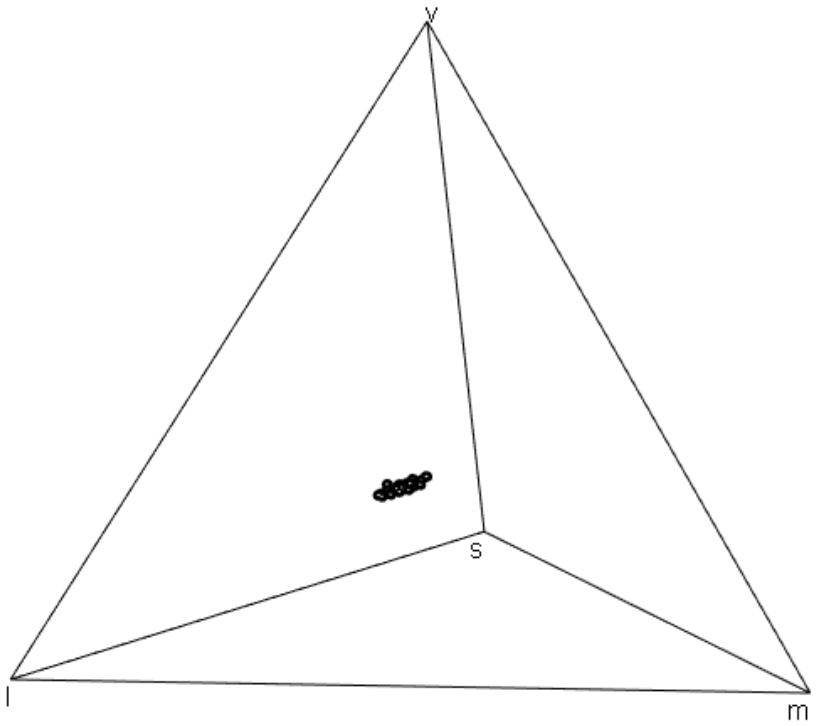
Zahavi, A. and Zahavi, A. 1997. The handicap principle; a missing piece of Darwin's puzzle.

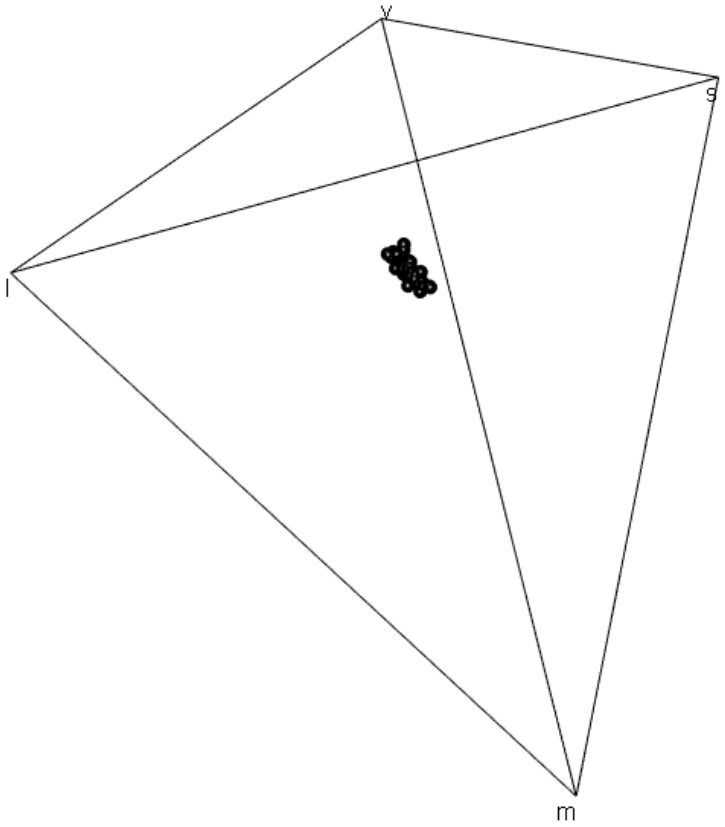
Zamzow, J. P. and Losey, G. S. 2002. Ultraviolet radiation absorbance by coral reef fish mucus: Photo-protection and visual communication. - Environ. Biol. Fishes 63: 41–47.

Zinter, M. S., Mayday, M. Y., Ryckman, K. K., Jelliffe-Pawlowski, L. L. and DeRisi, J. L. 2019. Towards precision quantification of contamination in metagenomic sequencing experiments. - Microbiome 7: 62.

SUPPLEMENTARY MATERIAL

Fig. S1. Distribution of eggshell colour patterns in the tetrahedron with different orientations. We used x, y z cartesian coordinates from Endler and Mielke (2005) model. The vertices are labeled with their cone types: violet, short (blue), medium (green) and long (red) wavelength sensitive (*v, s, m* and *l* respectively). Each point represents the average colour of eggs per nest depending on the estimation of each cone.

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CAPÍTULO III

CAPÍTULO III

Cosmetic coloration of cross-fostered eggs affects male parental investment in the Hoopoe (*Upupa epops*)

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Abstract

A signalling hypothesis suggests that avian eggshell coloration is a sexually selected female signal to advertise her quality (and hence the potential quality of her descent) to the male partner, thereby persuading him to increase his provisioning rate. This hypothesis has been tested for structural eggshell pigments, but not for cosmetic colorations such as that produced by the uropygial secretion on eggshells. During the breeding season, female hoopoes (*Upupa epops*) host in their uropygial glands symbiotic bacteria that are responsible of the brown colour of the secretion. They actively smear the eggshells with their secretion, protecting embryos from pathogenic trans-shell infections and changing eggshell' colour from bluish-grey to greenish-brown. Colour of the secretions is related to their antimicrobial potential and, thus, cosmetic eggshell coloration may act as a post-mating sexually selected signal if it affects provisioning rates of male partners. To test experimentally this hypothesis, we cross-fostered clutches between nests to disentangle effects of female quality and egg colour on male investment. In accordance with this hypothesis, males adjusted their provisioning rate to the colour of the experimental clutches. Our findings, therefore, suggest that males´ parental investment is affected by a cosmetic signal deposited by females on eggshells that reflects the characteristics of mutualistic bacteria. This is the first experimental demonstration that egg colour stained with uropygial secretion can act as a post-mating sexual signal of female quality for males.

Keywords: Eggshell colour, Female Signalling, Male investment, Symbiotic bacteria, *Upupa epops*, Uropygial secretion.

INTRODUCTION

Phenotypic or genetic quality of individuals can be reflected by some phenotypic characteristics that can influence mate choice preferences (Andersson 1994, Andersson and Simmons 2006) or differential reproductive investment after mating (Burley 1988, Sheldon 2000), being, therefore, sexually selected. Most of the studies have mainly explored these traits in males (Edward and Chapman 2011, Lyu et al. 2017), while the role of sexual selection in explaining the evolution of females traits have only been recently considered (Fitzpatrick and Servedio 2018, Soler et al. 2019).

One example of post-mating sexual signal of female reflecting her quality is the avian eggshell coloration (Moreno and Osorno 2003, Moreno et al. 2004, 2005, 2006a, Soler et al. 2018, 2005, 2008b, Siefferman et al. 2006, Krist and Grim 2007, Giordano et al. 2015, Hargitai et al. 2018a). Intra- and interspecific variation in eggshell colour have intrigued researchers for a long time. Several adaptive and non-adaptive explanation have been proposed (Wallace AR 1889, Swynnerton 1916, Gaston et al. 1993, Underwood and Sealy 2002, Moreno and Osorno 2003, Lahti 2008, Hanley et al. 2010), although not all of them received similar level of support (Underwood and Sealy 2002, Kilner 2006, Hanley et al. 2013). The possibility that eggshell coloration was sexually selected (SSEC hypothesis) (Moreno and Osorno 2003) implies two assumptions: (1) that females signal her quality (and hence the potential quality of her descent) to the male partner by means of egg colouration, and (2) that male partners adjust their reproductive investment to eggshell

colouration. Although there is general consensus on the association between eggshell colouration and quality of females (e.g. Moreno et al. 2006b, Siefferman et al. 2006, Krist and Grim 2007, Holveck et al. 2019), some controversy exits with support to the second assumption of the SSEC hypothesis. A relationship between parental investment and eggshell coloration has been found through some correlational (Hanley et al. 2008, Díaz-Lora et al. 2019 under review) and experimental studies (Soler et al. 2005, 2008a, Moreno et al. 2006, 2008, English and Montgomerie 2011, Poláček et al. 2017). However, not all of them have found such a relationship (Krist and Grim 2007, Honza et al. 2011, Johnsen et al. 2011, Stoddard et al. 2012, Bulla et al. 2012, Fronstin et al. 2016). The fact that the results found are mixed, makes necessary further experimental studies to explore such association, measuring parental investment during the incubation period to ensure that changes in male investment are caused by eggshell colour rather than the confounding variables of nestling traits (reviewed in Riehl 2011).

The SSEC hypothesis has been mainly focused on structural coloration due to antioxidant and physiological properties of biliverdin and/or protoporphyrin (Kaur et al. 2003, Moreno et al. 2004, 2006a, Hanley et al. 2008, Soler et al. 2008a, English and Montgomerie 2011, Stoddard et al. 2012), the responsible pigments of the eggshell colourations. However, there exist some other non-structural and external sources of eggshell coloration. This is for instance the case of the eggshell spots detected in some species caused by the activity of some ectoparasites (López-Rull et al. 2007, Avilés et al. 2009, Tomás et al. 2018). Deliberate application of cosmetic substances, such as the uropygial secretion (Soler et al. 2014), will also affect eggshell colouration and, thus, functioning as a signal of female quality that influences male parental investment (Díaz-Lora et al. 2019 under review). The effect of the cosmetic use of uropygial secretion on avian coloration has mainly been investigated for feathers (Montgomerie 2006, Delhey et

al. 2007, Piault et al. 2008, Amat et al. 2011, Pérez-Rodríguez et al. 2011) in scenarios of sexual selection (Negro et al. 1999, Delhey et al. 2007, López-Rull et al. 2010), but a pioneering correlative work in hoopoes suggest that it might also be the case for eggshells (Díaz-Lora et al. 2019 under review).

Female hoopoes cover their eggs with the uropygial secretion, which causes a colour change of the eggshell, from bluish-grey to greenish-brown (Soler et al. 2014). The dark colour of the uropygial secretion is similar to that of the coated eggshells and, at least partially, is caused by the antibiotic producing symbiotic bacteria living within the uropygial gland of females (Martín-Platero et al. 2006, Soler et al. 2008a, Martín-Vivaldi et al. 2010, Ruiz-Rodríguez et al. 2013). Thus, when the female stains the eggshell with the secretion, she transfers these bacteria to the egg surface (Martín-Vivaldi et al. 2014a), thus protecting embryos from pathogenic infections and increasing hatching success (Martín-Vivaldi et al. 2014a). Interestingly, previous studies have demonstrated an association between colouration of the uropygial secretion of hoopoes, the presence of bacterial symbionts and its antimicrobial properties. Secretions without bacteria are lighter in colour (Martín-Vivaldi et al. 2009), and the saturation in magenta hues is negatively related to the antimicrobial capacity (Soler et al. 2014). Similarly, saturation of previously coated eggshell is negatively related to the bacterial load of the uropygial secretion (Díaz-Lora et al. 2019 under review). All these findings suggest that the cosmetic coloration of the eggshells of hoopoes inform of antimicrobial properties of the symbiotic bacterial community hosted by females and, thus, open the opportunity of sexual selection acting on this characters provoking differential allocation in reproduction of mates (Soler et al. 2014). Benefits for males will included, not only larger hatching success (Martín-Vivaldi et al. 2014a) but also adaptive microbiota that their offspring will acquire from mothers (Ruiz-Rodríguez et al. 2014, Martínez-García et al. 2016b, Martín-Vivaldi et al. 2018). In

accordance with the hypothesis, previous correlative work pointed out that food provided by males to incubating females was higher in nests with lower saturation of the cosmetic colouration of the eggshells (Díaz-Lora et al. 2019 under review). However, egg colour might covariate with several characteristics including females and males' parental quality (Moreno et al. 2006) and, thus, an experimental approach is necessary to discern cause and consequence of the detected association.

Here, and for the first time in hoopoes, we test experimentally this hypothesis by monitoring male feeding behaviour to females incubating both, their own and cross-fostered eggs, naturally coated with uropygial secretion of their respective mothers. Our prediction is that male provisioning rate will be influenced by the differences in cosmetic coloration between both clutches. Given that birds perceive colours different from humans, we used spherical parameters obtained from a bird physiological visual system to quantify egg colour (Endler 1990, Renoult et al. 2017).

MATERIAL AND METHODS

Study species

The hoopoe is a cavity nester that readily nests in nest boxes and is distributed throughout Europe, Africa and Asia. Females lay one or two clutches of 6 to 8 eggs from February to July (Martín-Vivaldi et al. 1999, Plard et al. 2018). They start to incubate with the first or second egg, generating an asynchronous hatching and, therefore, a considerable size hierarchy within the brood (Cramp 1998). Only the female incubates and the male feeds her while she is into the nest (from the start of incubation until the first nestling is around 8 days old). Both sexes care for offspring, providing them food until fledglings abandon the nest with 24-30 days old (Martín-Vivaldi et al. 2014b). Within our study population,

there is variation in the colour of the uropygial secretions and eggshells among individuals (Soler et al. 2014).

Study area and general procedures

The fieldwork was carried out during the 2016 breeding season in the Hoya de Guadix (37 ºC 18´N, 11´W), Granada (southern Spain) in a hoopoe population studied over the last 25 years. They breed in nest boxes situated in trees and in natural cavities. Nest boxes are made in cork with the following dimensions: 24 cm (bottom-to-hole height), 35 × 18 × 21 cm (internal height × width × depth), 5.5 cm (entrance diameter).

Nest boxes were visited every five days from early March to the end of July. We considered laying date as the day when female laid her first egg, assuming that one egg was laid daily (Cramp 1998). We considered hatching date as the day on which the first nestling hatched. Females were captured by hand inside the nest boxes twice, during the cross-fostering experiment to mark them (see below), and 15 days after laying date to sample them. Males were also captured with a mist net located close to the nest entrance after the first nestling hatched. The first capture time, we measured tarsus length with a calliper (accuracy 1mm) and body mass with a hanging scale (Pesola 0-100 g, accuracy 1 g). All the individuals were marked with numbered aluminum rings (Spanish Institute for Nature Conservation, ICONA), and adults were provided with unique colour rings combinations. Disposable latex gloves previously cleaned with 96% ethanol were used during all the manipulations, to prevent contamination among nests and among individuals.

Body condition of the female was estimated as the residuals of body mass on tarsus length^3 (Senar and Pascual 1997, Peig and Green 2010, Labocha and Hayes 2012).

Experimental design

Eggs were cross-fostered between pairs of nests with a maximum laying-date difference of three days. After laying ceased, and at least two days after laying of the sixth egg, the browner six eggs (i.e. those that female had laid first and, therefore, harbored more uropygial secretion in their surface) of two synchronous nests were exchanged between experimental nests. Removed eggs were temporarily replaced with artificial plastic blue eggs until experimental hoopoes eggs arrived to the nest-box. Cross-fostering lasted less than half an hour and exchanged eggs were transported in a portable incubator (MARCA?). Once completed the cross-fostering, the eggs that were not exchanged were artificially incubated at 37.5ºC (Covatutto 24 Eco, Novital) and, as soon as they hatched, hatchlings were placed in a brood of its same age in which male feeding visits before and after the clutch exchange were already recorded. In the meantime, they were fed by hand with previously frozen fly larvae.

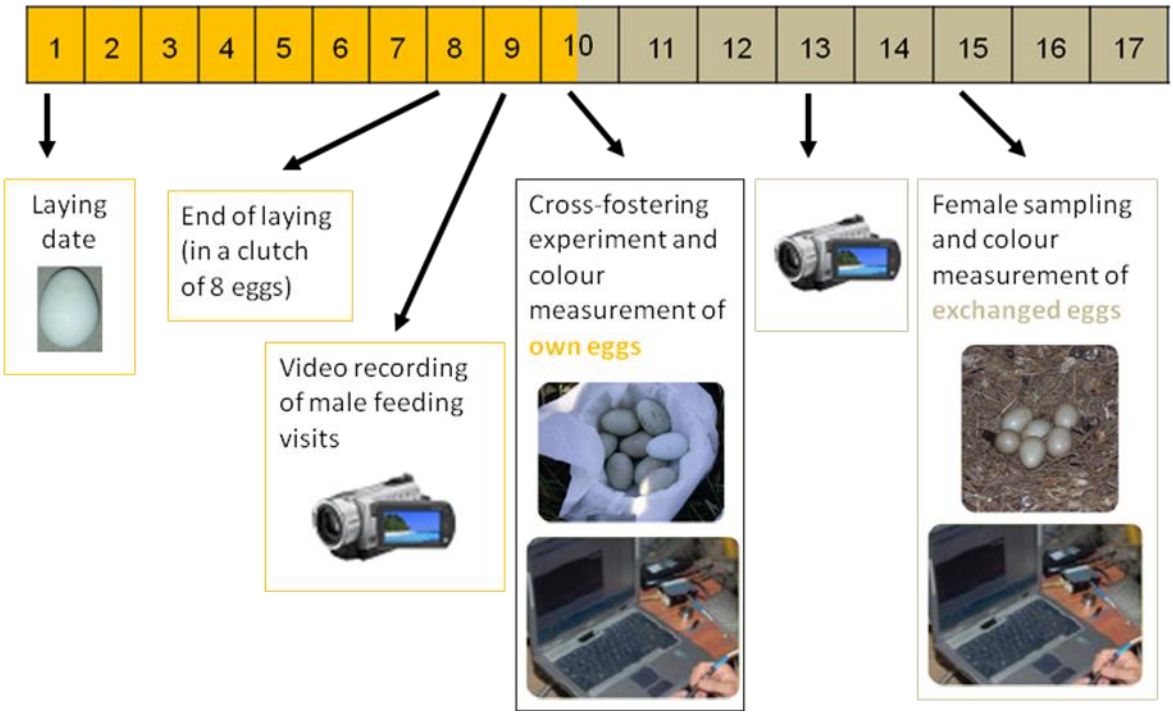
Nests in which less than 6 eggs were interchanged (2 nests with a clutch size lower than 6 eggs) and those for which some video-recording or the colour measurement was missing due to predation (18) or abandonment (12), were excluded from analysis. Finally, 61 out of 95 exchanged nests were analysed, including 41 first clutches, 8 replacement clutches, 11 second clutches and 1 third clutch.

Parental investment

The visits to the nest by parents were video-recoded twice at the end of the incubation period: first, 0-5 days after laying the last egg with the original clutch and, second, three days after the cross-fostering experiment, with the exchanged eggs (See Fig.1). For video recording, digital video cameras (Sony Handycam DCR SR190 and DCR-SR55 models) placed several meters away from the nest, camouflaged among stones, vegetation or trunks, were used. Each recording registered periods of approximately three hours starting

around 16.00 pm. Between 65 and 120 minutes were analyzed from the moment in which the behavior of the male and the female was normalized (i.e. after recording the second feeding visit) except for five cases, where between 31 and 57 minutes were analyzed. This was because in those cases, recording registered shorter periods of time and/or parents started to behave normally at the end of the video. Parents were identified by their colour ring combinations and/or other characteristics of their plumage or body. Male provisioning rate was calculated as the number of prey carried per hour (feeding rate) multiplied by the average relative size of all the prey carried. The relative size of prey was estimated in comparison to the length of the bill of the individual carrying it, in an ordinal scale from 1 to 3 (1 when the size of the prey was less than a quarter of the beak, 2 when it was between a quarter and a half and 3 when it was bigger than a half, Martín-Vivaldi et al. 1999). Prey size of some feeding attempts was not possible to estimate because of the relative position of arrived males and that of the video camera. In these cases, the average prey size of all studied males was considered to estimate conservatively the amount of food carried (it occurred for the completed video recorded feeding behaviour of one males in nest with the original eggs and of two males in nests with exchanged eggs). A total of 122 videos of 61 nests were analysed through VLC Media Player (version 2.2.6).

Fig. 1. Schematic representation of the experimental design in a nest with 8 eggs. The orange boxes represent the days when the nest contained the female´s own eggs and the brown ones the days in which it contained the exchanged eggs belonging to another female.



Egg colour and biometric measurements

To be able to analyze if the second female changed the eggshell colour of the exchanged eggs covering them with her own uropygial secretion, the colour of each egg was measured twice, before the interchange and after staying several days in the new nest (days 10 and 15 in the example of Fig. 1). Only the six eggs used for the cross-fostering from each nest were measured and marked with a permanent marker.

For measuring the eggshell coloration an Ocean Optics S2000 spectrometer connected to a deuterium-halogen light (D2-W, Mini) was used. A black bag wrapping the tip of the optical fiber and the egg was used to standardize ambient light conditions. All

measures were performed as quickly as possible, close to the nest box and blocking the nest box entrance in the meantime to prevent nest desertion by parents. Females were captured within the nest-box, kept inside a bag during egg measurements, and placed back in the nest after returning the eggs.

The spectrometer was calibrated using a standard white and black reference before measuring each clutch. Reflectance spectra at 10 nm intervals from 300 to 700 nm was obtained for the six experimental eggs of each clutch. Eggshell colour was measured on five equidistant points along the long egg axis. Each point was measured three times to estimate mean values. Prior to all analysis we applied the following corrections to our interpolated spectra data: reflectance curves were corrected for noise using triangular smoothing and negative values were set to zero (Gómez 2006). To verify that eggshell coloration was more variable among than within nests, repeatability was calculated for each point of the egg and among the eggs within the same clutch. We did so by using values of the three first factors of principal components analysis of colour information (reflectance at 1nm of the entire wavelength interval considered (300-700 nm)). (Repeatability of each zone of the egg: GLM: PCA1: $r = 0.69$, $F_{141,\ 565} = 12.35$, $P < 0.0001$; PCA2: $r = 0.85$, $F_{141,565} = 28.48$, $P < 0.0001$, PCA3: $r = 0.76$, $F_{141,\ 565} = 17.03$, $P < 0.0001$. Repeatability among the eggs of each nests: GLM: PCA1: $r = 0.97$, $F_{72,\ 365} = 16.54$, $P < 0.0001$; PCA2: $r = 0.58$, $F_{72,\ 365} = 9.19$, $P < 0.0001$, PCA3: $r = 0.78$, $F_{72,\ 365} = 14.04$, $P < 0.0001$). Thus, average values of clutches in subsequent analyses of the colour variables were used.

Trying to get information of animal vision in the signalling context considered (Endler 1990, Renoult et al. 2017), the visual parameters of a physiological model for a tetrachromatic violet vision were estimated using Avicol V.6 software (Gómez 2006). Specifically, Endler and Mielke (2005) model were used with some modifications

proposed by Stoddard and Prum (2008), such as, the correction for dark colours and the non-logarithmic transformation of the photoreceptor response values. The model was run considering ambient light conditions inside a nest-box, and the violet spectra sensitivity of peafowls (*Pavo cristatus*) (Hart 2002). Two variables were obtained with spherical coordinates: phi and tetha, and the variable chroma (r). Phi measure an angle (between -90º and 90º) in the ultraviolet/violet sensitive (UV/V) range and tetha (between -180º and 180º) in the red-green-blue plane (Endler and Mielke 2005). Both measures inform on hue, while r values inform on colour saturation (Stoddard and Prum 2008, Saino et al. 2013). Maximum potential chroma (r_{max}) depends on hue values since the colour space is a tetrahedral and not a sphere. For this reason, in our analyses achieved chroma (rA, hereafter saturation), computed as: $rA=r/r_{max}$ was used (Stoddard and Prum 2008).

Egg length and breadth were measured using a calliper (accuracy 1mm) and egg volume was estimated from Hoyt´s formula (size = 0.51 x length x breadth^2; Hoyt 1979). Finally average values of colour and size parameters for each egg and then for each clutch were used. All the eggs of the nest were considered to calculate the average size before the exchange, and after the exchange only the size of the six eggs exchanged.

Statistical Analysis

To verify that the initial colour of the clutch caused by the uropygial secretion of the original female was maintained after the cross-fostering experiment, a GRM was performed with the colour after the cross-fostering as a dependent variable and their initial colour before the exchange and the colour of the original eggs of the adoptive female as predictors. If eggs maintained their colour after the cross-fostering, the main predictor should be their initial colour.

To control for the possible effect of the type of clutch (i.e. breeding attempt and or replacement), it was considered as a numeric covariable in a scale indicating increasing levels of breeding effort already performed by pairs in the season. Thus, it was used 1 for first clutches, 1.5 for replacement clutches (when the previous clutch of the female was not successful, i.e. there were not nestlings on day 20th), 2 for second clutches (when the first one was successful), 2.5 for the replacement clutches after the second clutch and so on. One nest was evaluated as 1.75 since it had two replacement clutches after the first clutch.

To test the effect of the experiment on provisioning rate of males, we looked for a relationship between the differences in the colour of eggs (values of the three variables obtained in the physiological model) of the exchanged clutches and the differences in the amount of food carried per hour by males after and before the exchange. For this, the difference variables were calculated as values after the interchange minus values before it. In the case of Phi, given that this spherical parameter takes values between -90º and 90º, with maximum stimulation of the violet cone at 0º, positive values of the difference imply increases in violet cone stimulation between the first and second measurement. In the analyses, we controlled for other variables that could explain inter-individual differences in feeding effort in this phase as laying date, number of clutch, clutch size, female body condition and the differences between the egg sizes after and before the exchange. The expected associations were tested by means General Regression Model (GRM) selecting the best subset of predictors by mean of Mallow´s CP (Mallows 1973), equivalent to Akaike´s Information Criterion (AIC) (Boisbunon et al. 2013).

The residuals of all statistical models followed a normal distribution (K-S d=0.06, p>0.20). All statistical tests were performed with Statistica 7 software (Statsoft 2006).

RESULTS

The change in provisioning rate of males after the experimental exchange of clutches was related to differences in the colour between both clutches (Table 1). In nests that the rA and Phi values of experimental eggs (rA2 and Phi2) had lower values than original ones (rA1 and Phi1), males differentially increased feeding effort to females when incubating experimental eggs. Changes in rA and Phi resulted negatively related to changes in male provisioning rate (Figs 2 and 3, Table 1), while the changes in Theta did not explain additional variance (Table 1). These relationships indicate that an increase in saturation of egg colour (rA2-rA1 positive) produces a reduction of provisioning rate. In the case of Phi, the negative relationship found means a reduction in provisioning rates when the amount of violet in egg colour increases. In addition to colour variables, only difference in egg size entered in the best model, also with a negative trend, although it did not reach significance (Fig. 4, Table 1). In the nests in which the size of the experimental eggs (Egg size 2) was smaller than that of the originals ones (Egg size 1), males tend to increase differentially feeding effort to females when incubating experimental eggs.

Table 1. Results of a GRM model exploring the best subset of predictors explaining differences in the provisioning rate of males between after and before the experimental exchange of eggs with those of a different female (Male provisioning rate 2- Male provisioning rate 1). The numerical labels refer to values of the original (1) or adopted (2) clutch. Significant values are in bold. Whole model $F_{3,57} = 3.65$, $p = 0.018$, $R^2 = 0.161$.

Predictor		**In best subset**	**t**	**p**	**Beta**	**SE**
Intercept			0.59	0.558		
Eggshell colour changes:	Theta 2-Theta 1	Pooled				
	Phi 2 - Phi 1	Retained	2.72	**0.008**	-0.36	0.13
	rA 2 – rA 1	Retained	2.11	**0.040**	-0.28	0.13
Other predictors:	Laying date	Pooled				
	Nº of clutch	Pooled				
	Clutch size	Pooled				
	Egg size 2-1	Retained	1.72	0.091	-0.21	0.12
	Fem. body Cond.	Pooled				

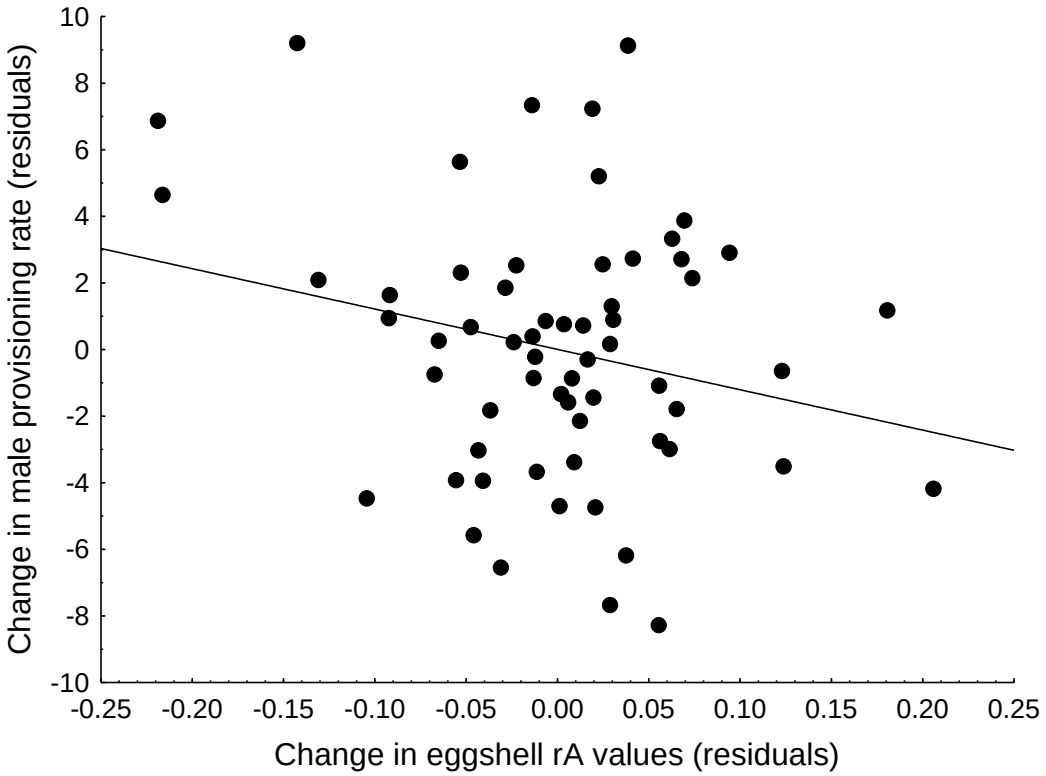


Fig. 2. Relationship between the residuals of change in male provisioning rate (Male provisioning rate 2-1), and the residuals of the change in rA values (rA2-rA1) of the eggshells after controlled for phi values changes and eggs size change.

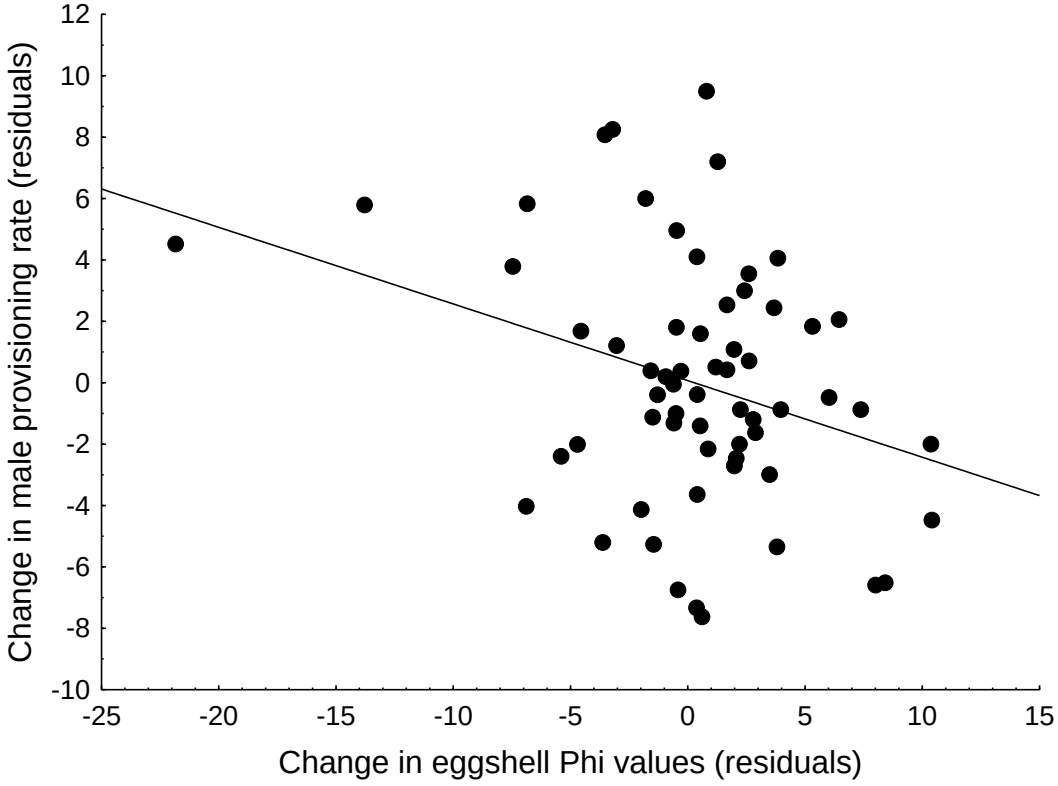


Fig. 3. Relationship between the residuals of change in male provisioning rate (Male provisioning rate 2-1), and the residuals of the change in phi values of the eggshells (Phi 2- Phi 1) after controlling for rA values changes and eggs size change.

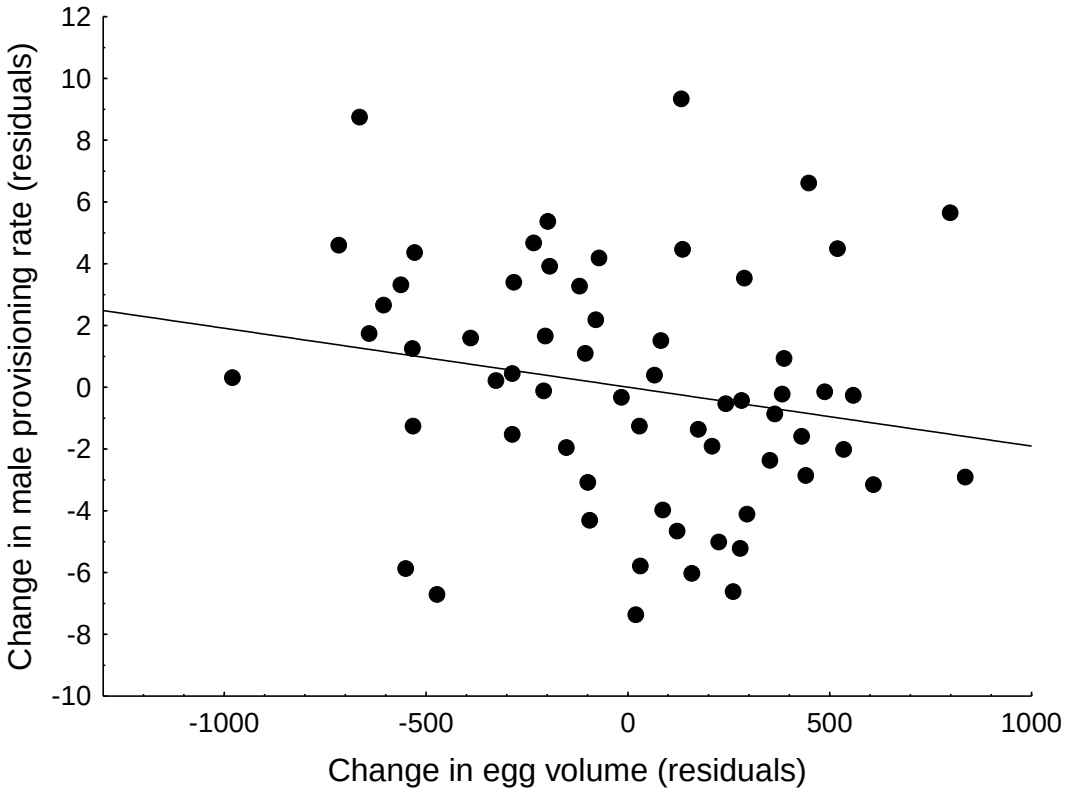


Fig. 4. Relationship between the residuals of change in male provisioning rate (Male provisioning rate 2-1), and the residuals of the change in egg volume (Egg size 2- Egg size 1).

DISCUSSION

We have found that provisioning rates of males to incubating female hoopoes depended on colorations of experimental exchange of clutches. Colouration of hoopoe eggshells is due to the full of bacteria secretion used by females to smear and colour their eggs, and reflects antimicrobial properties of female secretion (Martín-Vivaldi et al. 2014a, Soler et al. 2014). This is the first experimental demonstration of benefits associated with a cosmetic females' signal, therefore, suggesting that hoopoe eggshells' coloration is a post-

mating sexually selected indicator of female quality in term of antimicrobial capabilities of their secretion

Secretion deposited on the eggshells by females will change, not only colour, but also the odour of the clutch, and males might use one or both characteristics to adjust their feeding effort. We have previously shown that when bacteria are eliminated from uropygial glands by mean of antibiotics, secretions become lighter in colour, and, simultaneously, the amount of associated chemical volatiles drastically reduced (Martín-Vivaldi et al. 2009, 2010). Although the performed experiment does not allow to rule out the possibility that males cue on eggshells' odour, mainly because their own females are continuously producing a great amount of secretion daily and, probably, the intense odour of such fresh secretions (pers. Obs.) obscures the odour of dry secretions coming with the foster eggs. Therefore, although we cannot completely discard that other properties of the secretion covering eggs are the signals detected by males, the most probable explanation for our result is that they detected the change in egg colour.

Apart from egg colouration, in the final model also retained egg size. A non-significant reduction in provisioning rates was associate with increase egg size of experimental clutches (Brulez et al. 2015). This trend could be explained by females laying larger eggs when paired with high quality males (Cunningham and Russell 2000, Leitner et al. 2006), and males that do not use egg size to adjust feeding effort, a possibility that deserve further investigation.

The dim environment of hole nests (Wesolowski and Maziarz 2012) has been used by some authors to argument against visual signals such egg colouration working within those environments (Reynolds et al. 2009, but see Avilés et al. 2011). However, birds have impressive colour-discrimination abilities, being also able to discriminate in dim light conditions (Olsson et al. 2015). Moreover, some studies have shown how hole-nesting

birds are able to discriminate between colour parameters inside a nest cavity (Avilés and Soler 2009, Avilés et al. 2010, 2011, Holveck et al. 2010), and both avian visual modelling under low light condition and discrimination experiment in dim environment support the possibility that sexual signal in general, and those of egg colouration in particular, functions within nest environment (Holveck et al. 2010, Avilés et al. 2011) supporting the plausibility of the evolution of these colour signals of the birds´ eggs. In the present study, we have analyzed the visual parameters having into account the stimuli of the cones inside a nest hole, and therefore all evidences support such role also for the hoopoe.

Our results are consistent with the second assumption of the SSEC hypothesis that relates male investment with eggshell colouration (Moreno and Osorno 2003). As for the first one, we have not tested if eggshell cosmetic colour signal is a costly and reliable indicator of female quality, but it is known that uropygial secretions might be a limited resource of females and colouring the eggs is a time consuming activity. Thus, antimicrobial capacity of uropygial secretion could be an indicator of phenotypic quality of the female. Moreover, it is a difficult signal to deceive since the antimicrobial characteristic does not only depend on the female, but is the result of the characteristics of the microbial community of the secretion, something difficult to supplant to get colours. This, added to the fact that uropygial secretion protect embryos from pathogenic infections increasing hatching success (Martín-Vivaldi et al. 2014a) would make difficult to change the colour without changing the microbial community. All this indicates that the colour of the uropygial secretion in hoopoes could be an honest signal. Having into account this assumption, together with our findings about how eggshell coloration influence male provisioning rate, our results support the SSEC hypothesis. Moreover, they show that the signalling role of the colour of eggs can be produced by cosmetics, revealing thus a novel

mechanism by which sexual selection can produce post-mating signals on eggs (as suggested in Soler et al. 2014).

The possibility that egg colorations reflect antimicrobial capacity mediated by cosmetic uropygial secretions may also be applied to other bird species (Soler et al. 2014). That is because uropygial secretions of birds in general have antimicrobial substances (Jacob and Ziswiler 1982) that may reach eggshells during incubation due to the contact with belly feathers previously stained with it (Cook et al. 2005). Moreover, it is known how the uropygial secretion changes the coloration of different parts of the body surfaces of birds, such as the feathers and the bills (Kemp 2001, Montgomerie 2006, Delhey et al. 2007, Piault et al. 2008, Amat et al. 2011, Pérez-Rodríguez et al. 2011). Thus, it would be possible that they could also change the colour of the eggshells. Therefore, it might be worth to study the possibility that egg colouration due to cosmetic use of uropygial secretion has a sexually components in species other than hoopoes.

The use of cosmetics has been reported for a wide variety animals, including several species of fishes, mammals and birds (reviewed in Delhey et al. 2007). For instance, similarly to hoopoes, it has been shown how tropical reef fishes secrete biochemical compounds with antibiotics (Shephard 1994, Videler et al. 1999) into the epithelial mucus. These compounds are black at the UV-wavelength (Zamzow and Losey 2002) and, thus, its colour may signal not only characteristics of the mucus, but an individual quality in terms of capacity of obtaining food sources rich in UV-blockers (Zamzow and Losey 2002). In mammals, the red kangaroo (*Megaleia rufa*) and the grey possum (*Trichosurus vulpecula*), have coloured pigmented patches in the pelage due to the secretion of integumentary glands (Nicholls and Rienits 1971) and, thus is a sexually dimorphic character due to cosmetic colouration. Two studies in birds have shown how the plumage colour change due to deliberate staining of the cosmetic (by using iron red

soils or uropygial secretion) may help individuals to communicate their quality (Negro et al. 1999, Amat et al. 2011) in scenarios of social communication including mate choice (Amat et al. 2011). Differing from these and some others examples of cosmetic colourations, eggshell coloration of hoopoes are showing not only physiological or cultural abilities of individuals, but also properties of their microbial symbionts. As far as we know, hoopoes painting their eggs with their own uropygial secretion is the first example of animals using cosmetic coloration to show characteristics of their antimicrobial producing microbial symbionts. Thus, this behaviour is likely maintained and selected by differential feeding investment of males in a typically post-mating sexually selected process (Burley 1988, Sheldon 2000), as our experimental results demonstrated. In summary, the experimental evidence here presented strengthens the SSEC hypothesis and support the descriptive results of Díaz-Lora et al. 2019 (under review). Further investigation regarding the different determinants of hoopoe eggshell colour as a cosmetic post-mating signal, not only the combined effects of the different types of bacteria located in the uropygial secretion but also structural pigments, would be an interesting future line of research. Moreover, our results suggest that the colour of cosmetics produced in glands hosting symbionts may act as post-mating female quality signals in animals in general, thus increasing the kind of effects that the microbiomes hosted may affect animal evolution (McFall-Ngai et al. 2013).

ETHICAL NOTE

The study was conducted according to relevant Spanish national (Decreto 142/2013, 1 de octubre) and regional guidelines. The protocols adhered to the ASAB/ABS Guidelines for the Use of Animals in Research and it was approved by the ethics committee of the

University of Granada (Comité de Ética en Experimentación Animal, CEEA, Ref.:785). All necessary permits for hoopoe´s manipulations were provided by Consejería de Medio Ambiente de la Junta de Andalucía, Spain (Ref:SGYB/FOA/AFR/CFS and SGMN/GyB/JMIF). Our study area is not protected but privately owned, and the owners allowed us to work in their properties. The time spent in each hoopoe nest was the minimum necessary for the experiment.

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REFERENCES

Alberdi, A., Aizpurua, O., Bohmann, K., Zepeda-Mendoza, M. L. and Gilbert, M. T. P. 2016. Do Vertebrate Gut Metagenomes Confer Rapid Ecological Adaptation? - Trends Ecol. Evol. 31: 689–699.

Alexander, G. D., Houston, D. C. and Campbell, M. 1994. A possible acoustic function for the casque structure in hornbills (Aves: Bucerotidae). - J. Zool. 233: 57–67.

Amat, J. A., Rendón, M. A., Garrido-Fernández, J., Garrido, A., Rendón-martos, M. and

Pérez-gálvez, A. 2011. Greater flamingos Phoenicopterus roseus use uropygial secretions as make-up. - Behav. Ecol. 65: 665–673.

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Zech Xu, Z., Kightley, E. P., Thompson, L. R., Hyde, E. R., Gonzalez, A. and Knight, R. 2017. Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. - mSystems 2: e00191-16.

Amundsen, T. 2000. Why are female birds ornamented? - Trends Ecol. Evol. 15: 149–155.

Amundsen, T. 2018. Sex roles and sexual selection: Lessons from a dynamic model system. - Curr. Zool. 64: 363–392.

Andersson, M. 1994. Sexual selection (Princeton Univ. Press, Ed.).

Andersson, M. and Simmons, L. W. 2006. Sexual selection and mate choice. - Trends Ecol. Evol. 21: 296–302.

Archie, E. A. and Theis, K. R. 2011. Animal behaviour meets microbial ecology. - Anim. Behav. 82: 425–436.

Arlettaz, R., Schaub, M., Fournier, J., Reichlin, T. S., Sierro, A., Watson, J. E. M. and Braunisch, V. 2010. From Publications to Public Actions: When Conservation Biologists Bridge the Gap between Research and Implementation. - Bioscience 60: 835–842.

Aronesty, E. 2011. ea-utils: Command-line tools for processing biological sequencing data.: https://github.com/ExpressionAnalysis/ea-utils.

Avilés, J. M. and Soler, J. J. 2009. Nestling colouration is adjusted to parent visual performance in altricial birds. - J. Evol. Biol. 22: 376–386.

Avilés, J. M., Soler, J. J. and Pérez-Contreras, T. 2006. Dark nests and egg colour in birds: a possible functional role of ultraviolet reflectance in egg detectability. - Proc. Biol. Sci. 273: 2821–2829.

Avilés, J. M., Pérez-Contreras, T., Navarro, C. and Soler, J. J. 2009. Male spotless starlings adjust feeding effort based on egg spots revealing ectoparasite load. - Anim. Behav. 78: 993–999.

Avilés, J. M., Vikan, J. R., Fossøy, F., Antonov, A., Moksnes, A., Røskaft, E. and Stokke, B. G. 2010. Avian colour perception predicts behavioural responses to experimental brood parasitism in chaffinches. - J Evol Biol 23: 293–301.

Avilés, J. M., Soler, J. J. and Hart, N. S. 2011. Sexual selection based on egg colour: Physiological models and egg discrimination experiments in a cavity-nesting bird. -

Behav. Ecol. Sociobiol. 65: 1721–1730.

Azcárate-García, M., González-Braojos, S., Díaz-Lora, S., Ruiz-Rodríguez, M., Martin-Vivaldi, M., Martínez-Bueno, M., Moreno, J. and Soler, J. J. 2020. Interspecific variation in deterioration and degradability of avian feathers: The evolutionary role of the microbiome. - J. Avian Biol.: in press.

Baker, L. J., Freed, L. L., Easson, C. G., Lopez, J. V, Fenolio, D., Sutton, T. T., Nyholm, S. V and Hendry, T. A. 2019. Diverse deep-sea anglerfishes share a genetically reduced luminous symbiont that is acquired from the environment. - Elife 8: e47606.

Bandyopadhyay, A. and Bhattacharyya, S. P. 1996. Influence of fowl uropygial gland and its secretory lipid components on growth of skin surface bacteria of fowl. - Indian J. Exp. Biol. 34: 48–52.

Banning, J. L., Weddle, A. L., Wahl III, G. W., Simon, M. A., Lauer, A., Walters, R. L. and Harris, R. N. 2008. Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, Hemidactylium scutatum. - Oecologia 156: 423–429.

Barr, J. J., Auro, R., Furlan, M., Whiteson, K. L., Erb, M. L., Pogliano, J., Stotland, A., Wolkowicz, R., Cutting, A. S., Doran, K. S., Salamon, P., Youle, M. and Rohwer, F. 2013. Bacteriophage adhering to mucus provide a non–host-derived immunity. - Proc. Natl. Acad. Sci. 110: 10771–10776.

Beltran-Bech, S. and Richard, F.-J. 2014. Impact of infection on mate choice. - Anim. Behav. 90: 159–170.

Bennett, G. M. and Moran, N. A. 2015. Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. - Proc. Natl. Acad. Sci. 112: 10169–10176.

Bentley, D. R., Balasubramanian, S., Swerdlow, H. P., Smith, G. P., Milton, J., Brown, C. G., Hall, K. P., Evers, D. J., Barnes, C. L., Bignell, H. R., Boutell, J. M., Bryant, J., Carter, R. J., Keira Cheetham, R., Cox, A. J., Ellis, D. J., Flatbush, M. R., Gormley, N. A., Humphray, S. J., Irving, L. J., Karbelashvili, M. S., Kirk, S. M., Li, H., Liu, X., Maisinger, K. S., Murray, L. J., Obradovic, B., Ost, T., Parkinson, M. L., Pratt, M. R., Rasolonjatovo, I. M. J., Reed, M. T., Rigatti, R., Rodighiero, C., Ross, M. T., Sabot, A., Sankar, S. V., Scally, A., Schroth, G. P., Smith, M. E., Smith, V. P., Spiridou, A., Torrance, P. E., Tzonev, S. S., Vermaas, E. H., Walter, K., Wu, X., Zhang, L., Alam, M. D., Anastasi, C., Aniebo, I. C., Bailey, D. M. D., Bancarz, I. R., Banerjee, S., Barbour, S. G., Baybayan, P. A., Benoit, V. A., Benson, K. F., Bevis,

C., Black, P. J., Boodhun, A., Brennan, J. S., Bridgham, J. A., Brown, R. C., Brown, A. A., Buermann, D. H., Bundu, A. A., Burrows, J. C., Carter, N. P., Castillo, N., Catenazzi, M. C. E., Chang, S., Neil Cooley, R., Crake, N. R., Dada, O. O., Diakoumakos, K. D., Dominguez-Fernandez, B., Earnshaw, D. J., Egbujor, U. C., Elmore, D. W., Etchin, S. S., Ewan, M. R., Fedurco, M., Fraser, L. J., Fuentes Fajardo, K. V., Scott Furey, W., George, D., Gietzen, K. J., Goddard, C. P., Golda, G. S., Granieri, P. A., Green, D. E., Gustafson, D. L., Hansen, N. F., Harnish, K., Haudenschild, C. D., Heyer, N. I., Hims, M. M., et al. 2008. Accurate whole human genome sequencing using reversible terminator chemistry. - Nature 456: 53–59.

Boisbunon, A., Canu, S., Fourdrinier, D., Strawderman, W. and Wells, M. T. 2013. AIC , Cp and estimators of loss for elliptically symmetric distributions. - hal-00851206

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., Mills, D. A. and Caporaso, J. G. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. - Nat. Methods 10: 57–59.

Brandl, H. B., van Dongen, W. F. D., Darolová, A., Kristofík, J., Majtan, J. and Hoi, H. 2014. Composition of Bacterial Assemblages in Different Components of Reed Warbler Nests and a Possible Role of Egg Incubation in Pathogen Regulation. - PLoS One 9(12): 1–19.

Braun, M. S., Zimmermann, S., Danner, M., Rashid, H. and Wink, M. 2016. Corynebacterium uropygiale sp. nov., isolated from the preen gland of Turkeys (Meleagris gallopavo). - Syst. Appl. Microbiol. 39: 88–92.

Braun, M. S., Wang, E., Zimmermann, S. and Wink, M. 2018a. Corynebacterium heidelbergense sp. nov., isolated from the preen glands of Egyptian geese (Alopochen aegyptiacus). - Syst. Appl. Microbiol. 41: 564–569.

Braun, M. S., Wang, E., Zimmermann, S., Boutin, S. and Wink, M. 2018b. Kocuria uropygioeca sp. nov. and Kocuria uropygialis sp. nov., isolated from the preen glands of Great Spotted Woodpeckers (Dendrocopos major). - Syst. Appl. Microbiol. 41: 38–43.

Braun, M. S., Sporer, F., Zimmermann, S. and Wink, M. 2018c. Birds, feather-degrading bacteria and preen glands: The antimicrobial activity of preen gland secretions from turkeys (Meleagris gallopavo) is amplified by keratinase. - FEMS Microbiol. Ecol. 94: fiy117.

Braun, M. S., Wang, E., Zimmermann, S., Boutin, S., Wagner, H. and Wink, M. 2019a.

Kocuria tytonicola, new bacteria from the preen glands of American barn owls (Tyto furcata). - Syst. Appl. Microbiol. 42: 198–204.

Braun, M. S., Wang, E., Zimmermann, S., Wagner, H. and Wink, M. 2019b. Kocuria tytonis sp. nov., isolated from the uropygial gland of an American barn owl (Tyto furcata). - Int. J. Syst. Evol. Microbiol. 69: 447–451.

Bright, M. and Bulgheresi, S. 2010. A complex journey: transmission of microbial symbionts. - Nat. Rev. Microbiol. 8: 218–230.

Brulez, K., Pike, T. W. and Reynolds, S. J. 2015. Egg signalling: The use of visual, auditory, and chemical stimuli. - In: Deeming, D. C. and Reynolds, S. J. (eds), Nest, Egg, and Incubation. New ideas about avian reproduction. Oxford University Press, pp. 127–141.

Brunetti, A. E., Lyra, M. L., Melo, W. G. P., Andrade, L. E., Palacios-Rodríguez, P., Prado, B. M., Haddad, C. F. B., Pupo, M. T. and Lopes, N. P. 2019. Symbiotic skin bacteria as a source for sex-specific scents in frogs. - Proc. Natl. Acad. Sci. 116: 2124–2129.

Bulla, M., Šálek, M. and Gosler, A. G. 2012. Eggshell spotting does not predict male incubation but marks thinner areas of a shorebird's shells. - Auk 129: 26–35.

Burger, B. V, Reiter, B., Borzyk, O. and du Plessis, M. A. 2004. Avian Exocrine Secretions. I. Chemical Characterization of the Volatile Fraction of the Uropygial Secretion of the Green Woodhoopoe, Phoeniculus purpureus. - J. Chem. Ecol. 30: 1603–1611.

Burley, N. 1986. Sexual Selection for Aesthetic Traits in Species with Biparental Care. - Am. Nat. 127: 415–445.

Burley, N. 1988. The Differential Allocation hypothesis. An experimental test. - Am. Nat. 132: 611–628.

Callahan, B. J., McMurdie, P. J. and Holmes, S. P. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. - ISME J. 11: 2639–2643.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., González Peña, A., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., Mcdonald, D., Muegge, B., Pirrung, M., Reeder, J., Sevinsky, J., Turnbaugh, P., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J. and Knight, R. 2010. QIIME allows

analysis of high-throughput community sequencing data. - Nat Methods. 7: 335–336.

Caruso, V., Song, X., Asquith, M. and Karstens, L. 2019. Performance of Microbiome Sequence Inference Methods in Environments with Varying Biomass (SM Gibbons, Ed.). - mSystems 4: e00163-18.

Chapman, T., Arnqvist, G., Bangham, J. and Rowe, L. 2003. Sexual conflict. - Trends Ecol. Evol. 18: 41–47.

Cherry, M. I. and Gosler, A. G. 2010. Avian eggshell coloration: New perspectives on adaptive explanations. - Biol. J. Linn. Soc. 100: 753–762.

Chiarello, M., Villéger, S., Bouvier, C., Auguet, J. C. and Bouvier, T. 2017. Captive bottlenose dolphins and killer whales harbor a species-specific skin microbiota that varies among individuals. - Sci. Rep. 7: 15269.

Clayton, D. H. 1991. The influence of parasites on host sexual selection. - Parasitol. Today 7: 329–334.

Clutton-Brock, T. H. 1991. The evolution of parental care (Princeton University Press, Ed.).

Clutton-Brock, T. 2007. Sexual Selection in Males and Females. - Science (80-.). 318: 1882–1885.

Clutton-Brock, T. 2009. Sexual selection in females. - Anim. Behav. 77: 3–11.

Cook, M. I., Beissinger, S. R., Toranzos, G. A. and Arendt, W. J. 2005. Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection. - Ecol. Lett. 8: 532–537.

Corti, M., Romano, A., Costanzo, A., Bentz, A. B., Navara, K. J., Parolini, M., Saino, N. and Rubolini, D. 2018. Protoporphyrin-based eggshell pigmentation predicts hatching success and offspring sex ratio in the barn swallow. - J. Avian Biol. 49: 1–17.

Cramp, S. 1998. The complete birds of the western Palearctic on CD-ROM. - Oxford University Press.

Cunningham, E. J. A. and Russell, A. F. 2000. Egg investment is influenced by male attractiveness in the mallard. - Nature 404: 74–77.

Currie, C. R., Scott, J. A., Summerbell, R. C. and Malloch, D. 1999. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. - Nature 398: 701–704.

D'Alba, L. and Shawkey, M. D. 2015. Mechanisms of antimicrobial defense in avian eggs. - J. Ornithol. 156: S399–S408.

Darby, A. C. and Douglas, A. E. 2003. Elucidation of the Transmission Patterns of an

Insect-Borne Bacterium. - Appl. Environ. Microbiol. 69: 4403 LP – 4407.

Darwin, C. 1871. The descent of man and selection in relation to sex (J Murray, Ed.).

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A. and Callahan, B. J. 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. - Microbiome 6: 226.

de Goffau, M. C., Lager, S., Salter, S. J., Wagner, J., Kronbichler, A., Charnock-Jones, D. S., Peacock, S. J., Smith, G. C. S. and Parkhill, J. 2018. Recognizing the reagent microbiome. - Nat. Microbiol. 3: 851–853.

Delhey, K., Peters, A. and Kempenaers, B. 2007. Cosmetic Coloration in Birds: Occurrence, Function, and Evolution. - Am. Nat. 169: S145–S158.

Dequiedt, S., Thioulouse, J., Jolivet, C., Saby, N. P. A., Lelievre, M., Maron, P.-A., Martin, M. P., Prévost-Bouré, N. C., Toutain, B., Arrouays, D., Lemanceau, P. and Ranjard, L. 2009. Biogeographical patterns of soil bacterial communities. - Environ. Microbiol. Rep. 1: 251–255.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P. and Andersen, G. L. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. - Appl. Environ. Microbiol. 72: 5069–5072.

Devaynes, A., Antunes, A., Bedford, A. and Ashton, P. 2018. Progression in the bacterial load during the breeding season in nest boxes occupied by the Blue Tit and its potential impact on hatching or fledging success. - J. Ornithol. 159: 1009–1017.

Díaz-Lora, S., Pérez-Contreras, T., Azcárate-García, M., Peralta-Sánchez, J. M., Martínez-Bueno, M., Soler, J. J. and Martín-Vivaldi, M. Cosmetic coloration of cross-fostered eggs affects male parental investment in the Hoopoe (Upupa epops). - Under Rev. in press.

Díaz-Lora, S., Pérez-Contreras, T., Azcárate-García, M., Martínez-Bueno, M., Soler, J. J. and Martín-Vivaldi, M. Hoopoe (Upupa epops) male feeding effort is related to female cosmetic egg colouration. - Under Rev. in press.

Díaz-Lora, S., Martín-Vivaldi, M., Juárez-García-Pelayo, N., Azcárate-García, M., Rodríguez-Ruano, S., M., Martínez-Bueno, M. and Soler, J. J. 2019. Data from: experimental old nest material predicts hoopoe Upupa epops eggshell and uropygial gland microbiota. − Dryad Digital Repository, <http://dx.doi. org/10.5061/dryad.8574ft0>.

Dillon, R. J., Vennard, C. T., Buckling, A. and Charnley, A. K. 2005. Diversity of locust gut bacteria protects against pathogen invasion. - Ecol. Lett. 8: 1291–1298.

Douglas, A. E. 1998. Nutritional Interactions in Insect-Microbial Symbioses: Aphids and Their Symbiotic Bacteria Buchnera. - Annu. Rev. Entomol. 43: 17–37.

Dumbrell, A. J., Ferguson, R. M. W. and Clark, D. R. 2017. Microbial Community Analysis by Single-Amplicon High-Throughput Next Generation Sequencing: Data Analysis – From Raw Output to Ecology. - In: McGenity, T. J. et al. (eds), Springer. Hydrocarbon and Lipid Microbiology Protocols: Microbial Quantitation, Community Profiling and Array Approaches, pp. 155–206.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. - Bioinformatics 26: 2460–2461.

Edgar, R. C. 2017. UNCROSS: Filtering of high-frequency cross-talk in 16S amplicon reads. - PeerJ 5: e3889.

Edward, D. A. and Chapman, T. 2011. The evolution and significance of male mate choice. - Trends Ecol. Evol. 26: 647–654.

Eisenhofer, R., Minich, J. J., Marotz, C., Cooper, A., Knight, R. and Weyrich, L. S. 2019. Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. - Trends Microbiol. 27: 105–117.

Ellegaard, K. M. and Engel, P. 2016. Beyond 16S rRNA Community Profiling: Intra-Species Diversity in the Gut Microbiota . - Front. Microbiol. 7: 1475.

Endler, J. A. 1990. On the measurement and classification of color in studies of animal color patterns. - Biol. J. Linn. Soc. 41: 315–352.

Endler, J. A. and Mielke, P. W. 2005. Comparing color patterns as birds see them. - Biol. J. Linn. Soc. 86: 405–431.

English, P. A. and Montgomerie, R. 2011. Robin's egg blue: Does egg color influence male parental care? - Behav. Ecol. Sociobiol. 65: 1029–1036.

Erckmann, W., Beletsky, L., Orians, G., Johnsen, T., Sharbaugh, S. and D´Antonio, C. 1990. Old nests as cues for nest-site selection: an experimental test with Red-winged Blackbirds. - Condor 92: 113–117.

Ezenwa, V. O. and Williams, A. E. 2014. Microbes and animal olfactory communication: Where do we go from here? - BioEssays 36: 847–854.

Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M. and Xavier, J. B. 2012. Animal Behavior and the Microbiome. - Science (80-.). 338: 198–199.

Fitzpatrick, C. L. and Servedio, M. R. 2018. The evolution of male mate choice and female ornamentation: A review of mathematical models. - Curr. Zool. 64: 323–333.

Flórez, L. V. and Kaltenpoth, M. 2017. Symbiont dynamics and strain diversity in the defensive mutualism between *Lagria* beetles and *Burkholderia*. - Environ. Microbiol. 19: 3674–3688.

Flórez, L. V, Biedermann, P. H. W., Engl, T. and Kaltenpoth, M. 2015. Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. - Nat. Prod. Rep. 32: 904–936.

Folstad, I. and Karter, A. J. 1992. Parasites, bright males, and the immunocompetence handicap. - Am. Nat. 139: 603–622.

Fons, M., Gomez, A. and Karjalainen, T. 2000. Mechanisms of Colonisation and Colonisation Resistance of the Digestive Tract Part 2: Bacteria/Bacteria Interactions. - Microb. Ecol. Health Dis. 12: 240–246.

Fontaine, J. J. and Martin, T. E. 2006. Parent birds assess nest predation risk and adjust their reproductive strategies. - Ecol. Lett. 9: 428–434.

Fox, H. M. and Vevers, G. 1960. The nature of animal colors.

Friedman, J. and Alm, E. J. 2012. Inferring Correlation Networks from Genomic Survey Data. - PLoS Comput. Biol. 8: e1002687.

Fronstin, R. B., Doucet, S. M. and Christians, J. K. 2016. Haematocrit, eggshell colouration and sexual signaling in the European starling (Sturnus vulgaris). - BMC Ecol. in press.

Gamble, K. C. 2007. Internal Anatomy of the Hornbill Casque Described by Radiography , Contrast Radiography , and Computed Tomography. - J. Avian Med. Surg. 21: 38–49.

Gamble, K. C. 2012. Chapter 37. Squamous Cell Carcinoma in Buceros Hornbills. - In: Miller, R. E. and Fowler, M. (eds), Fowler's Zoo and Wild Animal Medicine. pp. 281–285.

Garamszegi, Z. and Møller, A. P. 2010. Effects of sample size and intraspecific variation in phylogenetic comparative studies : a meta-analytic review. - Biol. Rev. 85: 797–805.

Garamszegi, L. Z. and Møller, A. P. 2011. Nonrandom Variation in Within-Species Sample Size and Missing Data in Phylogenetic Comparative Studies. - Syst. Biol. 60: 876–880.

Gaston, A. J., De Forest, L. N. and G.Noble, D. 1993. Egg Recognition and Egg Stealing in Murres (Uria spp.). - Anim. Behav. 45: 301–306.

Gawryszewski, F. M. 2018. Color vision models: Some simulations, a general n-dimensional model, and the colourvision R package. - Ecol. Evol.: 8159–8170.

Giard, J.-C., Laplace, J.-M., Rincé, A., Pichereau, V., Benachour, A., Leboeuf, C., Flahaut, S., Auffray, Y. and Hartke, A. 2001. The stress proteome of *Enterococcus faecalis*. - Electrophoresis 22: 2947–2954.

Gil-Turnes, M. S. and Fenical, W. 1992. Embryos of Homarus americanus are Protected by Epibiotic Bacteria. - Biol. Bull. 182: 105–108.

Giordano, M., Costantini, D., Pick, J. L. and Tschirren, B. 2015. Female oxidative status, egg antioxidant protection and eggshell pigmentation: a supplemental feeding experiment in great tits. - Behav. Ecol. Sociobiol. 69: 777–785.

Goldstein, G., Flory, K. R., Browne, B. A., Majid, S., Ichida, J. M. and Burtt Jr., E. H. 2004. Bacterial Degradation of Black and White Feathers. - Auk 121: 656–659.

Golob, J. L., Margolis, E., Hoffman, N. G. and Fredricks, D. N. 2017. Evaluating the accuracy of amplicon-based microbiome computational pipelines on simulated human gut microbial communities. - BMC Bioinformatics 18: 283.

Gómez, D. 2006. AVICOL a program to analyse spectrometric data. Last update october 2011. Free executable available at http://sites.google.com/site/avicolprogram/ or from the author at dodogomez@yahoo.fr. in press.

González-Braojos, S., Vela, A. I., Ruiz-De-Castañeda, R., Briones, V., Cantarero, A. and Moreno, J. 2012. Is Nestling Growth Affected by Nest Reuse and Skin Bacteria in Pied Flycatchers *Ficedula hypoleuca?* - Acta Ornithol. 47: 119–127.

Gonzalez, J. C. T., Sheldon, B. C., Collar, N. J. and Tobias, J. A. 2013. A comprehensive molecular phylogeny for the hornbills (Aves: Bucerotidae). - Mol. Phylogenet. Evol. 67: 468–483.

Gosler, A. G., Higham, J. P. and Reynolds, S. J. 2005. Why are birds ' eggs speckled ? - Ecol. Lett. 8: 1105–1113.

Grether, G. F., Hudon, J. and Millie, D. F. 1999. Carotenoid limitation of sexual coloration along an environmental gradient in guppies. - Proc. R. Soc. London. Ser. B Biol. Sci. 266: 1317–1322.

Griggio, M., Valera, F., Casas, A. and Pilastro, A. 2005. Males prefer ornamented females: A field experiment of male choice in the rock sparrow. - Anim. Behav. 69:

1243–1250.

Grizard, S., Dini-Andreote, F., Tieleman, B. I. and Salles, J. F. 2014. Dynamics of bacterial and fungal communities associated with eggshells during incubation. - Ecol. Evol. 4: 1140–1157.

Grizard, S., Versteegh, M. A., Ndithia, H. K., Salles, J. F. and Tieleman, B. I. 2015. Shifts in Bacterial Communities of Eggshells and Antimicrobial Activities in Eggs during Incubation in a Ground-Nesting Passerine. - PLoS One: 10(4).

Gunderson, A. R. 2008. Feather-Degrading Bacteria: A New Frontier in Avian and Host–Parasite Research? - Auk 125: 972–979.

Gwinner, H. and Berger, S. 2005. European starlings: Nestling condition, parasites and green nest material during the breeding season. - J. Ornithol. 146: 365–371.

Haine, E. R. 2008. Symbiont-mediated protection. - Proc. R. Soc. B Biol. Sci. 275: 353–361.

Hamilton, W. D. and Zuk, M. 1982. Heritable true fitness and bright birds: a role for parasites? - Science (80-.). 218: 384–387.

Hanley, D. 2012. The function and evolution of egg colour in birds. - Electron. Theses Diss.: 382.

Hanley, D. and Doucet, S. M. 2009. Egg coloration in ring-billed gulls (Larus delawarensis): A test of the sexual signaling hypothesis. - Behav. Ecol. Sociobiol. 63: 719–729.

Hanley, D., Heiber, G. and Dearborn, D. C. 2008. Testing an Assumption of the Sexual-Signaling Hypothesis: Does Blue-Green Egg Color Reflect Maternal Antioxidant Capacity? - Condor 110: 767–771.

Hanley, D., Doucet, S. M. and Dearborn, D. C. 2010. A Blackmail Hypothesis for the Evolution of Conspicuous Egg Coloration in Birds. - Auk 127: 453–459.

Hargitai, R., Boross, N., Nyiri, Z. and Eke, Z. 2018a. Effects of food limitation on the intensity of blue-green and brown eggshell coloration: an experimental study with the canary. - J. Avian Biol.: e01486.

Hargitai, R., Herényi, M., Nagy, G. and Török, J. 2018b. Eggshell spotting pattern is related to hatching asynchrony, hematocrit value and growth of nestling great tits Parus major. - J. Avian Biol. 49: e01827.

Hart, N. S. 2001. The visual ecology of avian Photoreceptors. - Prog. Retin. Eye Res. 20, No. 5: 675 to 703.

Hart, N. S. 2002. Vision in the peafowl (Aves: Pavo cristatus). - J. Exp. Biol. 205: 3925–3935.

Harvey, P. H. and Pagel, M. D. 1991. The Comparative Method in Evolutionary Biology. - Oxford University Press.

He, Y., Caporaso, J. G., Jiang, X.-T., Sheng, H.-F., Huse, S. M., Rideout, J. R., Edgar, R. C., Kopylova, E., Walters, W. A., Knight, R. and Zhou, H.-W. 2015. Stability of operational taxonomic units: an important but neglected property for analyzing microbial diversity. - Microbiome 3: 20.

Heath-Heckman, E. A. C., Peyer, S. M., Whistler, C. A., Apicella, M. A., Goldman, W. E. and McFall-Ngai, M. J. 2013. Bacterial Bioluminescence Regulates Expression of a Host Cryptochrome Gene in the Squid-Vibrio Symbiosis (J Handelsman, Ed.). - MBio 4: e00167-13.

Heath, K. D. and Tiffin, P. 2007. Context dependence in the coevolution of plant and rhizobial mutualists. - Proc. R. Soc. B Biol. Sci. 274: 1905–1912.

Henry, L. P., Bruijning, M., Forsberg, S. K. G. and Ayroles, J. F. 2019. Can the microbiome influence host evolutionary trajectories? - bioRxiv: 700237.

Hill, M. J. 1997. Intestinal flora and endogenous vitamin synthesis. - Eur. J. Cancer Prev. 6 Suppl 1: S43-45.

Hill, G. E. 2000. Energetic constraints on expression of carotenoid-based plumage coloration. - J. Avian Biol. 31: 559–566.

Hill, G. E. 2002. A red bird in a brown bag: the function and evolution of ornamental plumage coloration in the house finch. - Oxford University Press.

Hill, G. E. 2006. Environmental Regulation of Ornamental Coloration. - In: Hill, G. E. and Mcgraw, K. J. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 507–560.

Hill, G. E., Farmer, K. L. and Beck, M. L. 2004. The effect of mycoplasmosis on carotenoid plumage coloration in male house finches. - J. Exp. Biol. 207: 2095–2099.

Hird, S. M. 2017. Evolutionary Biology Needs Wild Microbiomes. - Front. Microbiol. 8: 725.

Hoffmann, J., Postma, E. and Schaub, M. 2015. Factors influencing double brooding in Eurasian Hoopoes Upupa epops. - Ibis (Lond. 1859). 157: 17–30.

Holveck, M.-J., Doutrelant, C., Guerreiro, R., Perret, P., Gomez, D. and Grégoire, A. 2010. Can eggs in a cavity be a female secondary sexual signal? Male nest visits and

modelling of egg visual discrimination in blue tits. - Biol. Lett. 6: 453–457.

Holveck, M., Guerreiro, R., Perret, P., Doutrelant, C. and Grégoire, A. 2019. Eggshell coloration indicates female condition during egg-laying: a field experiment in blue tits. - Biol. J. Linn. Soc. XX: 1–20.

Honza, M., Požgayová, M., Procházka, P. and Cherry, M. I. 2011. Blue-green eggshell coloration is not a sexually selected signal of female quality in an open-nesting polygynous passerine. - Naturwissenschaften 98: 493–499.

Hornung, B. V. H., Zwittink, R. D. and Kuijper, E. J. 2019. Issues and current standards of controls in microbiome research. - FEMS Microbiol. Ecol. 95: fiz045.

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y. and Fukatsu, T. 2010. Wolbachia as a bacteriocyte-associated nutritional mutualist. - Proc. Natl. Acad. Sci. 107: 769–774.

Hoyt, D. F. 1979. Practical methods of estimating volume and fresh weight of bird eggs. - Auk 96: 73–77.

Jacob, J. and Ziswiler, V. 1982. The uropygial gland. - In: Farner, D. S. et al. (eds), Avian biology, vol VI. Academic Press, pp. 359–362.

Jacob, S., Parthuisot, N., Vallat, A., Ramon-Portugal, F., Helfenstein, F. and Heeb, P. 2015. Microbiome affects egg carotenoid investment, nestling development and adult oxidative costs of reproduction in Great tits. - Funct. Ecol. 29: 1048–1058.

Janzen, D. H. 1985. The natural history of mutualism. - In: Boucher, D. H. (ed), The Biology of Mutualism: Ecology and Evolution. Oxford University Press, pp. 40–99.

Javůrková, V. G., Kreisinger, J., Procházka, P., Požgayová, M., Ševčíková, K., Brlík, V., Adamík, P., Heneberg, P. and Porkert, J. 2019. Unveiled feather microcosm: feather microbiota of passerine birds is closely associated with host species identity and bacteriocin-producing bacteria. - ISME J.: 2363–2376.

Jervis-Bardy, J., Leong, L. E. X., Marri, S., Smith, R. J., Choo, J. M., Smith-Vaughan, H. C., Nosworthy, E., Morris, P. S., O'Leary, S., Rogers, G. B. and Marsh, R. L. 2015. Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. - Microbiome 3: 19.

Jetz, W., Thomas, G. H., Joy, J. B., Hartmann, K. and Mooers, A. O. 2012. The global diversity of birds in space and time. - Nature 491: 444–448.

Johnsen, A., Vesterkjær, K. and Slagsvold, T. 2011. Do Male Pied Flycatchers (Ficedula hypoleuca) Adjust Their Feeding Effort According to Egg Colour? - Ethology 117: 309–317.

Johnstone, R. A. 1995. Sexual selection, honest advertisement and the handicap principle: reviewing the evidence. - Biol. Rev. 70: 1–65.

Kaltenpoth, M., Göttler, W., Herzner, G. and Strohm, E. 2005. Symbiotic Bacteria Protect Wasp Larvae from Fungal Infestation. - Curr. Biol. 15: 475–479.

Kaltz, O. and Koella, J. C. 2003. Host growth conditions regulate the plasticity of horizontal and vertical transmission in holospora undulata, a bacterial parasite of the protozoan paramecium caudatum. - Evolution (N. Y). 57: 1535–1542.

Karstens, L., Asquith, M., Davin, S., Fair, D., Gregory, W. T., Wolfe, A. J., Braun, J. and McWeeney, S. 2019. Controlling for Contaminants in Low-Biomass 16S rRNA Gene Sequencing Experiments (JA Gilbert, Ed.). - mSystems 4: e00290-19.

Kaur, H., Hughes, M. N., Green, C. J., Naughton, P., Foresti, R. and Motterlini, R. 2003. Interaction of bilirubin and biliverdin with reactive nitrogen species. - FEBS Lett. 543: 113–119.

Keebaugh, E. S., Yamada, R., Obadia, B., Ludington, W. B. and Ja, W. W. 2018. Microbial Quantity Impacts Drosophila Nutrition, Development, and Lifespan. - iScience 4: 247–259.

Kemp, A. C. 2001. Family Bucerotidae (Hornbills). - In: Del Hoyo, J. et al. (eds), Handbook of the birds of the world, Piciformes to Bucerotiformes, vol 6. Lynx Editi. pp. 436–523.

Kemp, D. J., Herberstein, M. E., Fleishman, L. J., Endler, J. a., Bennett, A. T. D., Dyer, A. G., Hart, N. S., Marshall, J. and Whiting, M. J. 2015. An Integrative Framework for the Appraisal of Coloration in Nature. - Am. Nat. 185: 000–000.

Kikuchi, Y., Hayatsu, M., Hosokawa, T., Nagayama, A., Tago, K. and Fukatsu, T. 2012. Symbiont-mediated insecticide resistance. - Proc. Natl. Acad. Sci. 109: 8618–8622.

Kilner, R. M. 2006. The evolution of egg colour and patterning in birds. - Biol. Rev. Camb. Philos. Soc. 81: 383–406.

Kinnaird, M. F. and O'Brien, T. G. 2007. The Ecology and Conservation of Asian Hornbills: Farmers of the Forest.

Knight, R., Vrbanac, A., Taylor, B. C., Aksenov, A., Callewaert, C., Debelius, J., Gonzalez, A., Kosciolek, T., McCall, L. I., McDonald, D., Melnik, A. V., Morton, J. T., Navas, J., Quinn, R. A., Sanders, J. G., Swafford, A. D., Thompson, L. R., Tripathi, A., Xu, Z. Z., Zaneveld, J. R., Zhu, Q., Caporaso, J. G. and Dorrestein, P. C. 2018. Best practices for analysing microbiomes. - Nat. Rev. Microbiol. 16: 410–422.

Kopylova, E., Navas-molina, J. A., Mercier, C., Xu, Z., Mahé, F., He, Y., Zhou, H.-W., Rognes, T., Caporaso, J. G. and Knight, R. 2016. Open-Source Sequence Clustering Methods Improve the State Of the Art. mSystems. 1: 1–16.

Kose, M. and Møller, A. P. 1999. Sexual selection, feather breakage and parasites: the importance of white spots in the tail of the barn swallow (Hirundo rustica). - Behav. Ecol. Sociobiol. 45: 430–436.

Kraaijeveld, K., Kraaijeveld-Smit, F. J. L. and Komdeur, J. 2007. The evolution of mutual ornamentation. - Anim. Behav. 74: 657–677.

Krist, M. and Grim, T. 2007. Are blue eggs a sexually selected signal of female collared flycatchers? A cross-fostering experiment. - Behav. Ecol. Sociobiol. 61: 863–876.

Kuczynski, J., Stombaugh, J., Walters, W. A., González, A., Caporaso, J. G. and Knight, R. 2012. Using QIIME to analyze 16s rRNA gene sequences from microbial communities. - Curr. Protoc. Microbiol. in press.

Labocha, M. K. and Hayes, J. P. 2012. Morphometric indices of body condition in birds: A review. - J. Ornithol. 153: 1–22.

Lahti, D. C. 2008. Population Differentiation and Rapid Evolution of Egg Color in Accordance With Solar Radiation. - Auk 125: 796–802.

Larsson, A. J. M., Stanley, G., Sinha, R., Weissman, I. L. and Sandberg, R. 2018. Computational correction of index switching in multiplexed sequencing libraries. - Nat. Methods 15: 305–307.

Law-Brown, J. and Meyers, P. R. 2003. *Enterococcus phoeniculicola* sp . nov ., a novel member of the enterococci isolated from the uropygial gland of the Red-billed Woodhoopoe , *Phoeniculus purpureus*. - Int. J. Syst. Evol. Microbiol. 53: 683–685.

Leclaire, S., Nielsen, J. F. and Drea, C. M. 2014. Bacterial communities in meerkat anal scent secretions vary with host sex, age, and group membership. - Behav. Ecol. 25: 996–1004.

Leclaire, S., Jacob, S., Greene, L. K., Dubay, G. R. and Drea, C. M. 2017. Social odours covary with bacterial community in the anal secretions of wild meerkats. - Sci. Rep. 7: 3240.

Leftwich, P. T., Hutchings, M. I. and Chapman, T. 2018. Diet, Gut Microbes and Host Mate Choice. - BioEssays 40: 1800053.

Leitner, S., Marshall, R. C., Leisler, B. and Catchpole, C. K. 2006. Male song quality, egg size and offspring sex in captive canaries (Serinus canaria). - Ethology 112: 554–563.

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D. and Gordon, J. I. 2005. Obesity alters gut microbial ecology. - Proc. Natl. Acad. Sci. 102: 11070–11075.

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R. and Gordon, J. I. 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. - Nat. Rev. Microbiol. 6: 776–788.

Li, D., Chen, B., Zhang, L., Gaur, U., Ma, T., Jie, H., Zhao, G., Wu, N., Xu, Z., Xu, H., Yao, Y., Lian, T., Fan, X., Yang, D., Yang, M., Zhu, Q. and Satkoski Trask, J. 2016. The musk chemical composition and microbiota of Chinese forest musk deer males. - Sci. Rep. 6: 18975.

Lindquist, N., Barber, P. H. and Weisz, J. B. 2005. Episymbiotic microbes as food and defence for marine isopods: unique symbioses in a hostile environment. - Proc. R. Soc. B Biol. Sci. 272: 1209–1216.

Linville, S. U., Breitwisch, R. and Schilling, A. J. 1998. Plumage brightness as an indicator of parental care in northern cardinals. - Anim. Behav. 55: 119–127.

Lopetuso, L. R., Scaldaferri, F., Petito, V. and Gasbarrini, A. 2013. Commensal Clostridia: leading players in the maintenance of gut homeostasis. - Gut Pathog. 5: 23.

López-Rull, I., Gil, M. and Gil, D. 2007. Spots in starling Sturnus unicolor eggs are good indicators of ectoparasite load by Carnus hemapterus (Diptera: Carnidae). - Ardeola 54: 131–134.

López-Rull, I., Pagán, I. and Macías Garcia, C. 2010. Cosmetic enhancement of signal coloration: Experimental evidence in the house finch. - Behav. Ecol. 21: 781–787.

Lozupone, C. and Knight, R. 2005. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. - Appl. Environ. Microbiol. 71: 8228–8235.

Lyu, N., Servedio, M. R., Lloyd, H. and Sun, Y. 2017. The evolution of postpairing male mate choice. - Evolution (N. Y). 71: 1465–1477.

Macpherson, A. J. and Harris, N. L. 2004. Interactions between commensal intestinal bacteria and the immune system. - Nat. Rev. Immunol. 4: 478–485.

Maddison, W. . and Maddison, D. R. 2018. Mesquite: a modular system for evolutionary analysis. Version 3.6.: http://www.mesquiteproject.org.

Maier, R. M., Pepper, I. L. and Gerba, C. P. 2000. Environmental Microbiology. - Academic Press, San Diego, California.

Mainwaring, M. C., Hartley, I. R., Lambrechts, M. M. and Deeming, D. C. 2014. The design and function of birds' nests. - Ecol. Evol. 4: 3909–3928.

Mallows, C. L. 1973. Some Comments on Cp. - Technometrics 15: 661–675.

Maraci, Ö., Engel, K. and Caspers, B. A. 2018. Olfactory communication via microbiota: what is known in birds? - Genes (Basel). in press.

Martín-Platero, A. M., Valdivia, E., Ruíz-Rodríguez, M., Soler, J. J., Martín-Vivaldi, M., Maqueda, M. and Martínez-Bueno, M. 2006. Characterization of antimicrobial substances produced by *Enterococcus faecalis* MRR 10-3, isolated from the uropygial gland of the hoopoe (*Upupa epops*). - Appl. Environ. Microbiol. 72: 4245–4249.

Martín-Vivaldi, M., Palomino, J. J., Soler, M. and Soler, J. J. 1999. Determinants of reproductive success in the Hoopoe Upupa epops, a hole-nesting non-passerine bird with asynchronous hatching. - Bird Study 46: 205–216.

Martín-Vivaldi, M., Ruiz-Rodríguez, M., Soler, J. J., Peralta-Sánchez, J. M., Méndez, M., Valdivia, E., Martín-Platero, A. M. and Martínez-Bueno, M. 2009. Seasonal, sexual and developmental differences in hoopoe Upupa epops preen gland morphology and secretions: Evidence for a role of bacteria. - J. Avian Biol. 40: 191–205.

Martín-Vivaldi, M., Peña, A., Peralta-Sánchez, J. M., Sánchez, L., Ananou, S., Ruiz-Rodríguez, M. and Soler, J. J. 2010. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. - Proc. Biol. Sci. 277: 123–130.

Martín-Vivaldi, M., Soler, J. J., Peralta-Sánchez, J. M., Arco, L., Martín-Platero, A. M., Martínez-Bueno, M., Ruiz-Rodríguez, M. and Valdivia, E. 2014a. Special structures of hoopoe eggshells enhance the adhesion of symbiont-carrying uropygial secretion that increase hatching success. - J. Anim. Ecol. 83: 1289–1301.

Martín-Vivaldi, M., Doña, J., Romero Masegosa, J. and Soto Cárdenas, M. 2014b. Abubilla - Upupa epops Linnaeus, 1758. - En Encicl. Virtual los Vertebr. Españoles. Salvador. A., Morales, M. B. (Eds.). Mus. Nac. Ciencias Nat. Madrid. http//www.vertebradosibericos.org/

Martín-Vivaldi, M., Soler, J. J., Martínez-García, Á., Arco, L., Juárez-García-Pelayo, N., Ruiz-Rodríguez, M. and Martínez-Bueno, M. 2018. Acquisition of Uropygial Gland Microbiome by Hoopoe Nestlings. - Microb. Ecol. 76: 285–297.

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. - EMBnet.journal 17: 10–12.

Martin, T. E. and Badyaev, A. V 1996. Sexual dichromatism in birds: importance of nest predation and nest location for females versus males. - Evolution (N. Y). 50: 2454–2460.

Martínez-De La Puente, J., Merino, S., Moreno, J., Tomás, G., Morales, J., Lobato, E., García-Fraile, S. and Martínez, J. 2007. Are eggshell spottiness and colour indicators of health and condition in blue tits Cyanistes caeruleus? - J. Avian Biol. 38: 377–384.

Martínez-García, Á. 2015. Biodiversidad bacteriana de la secreción uropigial y la cáscara de los huevos de abubilla: estabilidad y adquisición.

Martínez-García, Á., Soler, J. J., Rodríguez-Ruano, S. M., Martínez-Bueno, M., Martín-Platero, A. M., Juárez-García, N. and Martín-Vivaldi, M. 2015. Preening as a Vehicle for Key Bacteria in Hoopoes. - Microb. Ecol. 70: 1024–1033.

Martínez-García, Á., Martín-Vivaldi, M., Rodríguez-Ruano, S. M., Peralta-Sánchez, J. M., Valdivia, E. and Soler, J. J. 2016a. Nest bacterial environment affects microbiome of hoopoe eggshells, but not that of the uropygial secretion. - PLoS One 11(7): e0158158.

Martínez-García, Á., Martín-Vivaldi, M., Ruiz-Rodríguez, M., Martínez-Bueno, M., Arco, L., Rodríguez-Ruano, S. M., Peralta-Sánchez, J. M. and Soler, J. J. 2016b. The Microbiome of the Uropygial Secretion in Hoopoes Is Shaped Along the Nesting Phase. - Microb. Ecol. 72: 252–261.

Massaro, M., Davis, L. S. and Darby, J. T. 2003. Carotenoid-derived ornaments reflect parental quality in male and female yellow-eyed penguins (Megadyptes antipodes). - Behav. Ecol. Sociobiol. 55: 169–175.

Mazgajski, T. D. 2007. Effect of old nest material on nest site selection and breeding parameters in secondary hole nesters — a review. - Acta Ornithol. 42: 1–14.

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., Desantis, T. Z., Probst, A., Andersen, G. L., Knight, R. and Hugenholtz, P. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. - ISME J. 6: 610–618.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E., Rawls, J. F., Reid, A., Ruby, E. G., Rumpho, M., Sanders, J. G., Tautz, D. and Wernegreen, J. J. 2013. Animals in a bacterial world, a new

imperative for the life sciences. - Proc. Natl. Acad. Sci. U. S. A. 110: 3229–3236.

Mcgraw, K. J. and Hill, G. E. 2004. Plumage color as a dynamic trait : carotenoid pigmentation of male house finches (Carpodacus mexicanus) fades during the breeding season. - Can. J. Zool 82: 734–738.

McGraw, K. J. and Ardia, D. R. 2003. Carotenoids, Immunocompetence, and the Information Content of Sexual Colors: An Experimental Test. - Am. Nat. 162: 704–712.

McKnight, D. T., Huerlimann, R., Bower, D. S., Schwarzkopf, L., Alford, R. A. and Zenger, K. R. 2019. microDecon: A highly accurate read-subtraction tool for the post-sequencing removal of contamination in metabarcoding studies. - Environ. DNA 1: 14–25.

Mikšík, I., Holáň, V. and Deyl, Z. 1996. Avian eggshell pigments and their variability. - Comp. Biochem. Physiol. 113: 607–612.

Miller, R. E., Trampel, D. W., Boever, W. J. and Kling, M. A. 1985. Carcinoma in the Casque of a Greater Indian Hornbill (Buceros bicornis). - J. Zoo Anim. Med. 16: 131–136.

Minich, J. J., Zhu, Q., Janssen, S., Hendrickson, R., Amir, A., Vetter, R., Hyde, J., Doty, M. M., Stillwell, K., Benardini, J., Kim, J. H., Allen, E. E., Venkateswaran, K. and Knight, R. 2018. KatharoSeq Enables High-Throughput Microbiome Analysis from Low-Biomass Samples (MJ McFall-Ngai, Ed.). - mSystems 3: e00218-17.

Møller, A. P. 1993. Sexual Selection in the Barn Swallow Hirundo rustica. III. Female Tail Ornaments. - Evolution (N. Y). 47: 417–431.

Møller, A. P. and Erritzoe, J. 1996. Parasite Virulence and Host Immune Defense : Host Immune Response is Related to Nest Reuse in Birds. - Evolution (N. Y). 50: 2066–2072.

Møller, A. P., Arriero, E., Lobato, E. and Merino, S. 2009. A meta-analysis of parasite virulence in nestling birds. - Biol. Rev. 84: 567–588.

Montgomerie, R. 2006. Cosmetic and adventitious colors. - In: Hill, E. and McGraw, K. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 399–427.

Morales, J., Sanz, J. J. and Moreno, J. 2006. Egg colour reflects the amount of yolk maternal antibodies and fledging success in a songbird. - Biol. Lett. 2: 334–336.

Morales, J., Moreno, J., Merino, S., Sanz, J. J., Tomás, G., Arriero, E., Lobato, E. and

Martínez-De La Puente, J. 2007. Female ornaments in the Pied Flycatcher Ficedula hypoleuca: Associations with age, health and reproductive success. - Ibis (Lond. 1859). 149: 245–254.

Morales, J., Velando, A. and Moreno, J. 2008. Pigment allocation to eggs decreases plasma antioxidants in a songbird. - Behav. Ecol. Sociobiol. 63: 227–233.

Moran, N. A. 2006. Symbiosis. - Curr. Biol. 16: R866–R871.

Moran, N. A., McCutcheon, J. P. and Nakabachi, A. 2008. Genomics and Evolution of Heritable Bacterial Symbionts. - Annu. Rev. Genet. 42: 165–190.

Moreau, R. E. 1934. Breeding Habits of Hornbills. - Nature 134: 899.

Moreno, J. and Osorno, J. L. 2003. Avian egg colour and sexual selection: Does eggshell pigmentation reflect female condition and genetic quality? - Ecol. Lett. 6: 803–806.

Moreno, J., Briones, V., Merino, S., Ballesteros, C., Sanz, J. J. and Tomás, G. 2003. Beneficial Effects of Cloacal Bacteria on Growth and Fledging Size in Nestling Pied Flycatchers (Ficedula Hypoleuca) in Spain. - Auk 120: 784–790.

Moreno, J., Osorno, J. L., Morales, J., Merino, S. and Tomás, G. 2004. Egg Colouration and Male Parental Effort in the Pied Flycatcher Ficedula Hypoleuca. - J. Avian Biol. 35: 300–304.

Moreno, J., Morales, J., Lobato, E., Merino, S., Tomás, G. and Martínez-De La Puente, J. 2005. Evidence for the signaling function of egg color in the pied flycatcher Ficedula hypoleuca. - Behav. Ecol. 16: 931–937.

Moreno, J., Morales, J., Lobato, E., Merino, S., Tomás, G. and Martínez-De La Puente, J. 2006a. More colourful eggs induce a higher relative paternal investment in the pied flycatcher Ficedula hypoleuca: A cross-fostering experiment. - J. Avian Biol. 37: 555–560.

Moreno, J., Lobato, E., Morales, J., Merino, S., Tomás, G., Martínez-De La Puente, J., Sanz, J. J., Mateo, R. and Soler, J. J. 2006b. Experimental evidence that egg color indicates female condition at laying in a songbird. - Behav. Ecol. 17: 651–655.

Moreno, J., Lobato, E., Merino, S. and Martínez-De La Puente, J. 2008. Blue-green eggs in pied flycatchers: An experimental demonstration that a supernormal stimulus elicits improved nestling condition. - Ethology 114: 1078–1083.

Morton, J. T., Sanders, J., Quinn, R. A., McDonald, D., Gonzalez, A., Vázquez-Baeza, Y., Navas-Molina, J. A., Song, S. J., Metcalf, J. L., Hyde, E. R., Lladser, M., Dorrestein, P. C. and Knight, R. 2017. Balance Trees Reveal Microbial Niche Differentiation (JK

Jansson, Ed.). - mSystems 2: e00162-16.

Mushegian, A. A., Walser, J.-C., Sullam, K. E. and Ebert, D. 2018. The microbiota of diapause: How host–microbe associations are formed after dormancy in an aquatic crustacean. - J. Anim. Ecol. 87: 400–413.

Naish, D. 2015. The Ecology and Conservation of Asian Hornbills: Farmers of the Forest. - Hist. Biol. 27: 954–956.

Navas-Molina, J. A., Peralta-Sánchez, J. M., González, A., McMurdie, P. J., Vázquez-Baeza, Y., Xu, Z., Ursell, L. K., Lauber, C., Zhou, H., Song, S. J., Huntley, J., Ackermann, G. L., Berg-Lyons, D., Holmes, S., Caporaso, J. G. and Knight, R. 2013. Advancing our understanding of the human microbiome using QIIME. - Elsevier Inc.

Negro, J. J., Margalida, A., Hiraldo, F. and Heredia, R. 1999. The function of the cosmetic coloration of bearded vultures : when art imitates life. - Anim. Behav. S8: 14–17.

Nicholls, E. M. and Rienits, K. G. 1971. Tryptophan derivatives and pigment in the hair of some Australian marsupials. - Int. J. Biochem. 2: 593–603.

Ödeen, A. and Håstad, O. 2003. Complex distribution of avian color vision systems revealed by sequencing the SWS1 opsin from total DNA. - Mol. Biol. Evol. 20: 855–861.

Oliver, K. M., Russell, J. A., Moran, N. A. and Hunter, M. S. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. - Proc. Natl. Acad. Sci. 100: 1803 LP – 1807.

Olson, V. A. and Owens, I. P. F. 1998. Costly sexual signals: are carotenoids rare, risky or required? - Trends Ecol. Evol. 13: 510–514.

Olsson, K. and Allander, K. 1995. Do Fleas, and/or Old Nest Material, Influence Nest-site preference in Hole-nesting Passerines? - Ethology 101: 160–170.

Olsson, P., Lind, O. and Kelber, A. 2015. Bird colour vision: behavioural thresholds reveal receptor noise. - J. Exp. Biol. 218: 184–193.

Orell, M., Rytkönen, S. and Ilomäki, K. 1993. Do pied flycatchers prefer nest boxes with old nest material? - Ann. Zool. Fennici 30: 313–316.

Pagel, M. 1997. Inferring evolutionary processes from phylogenies. - Zool. Scr. 26: 331–348.

Pagel, M. 1999. Inferring the historical patterns of biological evolution. - Nature 401: 877–884.

Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A. and Brown, P. O. 2007.

Development of the Human Infant Intestinal Microbiota. - PLoS Biol. 5: e177.

Paradis, E., Claude, J. and Strimmer, K. 2004. APE : Analyses of Phylogenetics and Evolution in R language. - Bioinformatics 20: 289–290.

Paz, L.-C., Schramm, A. and Lund, M. B. 2017. Biparental transmission of Verminephrobacter symbionts in the earthworm Aporrectodea tuberculata (Lumbricidae). - FEMS Microbiol. Ecol. 93: fix025.

Pearce, D. S., Hoover, B. A., Jennings, S., Nevitt, G. A. and Docherty, K. M. 2017. Morphological and genetic factors shape the microbiome of a seabird species (Oceanodroma leucorhoa) more than environmental and social factors. - Microbiome 5: 146.

Peig, J. and Green, A. J. 2010. The paradigm of body condition: A critical reappraisal of current methods based on mass and length. - Funct. Ecol. 24: 1323–1332.

Peralta-Sanchez, J. M., Møller, A. P., Martin-Platero, A. M. and Soler, J. J. 2010. Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: An experimental study. - Funct. Ecol. 24: 426–433.

Peralta-Sánchez, J. M., Martín-Vivaldi, M., Martín-Platero, A. M., Martínez-Bueno, M., Oñate, M., Ruiz-Rodríguez, M. and Soler, J. J. 2012. Avian life history traits influence eggshell bacterial loads: A comparative analysis. - Ibis (Lond. 1859). 154: 725–737.

Peralta-Sánchez, J. M., Soler, J. J., Martín-Platero, A. M., Knight, R., Martínez-Bueno, M. and Møller, A. P. 2014. Eggshell Bacterial Load Is Related to Antimicrobial Properties of Feathers Lining Barn Swallow Nests. - Microb. Ecol. 67: 480–487.

Pérez-Rodríguez, L., Mougeot, F. and Bortolotti, G. R. 2011. The effects of preen oils and soiling on the UV – visible reflectance of carotenoid-pigmented feathers. - Behav. Ecol. Sociobiol. 65: 1425–1435.

Piault, R., Gasparini, J., Bize, P., Paulet, M., McGraw, K. J. and Roulin, A. 2008. Experimental support for the makeup hypothesis in nestling tawny owls (Strix aluco). - Behav. Ecol. 19: 703–709.

Plard, F., Arlettaz, R. and Schaub, M. 2018. Hoopoe males experience intra-seasonal while females experience inter-seasonal reproductive costs. - Oecologia 186: 665–675.

Podofillini, S., Cecere, J. G., Griggio, M., Curcio, A., De Capua, E. L., Fulco, E., Pirrello, S., Saino, N., Serra, L., Visceglia, M. and Rubolini, D. 2018. Home, dirty home:

effect of old nest material on nest-site selection and breeding performance in a cavity-nesting raptor. - Curr. Zool.: 1–10.

Poláček, M., Griggio, M., Mikšík, I., Bartíková, M., Eckenfellner, M. and Hoi, H. 2017. Eggshell coloration and its importance in postmating sexual selection. - Ecol. Evol. 7: 941–949.

Poonswad, P., Kemp, A. and Strange, M. 2013. Hornbills of the world: A photographic guide.

Price, M. N., Dehal, P. S. and Arkin, A. P. 2009. Fasttree: Computing large minimum evolution trees with profiles instead of a distance matrix. - Mol. Biol. Evol. 26: 1641–1650.

Prum, R. O. 2006. Anatomy, Physics, and Evolution of Structural Colors. - In: Hill, G. E. and Mcgraw, K. J. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 295–353.

Refsnider, J. M. and Janzen, F. J. 2010. Putting Eggs in One Basket: Ecological and Evolutionary Hypotheses for Variation in Oviposition-Site Choice. - Annu. Rev. Ecol. Evol. Syst. 41: 39–57.

Reichlin, T. S., Hobson, K. A., Van Wilgenburg, S. L., Schaub, M., Wassenaar, L. I., Martín-Vivaldi, M., Arlettaz, R. and Jenni, L. 2013. Conservation through connectivity: Can isotopic gradients in Africa reveal winter quarters of a migratory bird? - Oecologia 171: 591–600.

Renoult, J. P., Kelber, A. and Schaefer, H. M. 2017. Colour spaces in ecology and evolutionary biology. - Biol. Rev. 92: 292–315.

Reynolds, S. J., Martin, G. R. and Cassey, P. 2009. Is sexual selection blurring the functional significance of eggshell coloration hypotheses? - Anim. Behav. 78: 209–215.

Richard, F.-J. 2017. Symbiotic Bacteria Influence the Odor and Mating Preference of Their Hosts. - Front. Ecol. Evol. 5: 1–9.

Rideout, J. R., He, Y., Navas-Molina, J. A., Walters, W. A., Ursell, L. K., Gibbons, S. M., Chase, J., McDonald, D., Gonzalez, A., Robbins-Pianka, A., Clemente, J. C., Gilbert, J. A., Huse, S. M., Zhou, H.-W., Knight, R. and Caporaso, J. G. 2014. Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. - PeerJ 2: e545.

Riehl, C. 2011. Paternal Investment and the "Sexually Selected Hypothesis" For the

Evolution of Eggshell Coloration: Revisiting the Assumptions. - Auk 128: 175–179.

Riley, M. A. and Wertz, J. E. 2002. Bacteriocins: Evolution, Ecology, and Application. - Annu. Rev. Microbiol. 56: 117–137.

Rizzo, C. and Lo Giudice, A. 2018. Marine Invertebrates: Underexplored Sources of Bacteria Producing Biologically Active Molecules. - Diversity 10: 52.

Rodríguez-Ruano, S. M. 2015. Diversidad bacteriana en la glándula uropigial de la abubilla: dinámica estacional y beneficios asociados.

Rodríguez-Ruano, S. M., Martín-Vivaldi, M., Martín-Platero, A. M., López-López, J. P., Peralta-Sánchez, J. M., Ruiz-Rodríguez, M., Soler, J. J., Valdivia, E. and Martínez-Bueno, M. 2015. The hoopoe's uropygial gland hosts a bacterial community influenced by the living conditions of the bird. - PLoS One 10: 1–18.

Rodríguez-Ruano, S. M., Martín-Vivaldi, M., Peralta-Sánchez, J. M., García-Martín, A. B., Martínez-García, Á., Soler, J. J., Valdivia, E. and Martínez-Bueno, M. 2018. Seasonal and Sexual Differences in the Microbiota of the Hoopoe Uropygial Secretion. - Genes (Basel). 9: 407.

Rognes, T., Flouri, T., Nichols, B., Quince, C. and Mahé, F. 2016. VSEARCH: a versatile open source tool for metagenomics. - PeerJ 4: e2584.

Rolig, A. S., Parthasarathy, R., Burns, A. R., Bohannan, B. J. M. and Guillemin, K. 2015. Individual Members of the Microbiota Disproportionately Modulate Host Innate Immune Responses. - Cell Host Microbe 18: 613–620.

Rollins-Smith, L. A., Ramsey, J. P., Pask, J. D., Reinert, L. K. and Woodhams, D. C. 2011. Amphibian Immune Defenses against Chytridiomycosis: Impacts of Changing Environments. - Integr. Comp. Biol. 51: 552–562.

Rosvall, K. A. 2011. Intrasexual competition in females: evidence for sexual selection? - Behav. Ecol. 22: 1131–1140.

Rubolini, D., Liker, A., Garamszegi, L. Z., Møller, A. P. and Saino, N. 2015. Using the birdtree.org website to obtain robust phylogenies for avian comparative studies: A primer. - Curr. Zool. 61: 959–965.

Ruiz-Castellano, C., Tomás, G., Ruiz-Rodríguez, M., Martín-Gálvez, D. and Soler, J. J. 2016. Nest material shapes eggs bacterial environment. - PLoS One 11: e0148894.

Ruiz-de-Castañeda, R., Burtt Jr, E. H., González-Braojos, S. and Moreno, J. 2012. Bacterial degradability of an intrafeather unmelanized ornament: a role for feather-degrading bacteria in sexual selection? - Biol. J. Linn. Soc. 105: 409–419.

Ruiz-De-Castañeda, R., Burtt, E. H., González-Braojos, S. and Moreno, J. 2015. Bacterial degradability of white patches on primary feathers is associated with breeding date and parental effort in a migratory bird. - Ibis (Lond. 1859). 157: 871–876.

Ruiz-Rodríguez, M., Azcárate-García, M., Ruiz-Castellano, C., Tomás, G., Díaz-Lora, S., Pérez-Contreras, T. and Soler, J. J. Antimicrobial capacity is related to body colouration and reproductive success in female spotless starlings. - Under Rev. in press.

Ruiz-Rodríguez, M., Valdivia, E., Soler, J. J., Martín-Vivaldi, M., Martín-Platero, A. M. and Martínez-Bueno, M. 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. - J. Exp. Biol. 212: 3621–6.

Ruiz-Rodríguez, M., Valdivia, E., Martín-Vivaldi, M., Martín-Platero, A. M., Martínez-Bueno, M., Méndez, M., Peralta-Sánchez, J. M. and Soler, J. J. 2012. Antimicrobial activity and genetic profile of enteroccoci isolated from hoopoes uropygial gland. - PLoS One 7: 1–6.

Ruiz-Rodríguez, M., Martínez-Bueno, M., Martín-Vivaldi, M., Valdivia, E. and Soler, J. J. 2013. Bacteriocins with a broader antimicrobial spectrum prevail in enterococcal symbionts isolated from the hoopoe's uropygial gland. - FEMS Microbiol. Ecol. 85: 495–502.

Ruiz-Rodríguez, M., Soler, J. J., Martín-Vivaldi, M., Martín-Platero, A. M., Méndez, M., Peralta-Sánchez, J. M., Ananou, S., Valdivia, E. and Martínez-Buenob, M. 2014. Environmental factors shape the community of symbionts in the hoopoe uropygial gland more than genetic factors. - Appl. Environ. Microbiol. 80: 6714–6723.

Ruiz-Rodríguez, M., Tomás, G., Martín-Gálvez, D., Ruiz-Castellano, C. and Soler, J. J. 2015. Bacteria and the evolution of honest signals. The case of ornamental throat feathers in spotless starlings. - Funct. Ecol. 29: 701–709.

Ruiz-Rodríguez, M., Møller, A. P., Mousseau, T. A. and Soler, J. J. 2016. Defenses against keratinolytic bacteria in birds living in radioactively contaminated areas. - Sci. Nat. 103: 71.

Sachs, J. L., Skophammer, R. G. and Regus, J. U. 2011. Evolutionary transitions in bacterial symbiosis. - Proc. Natl. Acad. Sci. 108: 10800 LP – 10807.

Saino, N., Romano, M., Rubolini, D., Ambrosini, R., Caprioli, M., Milzani, A., Costanzo, A., Colombo, G., Canova, L. and Wakamatsu, K. 2013. Viability Is Associated with Melanin-Based Coloration in the Barn Swallow (Hirundo rustica). - PLoS One 8:

e60426.

Sanz, J. J. and García-Navas, V. 2009. Eggshell pigmentation pattern in relation to breeding performance of blue tits Cyanistes caeruleus. - J. Anim. Ecol. 78: 31–41.

Scott, J. J., Oh, D.-C., Yuceer, M. C., Klepzig, K. D., Clardy, J. and Currie, C. R. 2008. Bacterial Protection of Beetle-Fungus Mutualism. - Science (80-.). 322: 63.

Searcy, W. A. and Nowicki, S. 2005. The evolution of animal communication. - Princeton Univ. Press, Princeton, NJ.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S. and Huttenhower, C. 2011. Metagenomic biomarker discovery and explanation. - Genome Biol. 12: R60.

Senar, J. C. and Pascual, J. 1997. Tarsus lenght predictor body size. - Ardea 85: 269–274.

Sender, R., Fuchs, S. and Milo, R. 2016. Revised Estimates for the Number of Human and Bacteria Cells in the Body. - PLOS Biol. 14: e1002533.

Sergio, F. and Newton, I. 2003. Occupancy as a measure of habitat quality. - J Anim Ecol. 72: 857–865.

Shapira, M. 2016. Gut Microbiotas and Host Evolution: Scaling Up Symbiosis. - Trends Ecol. Evol. 31: 539–549.

Sharon, G., Segal, D., M.Ringo, H., Hefetz, A., Zilber-Rosenberg, I. and Rosenberg, E. 2010. Commensal bacteria play a role in mating preference of Drosophila melanogaster. - PNAS 107: 20051–20056.

Shawkey, M. D., Pillai, S. R. and Hill, G. E. 2003. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. - J. Avian Biol. 34: 345–349.

Shawkey, M. D., Pillai, S. R. and Hill, G. E. 2009. Do feather-degrading bacteria affect sexually selected plumage color? - Naturwissenschaften 96: 123–128.

Sheldon, B. C. 2000. Differential allocation: Tests, mechanisms and implications. - Trends Ecol. Evol. 15: 397–402.

Shephard, K. L. 1994. Functions for fish mucus. - Rev. Fish Biol. Fish. 4: 401–429.

Sherwin, E., Bordenstein, S. R., Quinn, J. L., Dinan, T. G. and Cryan, J. F. 2019. Microbiota and the social brain. - Science (80-.). 366: eaar2016.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. and Ishikawa, H. 2000. Genome sequence of the endocellular bacterial symbiont of aphids Buchnera sp. APS. - Nature 407: 81–86.

Siefferman, L., Navara, K. J. and Hill, G. E. 2006. Egg coloration is correlated with

female condition in eastern bluebirds (Sialia sialis). - Behav. Ecol. Sociobiol. 59: 651–656.

Singleton, D. R. and Harper, R. G. 1996. Bacteria in Old House Wren nests. - J F. Ornithol 69: 71–74.

Smith, H. G. and Montgomerie, R. 1991. Nestling American robins compete with siblings by begging. - Behav. Ecol. Sociobiol. 29: 307–312.

Soini, H. A., Whittaker, D. J., Wiesler, D., Ketterson, E. D. and Novotny, M. V. 2013. Chemosignaling diversity in songbirds: Chromatographic profiling of preen oil volatiles in different species. - J. Chromatogr. A 1317: 186–192.

Soler, J. J. and Møller, A. P. 1996. A comparative analysis of the evolution of variation in appearance of eggs of European passerines in relation to brood parasitism. - Behav. Ecol. 7: 89–94.

Soler, J. J., Moreno, J., Aviles, J. M. and Møller, A. P. 2005. Blue and green egg-color intensity is associated with parental effort and mating system in passerines: support for the sexual selection hypothesis. - Evolution (N. Y). 59: 636–644.

Soler, J. J., Martín-Vivaldi, M., Ruiz-Rodríguez, M., Valdivia, E., Martín-Platero, A. M., Martínez-Bueno, M., Peralta-Sánchez, J. M. and Méndez, M. 2008a. Symbiotic association between hoopoes and antibiotic-producing bacteria that live in their uropygial gland. - Funct. Ecol. 22: 864–871.

Soler, J. J., Navarro, C., Contreras, T. P., Avilés, J. M. and Cuervo, J. J. 2008b. Sexually Selected Egg Coloration in Spotless Starlings. - Am. Nat. 171: 183–194.

Soler, J. J., Martín-vivaldi, M., Peralta-sánchez, J. M. and Ruiz-rodríguez, M. 2010. Antibiotic-Producing Bacteria as a Possible Defence of Birds against Pathogenic Microorganisms. - Open Ornithol. J. 3: 93–100.

Soler, J. J., Martín-Vivaldi, M., Peralta-Sánchez, J. M., Arco, L. and Juárez-García-Pelayo, N. 2014. Hoopoes color their eggs with antimicrobial uropygial secretions. - Naturwissenschaften 101: 697–705.

Soler, J. J., Martínez-García, Á., Rodríguez-Ruano, S. M., Martínez-Bueno, M., Martín-Platero, A. M., Peralta-Sánchez, J. M. and Martín-Vivaldi, M. 2016. Nestedness of hoopoes' bacterial communities: symbionts from the uropygial gland to the eggshell. - Biol. J. Linn. Soc. 118: 763–773.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martín-Vivaldi, M., Martínez-de la Puente, J., Ruiz-Rodríguez, M. and Tomás, G. 2017. Telomere length and dynamics of

spotless starling nestlings depend on nest-building materials used by parents. - Anim. Behav. 126: 89–100.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martínez-de la Puente, J., Ruiz-Rodríguez, M. and Tomás, G. 2018. Egg colouration predicts brood size, telomere length and body condition of spotless starling fledglings. - J. Avian Biol. 49: 1–12.

Soler, J. J., Morales, J., Cuervo, J. J. and Moreno, J. 2019. Conspicuousness of passerine females is associated with the nest-building behaviour of males. - Biol. J. Linn. Soc. 126: 824–835.

Solís, J. C. and de Lope, F. 1995. Nest and Egg Crypsis in the Ground-Nesting Stone Curlew Burhinus oedicnemus. - J. Avian Biol. 26: 135–138.

Spicer, M. E., Pruitt, J. N. and Keiser, C. N. 2019. Spiders, microbes and sex: Bacterial exposure on copulatory organs alters mating behaviour in funnel-web spiders. - Ethology 125: 677–685.

Spor, A., Koren, O. and Ley, R. 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. - Nat. Rev. Microbiol. 9: 279–290.

Stanback, M. T. and Rockwell, E. K. 2003. Nest-site fidelity in Eastern Bluebirds (Sialia sialis) depends on the quality of alternative cavities. - Auk 120: 1029–1032.

Statsoft, I. 2006. STATISTICA (data analysis software system), version 7. Available at www.statsoft.com.

Steinert, M., Hentschel, U. and Hacker, J. 2000. Symbiosis and pathogenesis: evolution of the microbe-host interaction. - Naturwissenschaften 87: 1—11.

Stoddard, M. C. and Prum, R. O. 2008. Evolution of avian plumage color in a tetrahedral colour space: a phylogenetic analysis of New World buntings. - Am. Nat. 171: 755–776.

Stoddard, M. C. and Osorio, D. 2019. Animal Coloration Patterns: Linking Spatial Vision to Quantitative Analysis. - Am. Nat. 193: 000–000.

Stoddard, M. C., Fayet, A. L., Kilner, R. M. and Hinde, C. A. 2012. Egg speckling patterns do not advertise offspring quality or influence male provisioning in great tits. - PLoS One 7: e40211.

Suedmeyer, W. K., McCaw, D. and Turnquist, S. 2001. Attempted Photodynamic Therapy of Squamous Cell Carcinoma in the Casque of a Great Hornbill (Buceros bicornis). - J. Avian Med. Surg. 15: 44–49.

Sumasgutner, P., Vasko, V., Varjonen, R. and Korpimäki, E. 2014. Public information

revealed by pellets in nest sites is more important than ecto-parasite avoidance in the settlement decisions of Eurasian kestrels. - Behav. Ecol. Sociobiol. 68: 2023–2034.

Surmacki, A. and Nowakowski, J. K. 2007. Soil and preen waxes influence the expression of carotenoid-based plumage coloration. - Naturwissenschaften 94: 829–835.

Suzuki, T. A. 2017. Links between Natural Variation in the Microbiome and Host Fitness in Wild Mammals. - Integr. Comp. Biol. 57: 756–769.

Swynnerton, C. F. M. 1916. On the coloration of the mouths and eggs of birds. II. On the coloration of eggs. - Ibis (Lond. 1859). 4: 529–606.

Swynnerton, C. F. M. 1918. Rejections by birds of eggs unlike their own: with remarks on some of the cuckoo problems. - Ibis (Lond. 1859).: 127–154.

Team, R. D. C. 2019. R: A Language and Environment for Statistical Computing (RDC Team, Ed.). - R Found. Stat. Comput. in press.

Theis, K. R., Venkataraman, A., Dycus, J. A., Koonter, K. D., Schmitt-Matzen, E. N., Wagner, A. P., Holekamp, K. E. and Schmidt, T. M. 2013. Symbiotic bacteria appear to mediate hyena social odors. - Proc. Natl. Acad. Sci. 110: 19832–19837.

Tomás, G., Merino, S., Moreno, J., Sanz, J. J., Morales, J. and García-Fraile, S. 2006. Nest Weight and Female Health in the Blue Tit (Cyanistes Caeruleus). - Auk 123: 1013–1021.

Tomás, G., Merino, S., Moreno, J. and Morales, J. 2007. Consequences of nest reuse for parasite burden and female health and condition in blue tits, Cyanistes caeruleus. - Anim. Behav. 73: 805–814.

Tomás, G., Martín-Gálvez, D., Ruiz-Castellano, C., Ruiz-Rodríguez, M., Peralta-Sánchez, J. M., Martín-Vivaldi, M. and Soler, J. J. 2018. Ectoparasite Activity During Incubation Increases Microbial Growth on Avian Eggs. - Microb. Ecol. 76: 555–564.

Trail, P. W. 2007. African hornbills: keystone species threatened by habitat loss, hunting and international trade. - Ostrich 78: 609–613.

Trivers, R. L. 1972. Parental investment and sexual selection. - In: Campbell, B. (ed), Sexual selection and the descent of man 1871-1971. pp. 136–179.

Tschumi, M., Schaub, M. and Arlettaz, R. 2014. Territory occupancy and parental quality as proxies for spatial prioritization of conservation areas. - PLoS One 9: e97679.

Uchida, Y. 1970. On the color change in Japanese crested ibis. A new type of cosmetic coloration in birds. - Misc Rep Yamashina Inst Ornithol 6: 54–72. In Japanese, with English summary.

Umesaki, Y., Setoyama, H., Matsumoto, S., Imaoka, A. and Itoh, K. 1999. Differential Roles of Segmented Filamentous Bacteria and Clostridia in Development of the Intestinal Immune System. - Infect. Immun. 67: 3504 LP – 3511.

Underwood, T. and Sealy, S. 2002. Adaptive significance of egg coloration. - In: Deeming DC (ed), Avian incubation: behaviour, environment, and evolution. Oxford Uni. pp. 280–298.

van Dongen, W. F., White, J., Brandl, H. B., Moodley, Y., Merkling, T., Leclaire, S., Blanchard, P., Danchin, É., Hatch, S. A. and Wagner, R. H. 2013. Age-related differences in the cloacal microbiota of a wild bird species. - BMC Ecol. 13: 11.

van Veelen, H. P. J., Falcao Salles, J. and Tieleman, B. I. 2017. Multi-level comparisons of cloacal, skin, feather and nest-associated microbiota suggest considerable influence of horizontal acquisition on the microbiota assembly of sympatric woodlarks and skylarks. - Microbiome 5: 156.

van Veelen, H. P. J., Salles, J. F. and Tieleman, B. I. 2018. Microbiome assembly of avian eggshells and their potential as transgenerational carriers of maternal microbiota. - ISME J. 12: 1375–1388.

van Wijk, R. E., Schaub, M. and Bauer, S. 2017. Dependencies in the timing of activities weaken over the annual cycle in a long-distance migratory bird. - Behav. Ecol. Sociobiol. 71: 73.

van Wijk, R. E., Schaub, M., Hahn, S., Juárez-García-Pelayo, N., Schäfer, B., Viktora, L., Martín-Vivaldi, M., Zischewski, M. and Bauer, S. 2018. Diverse migration strategies in hoopoes (Upupa epops) lead to weak spatial but strong temporal connectivity. - Sci. Nat. 105: 42.

Victoria, J. K. 1972. Clutch characteristics and egg discriminative ability of African village weaverbird Ploceus cucullatus. - Ibis (Lond. 1859). 114: 367–376.

Videler, H., Geertjes, G. J. and Videler, J. J. 1999. Biochemical characteristics and antibiotic properties of the mucous envelope of the queen parrotfish. - J. Fish Biol. 54: 1124–1127.

Viseshakul, N., Charoennitikul, W., Kitamura, S., Kemp, A., Thong-Aree, S., Surapunpitak, Y., Poonswad, P. and Ponglikitmongkol, M. 2011. A phylogeny of frugivorous hornbills linked to the evolution of Indian plants within Asian rainforests. - J. Evol. Biol. 24: 1533–1545.

Wallace AR 1889. Darwinism: an exposition of the theory of natural selection, with some

of its applications.

Walters, L. A. and Getty, T. 2010. Are brighter eggs better? Egg color and parental investment by House Wrens. - J. F. Ornithol. 81: 155–166.

Wang, B., Wan, L., Wang, A. and Li, L. M. 2017. An adaptive decorrelation method removes Illumina DNA base-calling errors caused by crosstalk between adjacent clusters. - Sci. Rep. 7: 41348.

Warnecke, L., Turner, J. M., Bollinger, T. K., Lorch, J. M., Misra, V., Cryan, P. M., Wibbelt, G., Blehert, D. S. and Willis, C. K. R. 2012. Inoculation of bats with European Geomyces destructans supports the novel pathogen hypothesis for the origin of white-nose syndrome. - Proc. Natl. Acad. Sci. 109: 6999–7003.

Weiß, B. M., Kücklich, M., Thomsen, R., Henkel, S., Jänig, S., Kulik, L., Birkemeyer, C. and Widdig, A. 2018. Chemical composition of axillary odorants reflects social and individual attributes in rhesus macaques. - Behav. Ecol. Sociobiol. 72: 65.

Werren, J. H. 2012. Symbionts provide pesticide detoxification. - Proc. Natl. Acad. Sci. 109: 8364–8365.

Werren, J. H., Baldo, L. and Clark, M. E. 2008. Wolbachia: master manipulators of invertebrate biology. - Nat. Rev. Microbiol. 6: 741–751.

Wesolowski, T. and Maziarz, M. 2012. Dark tree cavities – a challenge for hole nesting birds? - J. Avian Biol. 43: 454–460.

Westcott, S. L. and Schloss, P. D. 2015. De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. - PeerJ 3: e1487.

Whittaker, D. J. and Theis, K. R. 2016. Bacterial Communities Associated with Junco Preen Glands : Preliminary Ramifications for Chemical Signaling. - In: Schulte, B. A. et al. (ed), Chemical Signals in Vertebrates 13. Springer International Publishing, pp. 105–117.

Whittaker, D. J., Soini, H. A., Atwell, J. W., Hollars, C., Novotny, M. V and Ketterson, E. D. 2010. Songbird chemosignals: volatile compounds in preen gland secretions vary among individuals, sexes, and populations. - Behav. Ecol. 21: 608–614.

Whittaker, D. J., Richmond, K. M., Miller, A. K., Kiley, R., Bergeon Burns, C., Atwell, J. W. and Ketterson, E. D. 2011. Intraspecific preen oil odor preferences in dark-eyed juncos (Junco hyemalis). - Behav. Ecol. 22: 1256–1263.

Whittaker, D. J., Gerlach, N. M., Slowinski, S. P., Corcoran, K. P., Winters, A. D., Soini,

H. A., Novotny, M. V., Ketterson, E. D. and Theis, K. R. 2016. Social Environment Has a Primary Influence on the Microbial and Odor Profiles of a Chemically Signaling Songbird. - Front. Ecol. Evol. 4: 1–15.

Whittaker, D. J., Slowinski, S. P., Greenberg, J. M., Alian, O., Winters, A. D., Ahmad, M. M., Burrell, M. J. E., Soini, H. A., Novotny, M. V., Ketterson, E. D. and Theis, K. R. 2019. Experimental evidence that symbiotic bacteria produce chemical cues in a songbird. - J. Exp. Biol.: jeb.202978.

Wright, E. S. and Vetsigian, K. H. 2016. Quality filtering of Illumina index reads mitigates sample cross-talk. - BMC Genomics 17: 876.

Zahavi, A. 1975. Mate selection-A selection for a handicap. - J. Theor. Biol. 53: 205–214.

Zahavi, A. and Zahavi, A. 1997. The handicap principle; a missing piece of Darwin's puzzle.

Zamzow, J. P. and Losey, G. S. 2002. Ultraviolet radiation absorbance by coral reef fish mucus: Photo-protection and visual communication. - Environ. Biol. Fishes 63: 41–47.

Zinter, M. S., Mayday, M. Y., Ryckman, K. K., Jelliffe-Pawlowski, L. L. and DeRisi, J. L. 2019. Towards precision quantification of contamination in metagenomic sequencing experiments. - Microbiome 7: 62.

CAPÍTULO IV

CAPÍTULO IV

Symbioses with bacteria in hornbill (*Bucerotiformes*) uropygial glands

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Abstract

Hornbills (*Bucerotiformes*) are sub-Saharan African and tropical Asian birds closely related to woodhoopoes and hoopoes (*Upupiformes*). *Upupiformes* are known to present a tight symbiotic relationship with mutualistic bacteria living within their uropygial gland and providing chemical defences against parasites and predators. The uropygial secretion of many species of hornbills is brightly coloured in yellow-orange tones, and we hypothesised that their glands may also harbour symbiotic bacteria responsible for some of the properties of their secretions, which have never been studied in this regard. To test this hypothesis we took samples of secretions from the uropygial glands and other body parts of live specimens of 13 hornbill species hosted in different zoological parks in Spain, Portugal and France. Secretion samples were inoculated in culture media to detect bacteria and extended on slides and observed in a microscope to estimate their abundance. In addition, sterile cotton swabs were used to take samples of the surfaces of several body parts, including the tuft and surroundings of uropygial glands, and compare the composition of their microbiomes by means of Illumina sequencing of bacterial rRNA. We found that bacterial growth from secretion samples was common in all African species and one Asian species. Moreover, several of these hornbills

species had secretions densely populated with bacteria when observed in a microscope. Finally, despite a strong effect of the zoo of origin on the composition of microbiomes, by means of GNEISS analysis there was a clear association of particular bacterial taxa to the uropygial glands of several species or the body areas specifically covered with secretions. Several of those bacterial taxa are known producers of antibiotic substances and are commonly associated with several groups of animals in mutualistic relationships. Moreover, one group of pigmented bacteria producing carotenoids is associated with the uropygial gland producing coloured secretions, and the areas stained with it in an Asian hornbill species. This evidence suggests that hornbills are a group of birds engaged in coevolutionary interactions with bacteria living in or around their uropygial secretions which deserves further research.

INTRODUCTION

Associations between animals and symbiotic bacteria hosted in their glands are widespread in nature (McFall-Ngai et al., 2013). In these systems, bacteria provide animals with a variety of biological products (Piel, 2004; Florez et al., 2015) that are beneficial for hosts either as nutrition (Sorgo et al., 2002), light (Nyholm and McFall-Ngai, 1998; Kimbell and McFall-Ngai, 2003; Dunlap et al., 2007; Schwartzman et al., 2015) or chemical defences against predators (Hwang et al., 1989; Law-Brown, 2001; Chau et al., 2011; Lago et al., 2015) or parasites (Barbieri et al., 1997; Currie et al., 1999; Kaltenpoth et al., 2005; Currie et al., 2006; Little et al., 2006; Kaltenpoth and Strohm, 2007), or even used in light (Herring, 2007; Widder, 2010) and olfactory communication (Theis et al., 2013; reviewed in Ezenwa and Williams, 2014; Leclaire et al., 2017). In the case of birds, the uropygial gland is the most

important exocrine gland, whose secretion is used for covering mainly feathers (Jacob and Ziswiler, 1982), but also eggs (Martín-Vivaldi et al., 2014; Soler et al., 2014) conferring protection against a variety of environmental agents (reviewed in Jacob and Ziswiler, 1982). In the case of species of the order *Upupiformes*, the protection against pathogens and predators by means of the uropygial secretion is mediated by the action of mutualistic bacteria living within their glands (Law-Brown, 2001; Law-Brown and Meyers, 2003; Burger et al., 2004; Martin-Platero et al., 2006; Soler et al., 2008; Ruiz-Rodríguez et al., 2009; Martín-Vivaldi et al., 2010; Ruiz-Rodríguez et al., 2012). In this group of species, secretions are dark and malodorous, their final colour and the chemical volatiles present, produced by the action of bacteria living within the gland (Burger et al., 2004; Martín-Vivaldi et al., 2009; Martín-Vivaldi et al., 2010). Until very recently, only woodhoopoes and hoopoes had been shown to host symbiotic bacteria within the uropygial gland, although the theoretical background would suggest it could be a common phenomenon (reviewed in Soler et al., 2010). The existence of groups of birds whose uropygial secretions are special for some reason, such as including chemical compounds similar to bacterial metabolites, particular odours, or special colours, are prime candidates as systems where birds could be obtaining benefits from bacteria by their action on the uropygial secretion. For example, the use of uropygial secretions as olfactory markers for intraspecific communication is known for several bird species (Whittaker et al., 2010; Whittaker et al., 2011; Soini et al., 2013). However, only in the last years has it been suggested (Whittaker and Theis, 2016), or experimentally tested in *Junco hyemalis*(Whittaker et al., 2019), the possible role of bacteria as mediators of such a function by the production of the volatiles used by birds. Given that in these studies with juncos the samples are taken without differentiating between secretions present around or coming from inside the gland, they do not distinguish whether in this species the action of these bacteria takes place within or outside the uropygial gland. Nevertheless, the last study shows several pieces of evidence

suggesting that particular bacterial groups associated with the gland may be responsible for some of the compounds important for intra-specific social communication (Whittaker et al., 2019). Moreover, in recent years, a number of new bacterial species have been isolated and designated from the uropygial glandin different birds, suggesting that it is a special environment causing specialization of microorganisms. Besides *Enterococcus phoeniculicula* typical of the uropygial gland of woodhoopoes (Law-Brown and Meyers, 2003), new species are *Corynebacterium uropygiale* in *Meleagris gallopavo*(Braun et al., 2016); *Corynebacterium heidelbergense* in *Alopochen aegyptiacus*(Braun et al., 2018b); or *Kocuria uropygialis* and *K. uropygioeca* in *Dendrocopos major*(Braun et al., 2018c), and *K. tytonicola* and *K. tytonis* in *Tyto furcata*(Braun et al., 2019a; Braun et al., 2019b). The *Corynebacterium* species are facultatively anaerobic *Actinobacteria* which, according to described methods in those studies have undoubtedly been isolated from within the uropygial gland by excising the gland lobes or taking the secretion with sterile pipette tips after washing the gland surface with special caution to avoid contamination from other sources (Braun et al., 2016; Braun et al., 2018b). *Kocuria* species from *Dendrocopos* and *Tyto* secretions were also isolated with similar methods, suggesting again that they are inhabitants of the interior of the uropygial gland. Although most *Kocuria* taxa are considered strictly aerobic, the strains isolated from the uropygial glands of *Tyto furcata* showed reduced growth, but not inhibition in anaerobic conditions (Braun et al., 2019a), suggesting they are indeed adapted to living within the gland. In the only case in which the antimicrobial effects of these symbionts have been studied (*C. uropygiale*in *Meleagris gallopavo*) the results were that the bacteria do not contribute to the antimicrobial effects of the secretion of the bird (Braun et al. 2018c). Therefore, the possible function of the association for the host is unknown. Nevertheless, all these studies suggest that the association with bacteria within bird uropygial glands may be common, as previously suggested (Soler et al., 2010). In none of these cases, however, are the secretions

conspicuously altered by bacteria, except in the cases of hoopoes and woodhoopoes. Nevertheless, the sister group of *Upupiformes*: the hornbills (*Bucerotiformes*) are orders with very special uropygial secretions, since in several species it is brightly coloured in yellow, orange or red tones, and used to stain several body parts, apparently linked to an ornamental role (Fig. 1, Kemp, 2001; Delhey et al., 2007; Poonswad et al., 2013). It is assumed that such coloration of secretions is caused by incorporation of carotenoids obtained in the diet (Gamble, 2012), as in other bird groups with carotenoid derived cosmetic coloration as flamingos (Amat et al., 2011). However, the possibility that such pigmented secretions may be linked to associations with symbiotic bacteria as in the closely related hoopoes and woodhoopoes has not been investigated.

Here we explore the hypothesis that hornbills host symbiotic bacteria in their uropygial glands, and that the special colorations found in the secretions of some species of the Asian lineage are caused by such symbionts. If that is the case, we should find that uropygial secretions obtained in sterile conditions are loaded with bacteria, and that those of species with cosmetic coloration has bacterial taxa producing coloured metabolites. With this purpose we have inoculated secretions in culture media, examined secretions extensions in a microscope and compared the taxonomic composition of microbiomes found in uropygial glands and other body parts for species of hornbills belonging to the African (mainly carnivorous and without known cosmetic coloration) and Asian (mainly frugivorous lineages with known cosmetic coloration) lineages.


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Fig. 1. Several of the species of hornbills included in the study and their uropygial glands. Three species of the African lineage: a) *Bucorvus leadbeateri*, c) Pair of *Bycanistes brevis*, e) Pair of *Tockus deckeni*; and three species of the Asian lineage: b) Male *Buceros bicornis*, d) Male *Buceros rhinoceros*, f) Male *Aceros corrugatus*. Yellow stains of uropygial secretions are patent in white feather areas in (b) and (d). The glands of (g) a *Bycanistes*, (i) a *Buceros bicornis* and (j) a *Bucorvus leadbeateri* individual showing different types of tufts of hornbill glands. (h) Extraction of secretion from a *Bycanistes* gland, (i) extraction from inside the ampulla of a female hoopoe uropygial secretion for comparison. (k) The densely feathered tufts as (j) had to be cut in a small area with sterile scissors before secretion extraction.

MATERIAL AND METHODS

StudyArea and Species

Hornbills (Order *Bucerotiformes*, Fig. 1) are large-sized and long-living birds closely related to Hoopoes and Woodhoopoes (Sibley and Ahlquist, 1990; Hackett et al., 2008) distributed throughout Tropical Asia and Africa (Poonswad et al. 2013). These two separated areas are occupied by two main lineages, the Asian derived from the ancient origin in Africa, although the position of *Berenicornis* in the African clade suggests two possible events of colonization of the Asian continent (Gonzalez et al., 2013). All hornbills are cavity nesters, and except for the terrestrial basal clade *Buceros* have the striking behaviour of sealing the nest entrance with mud, excrements and crushed arthropods such as millipedes, only leaving a small opening for the male to deliver food to the female, which stays enclosed inside since laying until the age when nestlings are feathered (Kemp, 2001). Asian hornbills are mainly frugivorous, while the African clade mostly is faunivorous except for the *Bycanistes* and *Ceratogymna* genera closely related to the Asian *Benericornis* in the last phylogenetic analyses (Gonzalez et al., 2013).

The study was carried out with 54captive individuals of 13 species of hornbills from different zoological parks distributed across Spain, Portugal and France (Table 1 in Supplementary Material). During 2015 and 2016, 32 individuals belonging to 11 species and 6 genera were sampled in Spain and Portugal in the following parks: Barcelona Zoo, Oasys de Tabernas Theme Park, Lourosa Zoo, Palmitos Park and Jungle Park. During November and December of 2017, 22 individuals of 9 species were sampled in the following zoos across France: Parczoologique de Fréjus, Zoo de la Boissiere du Doré, Zoo d'Upie, ZooParc de Beauval and Zoo du Bois d'Attilly.

Sampling Procedure

To obtain comparable samples of microbial communities from different body parts (see below), sterile cotton swabs (EUROTUBO® DeltaLab) previously moistened with sterile sodium phosphate buffer (PBS, 0.2 M; pH = 7.08) were used to clean a square of about 2x2 cm in each of the surfaces to sample, avoiding touching any other surface. Afterwards, the swab was introduced into a sterile microcentrifuge tube with sterile phosphate buffer and transported to the laboratory in a portable refrigerator at 4–6°C. Samples were stored at -20°C until being processed.

Each individual was sampled at different sites with a different swab: (1) Uropygial gland tuft (Fig. 1, in this case usually there was less than 4cm^2 available, and, therefore, we sampled the whole tuft).(2) Beak, at two different sites: on the basal third (beak base) or on the distal third (beak tip) half of the surface on its mandible in each case. (3) Casque, which is a unique character of this group of birds, constituted by a hollow horn structure covered in keratine placed dorsally to the beak (see Fig. 1), was sampled on one of their sides in the middle of its length. (4) Wing white feathers, (5) wing black feathers, (6) white tail feathers, (7) black tail feathers and (8) nape.

To check for the presence of bacteria within the uropygial gland secretions, after sampling the tuft with a swab, we cleaned the tuft and surroundings of the gland pores with a cotton soaked in 96º ethanol, and gently pressed the gland ducts towards the openings (Fig. 1h) taking the secretion with sterile capillary tubes. When tufts completely occluded the ducts openings (Fig. 1j), a small area of the gland surface was cleared using scissors previously cleaned with 70% ethanol (Fig. 1k). Secretions thus obtained were either smeared on Polysine adhesion microscope slides (Thermo Scientific, Waltham, MA, USA)by extending the drop to form a layer as thin as possible with the help of a second clean slide rubbed over the secretion, or stored in sterile microfuge tubes for bacteria cultivation and DNA extraction. We gave priority to sampling for bacterial cultivation and frequently we did not get any secretions, and, therefore, samples for cultivation/microscopic study of secretions were not always available.Microscope slides were immediately fixed in 4% paraformaldehyde; washed in sterile distilled water; dehydrated in successive 50%, 80%, and 96% ethanol baths; air-dried; and stored in darkness at 4º C until microscopic observation.

Before sampling each individual, we used latex gloves previously washed with ethanol 70% to prevent bacterial contamination between individuals.

Microbiological study

Presence of cultivable bacteria in secretions

The presence of cultivable bacteria in secretions was tested by inoculating plates with a general medium used to grow heterotrophic bacteria (Tryptone Soya Agar, TSA), by directly sinking or streaking the extreme of the capillary tubes used to take the secretion. Growing was measured qualitatively as a dichotomous (presence/absence) variable reflecting whether there were bacterial colonies after incubation in aerobic conditions for 24 h at37ºC.

Microscopic study

Slides with fixed secretions were used to study presence and abundance of bacteria by several different methods. Some of them were stained with crystal violet for two minutes after re-fixation in ethanol-chloroform, washed in ethanol 96º and air dried. The remaining were marked for observation at fluorescence microscopy. For this, cell walls were permeated adding 10 µL of lysozymesolution (100 mM Tris-HCl, 50 mMEDTA), pH 8-2 mg/mL lysozyme, USB Corporation, Cleveland, OH, USA) and incubated at 37º C during 60 min in a wet atmosphere. Then, slides were washed with filtered distilled water; air-dried; and dehydrated in successive 50%, 80%, and 96% ethanol baths. Fluorescent marking of DNA was performed by covering fixed secretions with a hybridization mixture (20 mM Tris-HCl; 0.9 M NaCl; SDS 0.1%; formamide 25%,Sigma-Aldrich; pH 7.2–7.4),with 10 µL of Hoechst added (10 µg/mL, Sigma-Aldricht, St. Louis, MO, USA), maintained for 30 min in a wet atmosphere, and afterwards washed with phosphate-buffered saline (PBS) for 2 min and dried at room temperature. Finally, slides were mounted applying a drop of antifading solution (Vectashield Mounting Medium H-1000, Vector Laboratories, Burlingame, CA, USA) and a coverslip, and then sealed with varnish. Observations were performed through an Olympus BX51 fluorescence microscope at 100x magnification with DAPI (blue) filter. The whole process was performed maintaining slides in the dark to avoid fading of the Hoechst dye, and they were stored in dark boxes at 4º C until visualization under fluorescence microscopy.

Microbiome composition

To study the composition of microbiomes of the different body parts, we sequenced the bacterial 16S rRNA from the samples. Before DNA extraction, samples were sonicated in an ultrasonic bath for 90 seconds to free cells from the cotton swab. Afterwards vials were centrifuged for 5 minutes at maximum speed. The pellets were treated with a lysozyme

solution (10 mg/ml, 37ºC for30 min) and extracted with the commercial kit FavorPrep Blood Genomic DNA Extraction Mini Kit (Favorgen Biotech), following manufacturer instructions. DNA thus obtained was stored at -20ºC until processed for sequencing. In parallel with hornbill samples we ran the same protocol for positive (cheese extracts to check the right amplification of samples)and several types of negative controls (Hornung et al., 2019) to test for possible sources of contamination (controls for the extraction kit, for the first and the second amplification). Samples were processed in two different batches from DNA extraction to sequencing, and we included controls for both batches.Amplicon PCR was performed from bacterial total DNA of the V6-V8 region of the 16S rRNA gene using the primer pair B969F (5´-ACGCGHNRAACCTTACC-3´) and BA1406R (5´- ACGGGCRGTGWGTRCAA-3´).A second PCR was applied to add barcodes to individual samples, so that the derived sequences can be sorted into respective samples in downstream analysis. These barcodes overlap with the sequence of the primers used in the first PCR. Purification steps were carried out using HigherPurity™ DNA Purification SPRI Magnetic Beads (Canvax®). Then, DNA concentration was measured using a Qubit® Fluorimeter (Invitrogen^{TM}) and standardized to 25 ng of DNA per sample in the sequencing mix. When samples or controls showed undetectable very low DNA concentrations, we used 25 µl of their stored solution. High throughput sequencing were carried out in IlluminaMiSeq platform in the Institute of Parasitology and Biomedicine López Neyra (CSIC, Granada, Spain).

The processing of the sequences obtained from IlluminaMiSeqwas carried out with QIIME2 v2019.4 (Quantitative Insights in Microbial Ecology, Bolyen et al., 2019) for the results of the two batches together. First, primer trimming was performed using cutadapt plugin with default parameters (Martin, 2011). Forward and reverse reads were joined using VSEARCH (Rognes et al., 2016). Quality filtering was performed using default parameters, and, afterwards, we used Deblur, a sub-operational-taxonimic-unit (sOTU) approach, in order

to create the sOTU table and remove sequencing errors (Amir et al., 2017). Sequences which passed quality filters were truncated to 380 pb, using 20 as a criterion of quality limit, giving a dataset of 11,430,874 total reads. Then, we used the fragment insertion plugin, a script that performs the sequences alignment and create the bacterial phylogenetic tree (Janssen et al., 2018). Taxonomic assignment was based on Greengenes 13.08 with a similarity of 99% (DeSantis et al., 2006). Finally, chloroplasts, mitochondria and non-bacterial DNA were removed from the sOTU table, retaining only bacterial DNA.All plugins and scripts were implemented in QIIME2.

The sOTU table for the whole set of samples and controls included 31,225 features. A great percentage of those features showed a very low prevalence including control samples (prev=1 (13,691); prev=2 (9,256), prev=3 (6,852), prev=4 (5,396)). For several types of sequence analyses it is recommended to work only with features over a minimum prevalence (Morton et al., 2017) so we decided to retain only those present in at least 5 samples. Moreover, several of our negative controls showed detectable DNA concentrations, and sequencing batches presented different taxonomic profiles, with some single sOTUs showing high abundances along the batch (Fig. 1 in Supplementary Material). This evidence suggests the existence of contaminants in our list of sOTUs. Today it is widely accepted that even following strict sampling and laboratory processing protocols (Eisenhofer et al., 2019; Hornung et al., 2019), some contamination in sequencing products is unavoidable (de Goffau et al., 2018; Eisenhofer et al., 2019). Contaminants can come from different sources, including reagents, sampling procedures, or even commercial extraction kits (Minich et al., 2018; Eisenhofer et al., 2019; Zinter et al., 2019) and cross-contamination among samples is also possible (Edgar, 2016; Wright and Vetsigian, 2016; Larsson et al., 2017; Wang et al., 2017). Therefore, although elimination of all contaminants is not possible and

decontamination can eliminate some valid features from samples (Karstens et al., 2019), several post sequencing algorithms have been proposed to improve the quality of the data used for downstream analyses (Jervis-Bardy et al., 2015; Davis et al., 2018; Caruso et al., 2019; Karstens et al., 2019; McKnight et al., 2019; Zinter et al., 2019). To clean possible contaminants from our sOTU table we used Decontam (Davis et al 2018), as other recent studies on bacterial communities found in birds (e.g. Whittaker et al., 2019). First we used the frequency method to discard features showing negative relationships between abundance and sample DNA concentration (Fig. 2a in Supplementary Material), with a threshold pFreq = 0.1. Second, we used the prevalence method to discard sOTUs showing higher prevalence in negative controls than in samples (Fig. 2b in Supplementary Material), with the threshold pPrev = 0.5. In both cases we used the per batch option in order to identify contaminants being performed in each batch separately. By using both methods, we identified 741 sOTUs as probable contaminants that were discarded from samples. In this way a total of 4,515 sOTUs were retained for subsequent analyses. After examination of the total reads available per sample, we rarefied samples to 1,100 reads for estimates of diversity and comparison of the composition of sample types.

Statistical analysis

QIIME2v.2019.10 was used to generate alpha-diversity estimates forsamples (richness of sOTUs and Shannon index), and beta-diversity matrices of distancesamong samples (weighted and unweightedUniFracdistances (Lozupone and Knight, 2005)). Comparisonsamong types of samples were performed with Primer 7.0.13(PRIMER-e) and Statistica ver. 7.1. Comparison of Shannonindex and nº of sOTUs were performed by means of nonparametric Kruskal-Wallis tests, since the distribution of values differed from Gaussian distributions (Kolmogorov-Smirnov tests $p < 0.01$). Nevertheless, when needing complex

designs, with random factors, we used GLMs. The composition of bacterial communitywere compared among types of samples by means ofPERMANOVA in Primer ver. 7.0.13 using unweighed UniFrac distance matrices generated with QIIME2 for the sOTU level.

Principal coordinates analyses (PCoA) were used to visualizethe relative position of the different types of samples in themultidimensional space of bacterial community composition.

In order to determine which particular sOTUs differed among groups of samples, we used Gneiss analysis (Morton et al. 2017). In essence, this method tests associations (by mean of ordinal least square regression (ols) or mixed effects linear regression (lme)) between variables identifying environmental factors or other properties grouping samples, and the values of balances among groups of features clustered, by a known gradient, or by their co-occurrence (hierarchical clustering using feature correlation). By using balances, the analysis avoids errors related to inter-dependence of the relative abundances of different features, caused by the compositional nature of sequencing data (Morton et al., 2017). All analyses were performed using the ols regression method for one multilevel factor at a time. Analyses were considered valid when the predicted mse were smaller than the model mse. For each Gneiss analysis we explored the heatmap showing the abundances of the sOTUs per sample as good as the FDR corrected p values for all the balances for the different levels of factor tested. As we are especially interested in determining whether hornbill glands are associated with special bacterial communities, we paid attention not only to significant coefficients in the first nine balances, but also to those significant for glands after $y_{9.}$ For balances including significant coefficients for the factor-levels of interest we examined the direction of differences and the identification of sOTUs in the denominator, thus inferring the features that are comparatively more abundant in the balance.

RESULTS

Inoculation of hornbill uropygial secretions on TSA solid medium resulted in bacterial growth (in at least one individual) for the eight African species with available samples and only one out four of the Asian hornbill species tested (Fig. 2). For all except the African *Ceratogymna atrata*, the frequency of growing was at least 50%. These results suggest that uropygial secretion of hornbills may host symbiotic bacteria and that in the case of the Asian lineage this property may be less common, although small sample sizes does not allow tests of such possible difference.

Bacterial growth may result from contamination from external sources at the moment of sampling, especially in the species with difficult access to the interior of the gland ducts (see Methods section), therefore, additional evidence is needed to ensure that the secretions are loaded with bacteria. For this, we examined in a microscope the secretion-extensions of 13 individuals of six species in total to observe bacteriadirectly. With this method we found clear evidence of the existence of abundant bacteria throughout the whole extension of the secretion for several individuals of different species. That was the case for two out of three *Bucorvus leadbeateri* individuals (Fig. 3a-b, see also Video-1 in Supplementary Material), and two out of three *Bycanistes brevis* individuals (Fig. 3c-d, see also Video-2 in Supplementary Material). In the only *Tockus nasutus* sample examined, there were some areas with abundant dispersed bacteria and others where they were less frequent (Fig. 3e). In the remaining cases, it was possible to find some areas with small aggregations of bacteria in a general field without them (the four individuals of *Ceratogymna atrata*, e.g. Fig. 3f) or bacteria were everywhere, but very scarce and dispersed (the only individual of *Aceros corrugatus*Fig. 3g, also the third individual of *Buceros leadbeateri* and *Bycanistes brevis*, not shown). Finally in the only secretion of *Tockus deckeni* available there were no bacteria in most of the surface, but some can be found very dispersed and sporadically some small groupings (Fig. 3h).

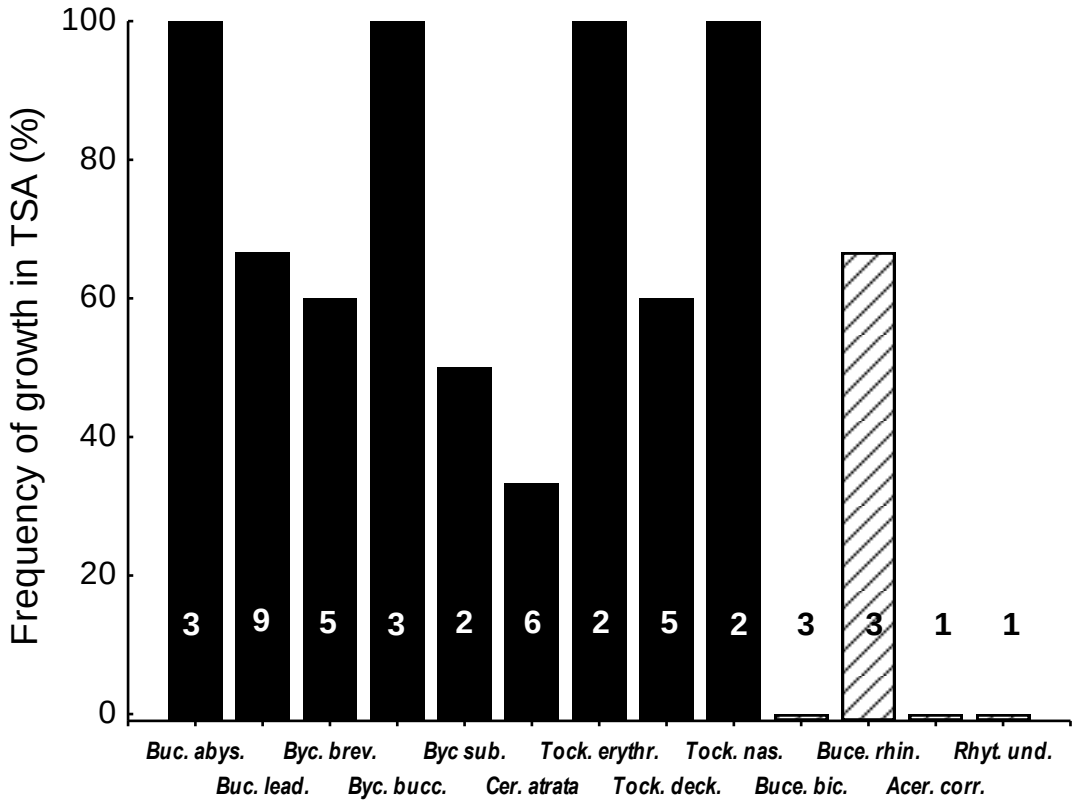
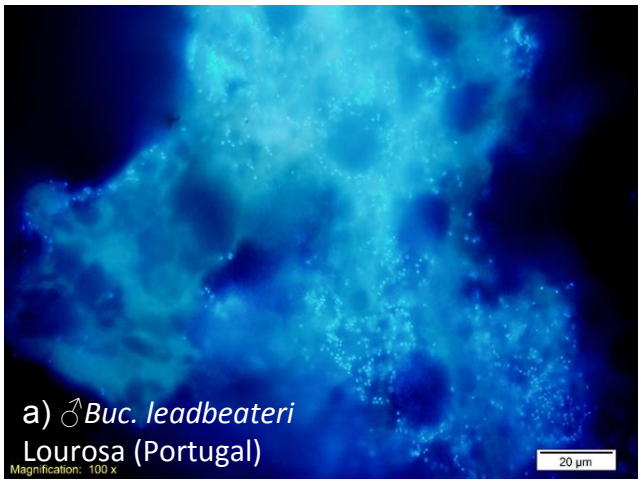
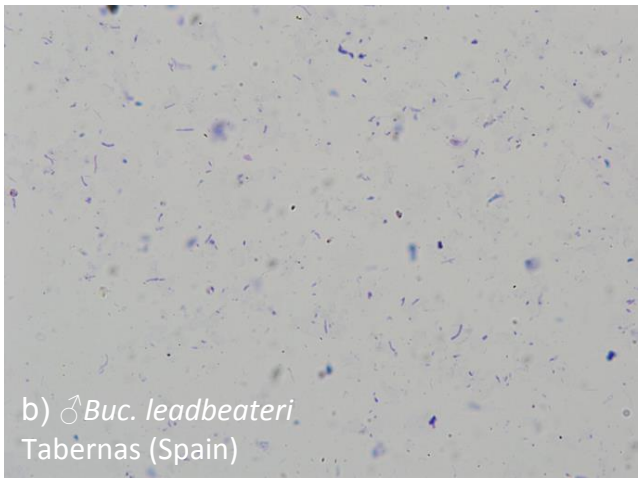
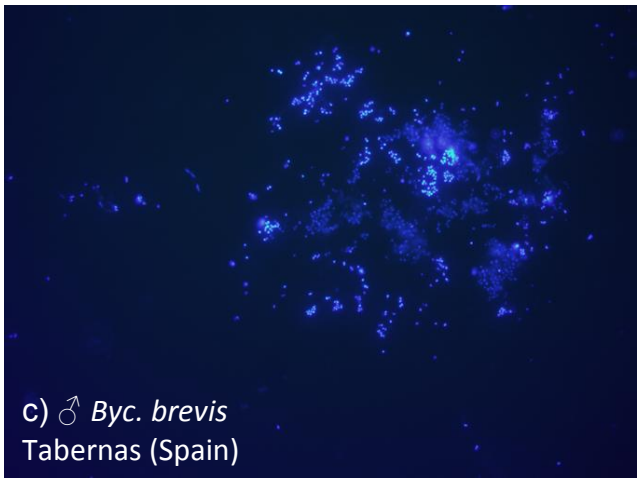
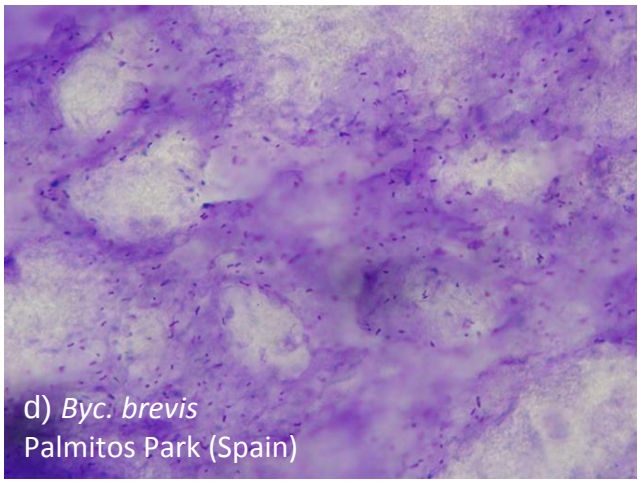


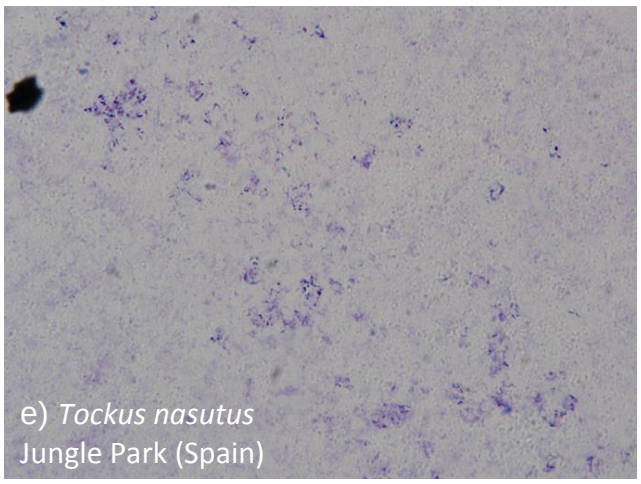
Fig. 2. Percentage of secretions inoculated in TSA medium with positive bacterial growth per hornbill species. Numbers in bars indicate the sample size (number of individuals) per species. Black bars are from species of the African lineage, dashed bars from those of the Asian lineage.

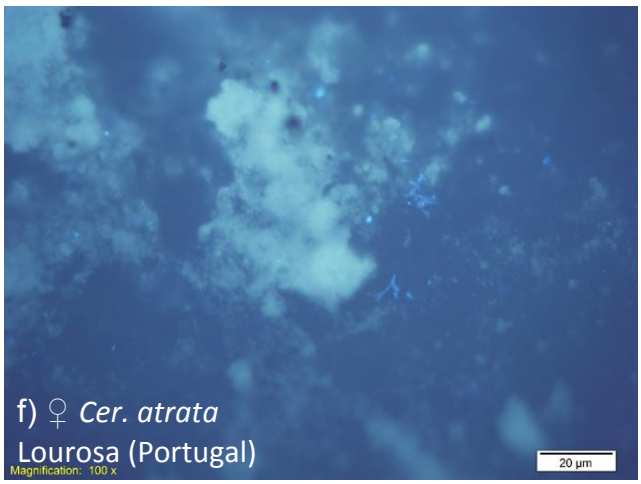

a) ♂*Buc. leadbeateri*
Lourosa (Portugal)
Magnification: 100 x
20 µm


b) ♂*Buc. leadbeateri*
Tabernas (Spain)


c) ♂ *Byc. brevis*
Tabernas (Spain)


d) *Byc. brevis*
Palmitos Park (Spain)


e) *Tockus nasutus*
Jungle Park (Spain)


f) ♀ *Cer. atrata*
Lourosa (Portugal)
Magnification: 100 x
20 µm

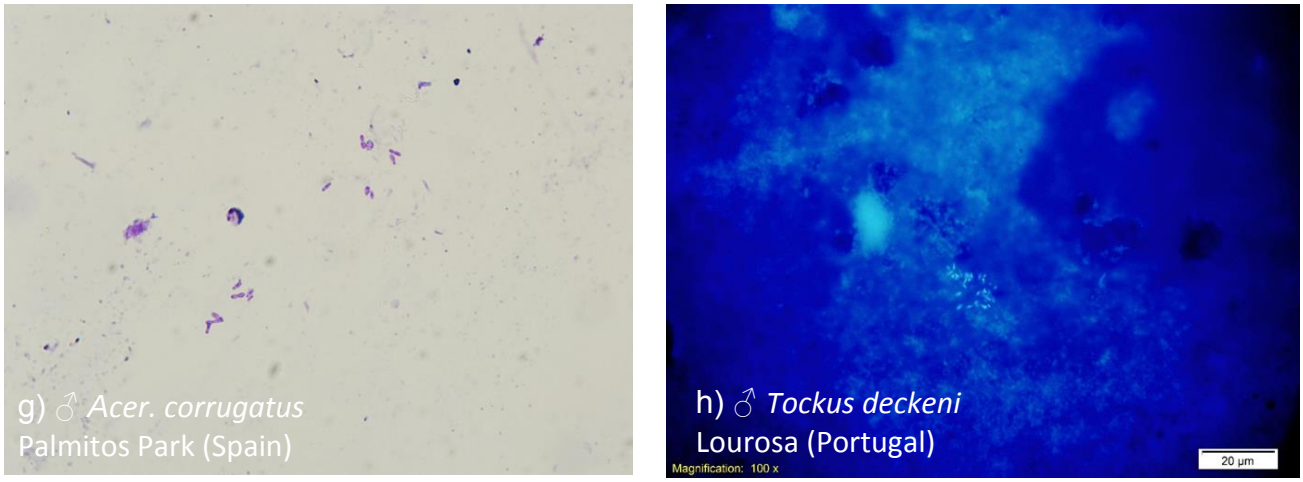


Fig. 3. Microscopic images of uropygial secretions of different species of hornbills for the study of the existence and abundance of bacteria. In a, c, f and h bacterial DNA is marked with hoescht and observed at fluorescence microscope. In b, d, e and g, violet crystal is used to stain bacteria and are observed at optic microscope.

Analysis of bacteriomes

The sequencing of the 16S bacterial DNA present in biological samples can be used to infer the presence and composition of bacterial communities hosted in bird tissues. Despite we do not still have sequences of secretions taken from inside the uropygial gland of hornbills, cotton swabs taken form the surroundings of the glands can provide insight into the communities associated with this organ in this group of birds. Here we analyzed several indices of diversity and similarities in composition among samples, to test the possible existence of groups of sOTUs linked differentially to uropygial glands in comparison to other body parts. Given the complex matrix of samples obtained from different species coming from different zoos, with different number of individuals and kind of samples per individual (Table 1 in Supplementary Material), we first tested for general patterns of effects from zoos, large lineages and species, and afterwards tested for differences among body parts taking into account other general effects.

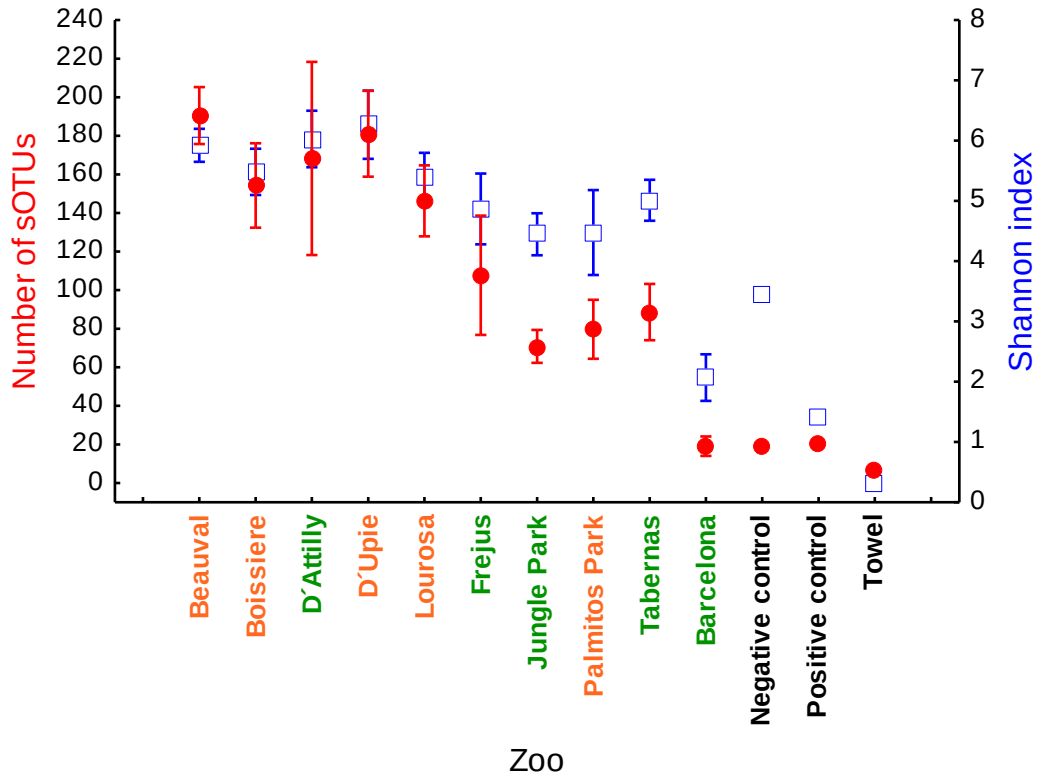


Fig. 4. Differences among zoos in two estimates of alphadiversity:number of sOTUs (red filled circles) and Shannon index (empty blue squares) after decontamination and rarefaction to 1,100 sequences per sample. Values for one negative and one positive control as good as a sample taken from a towel used to cover a hornbill head in a zoo are also included. Colors of zoo labels indicate whether they include only African hornbill species (green) or both African and Asian species (orange). Means and 95% confidence intervals are shown.

Considering all body parts and species together, there were clear effects of the zoos from which samples came on alpha-diversity estimates, mainly when considering the number of sOTUs per sample (Kruskal Wallis, H (9, N= 312) =176.20 p <0.001, Fig. 4). The most different zoo was Barcelona, with a very low number of sOTUs per sample and very similar to those in the two controls and a sample from a towel that were retained after decontamination and rarefaction to 1,100 sequences. In addition, a group of four zoos (Tabernas, Palmitos Park, Jungle Park and Frejus) presented fewersOTUs per sample than the remaining (all post hocs multiple comprison tests between these two groups of zoos with p <

0,05 except for Frejus in an intermediate position, Fig. 4). When considering Shannon index, the differences (Kruskal Wallis, H (9, N= 312) =125.58, Fig. 4) were mainly due again to the very low diversity in Barcelona, but also to differences between the three with the highest values (D´Upie, Beauval and D´Atilly) and those in the lower group (Frejus, Jungle Park, Palmitos Park and Tabernas). These analyses suggest that samples from Barcelona present abnormally low diversity. Indeed, when we plotted differences among zoos in the composition of their microbiomes using unweighed unifrac distances in a PCoA, all samples from Barcelona grouped together very far from most of those of the remaining zoos and close from controls and non-biological samples (the towel), Fig. 5a). An examination of the summarized taxa of samples from different zoos (Fig. 5b) also showed that those from Barcelona were dominated by very few features both after decontamination (mainly an unknown *Xantomonadaceae*, 95%, and *Mycrobacterium maritypicum*) and before that (the same two (53.3%) plus one *Rhodococcus* sp. 30%, *Ralstonia* sp. 10% and one *Sphingomonas* sp. 6.7%). All this evidence suggests that samples from Barcelona have been damaged during storage, and therefore we will discard them from subsequent analyses.

Factors affecting alphadiversity

From Fig. 4 is evident that zoos including samples of both African and Asian species (orange labels in Fig. 4) have higher alpha-diversity. Indeed, a comparison between samples of these two groups of zoos showed clear difference in the number of sOTUs (Kruskal Wallis H(1, N= 295) = 84.43, $p < 0.001$; only Africans Mean(SE) = 89.3(5.4), both lineages Mean(SE) = 160.2(4.5)) and in the Shannon index (Kruskal Wallis H(1, N= 295) =42.44 $p < 0.0001$); only Africans Mean(SE) = 4.8(0.1), both lineages Mean(SE) = 5.6(0.1)). Given that we are analyzing features per sample and not considering the total different sOTUs present in each zoo, this effect could be caused by a higher diversity in samples from Asian species.

Considering all zoos together, samples from Asian species presented a higher number of sOTUs (Kruskal-Wallis H(1, N= 295) =12.51 $p < 0.0005$); Africans Mean(SE) = 124.6(4.4), Asians Mean(SE) = 157.2(8.8)), but not a higher Shannon index (Kruskal-Wallis test: H(1, N= 295) = 0.03 $p = 0.858$). When we compared within zoos, only for those with both kinds of species, the effect of the lineage on number of sOTUs disappeared, suggesting that the effect was in fact caused by the zoo and not by the lineage of the species considered (GLM with zoo as random factor, effect of lineage $F(1,168) = 0.15$, $p = 0.703$). Indeed, differences between lineages in the Shannon index approached significance in the opposite direction (GLM with zoo as random factor, effect of lineage $F(1,168) = 3.79$, $p = 0.053$; Africans Mean(SE) = 5.8(0.1), Asians Mean(SE) = 5.3(0.2)).

Given the strong effect of zoos in the diversity found in samples, and the different assemblages of hornbill species available in different zoos, comparison among body parts were performed within individuals nested within species. With this design there was a general trend of uropygial glands and casque being the body parts with more sOTUs, differing significantly from samples taken from wing and tail black and white areas (Fig. 6). Beak tip presented also significantly more sOTUs than the black wing feathers, and the remaining body parts presented intermediate values (Fig. 6). The same result was obtained when analyzed separately for African species. However, for Asian species the only difference was between the nape, with the highest richness and wing areas with the lowest. In the case of the Shannon index, there were no significant differences among body parts (results not shown). The interaction of species identity and body part did not result in significantlydifferent numbers of sOTUs,as it was for the Shannon index, although this should be taken with caution, since we did not have the same body parts sampled for all species, and in some species we only had one individual.

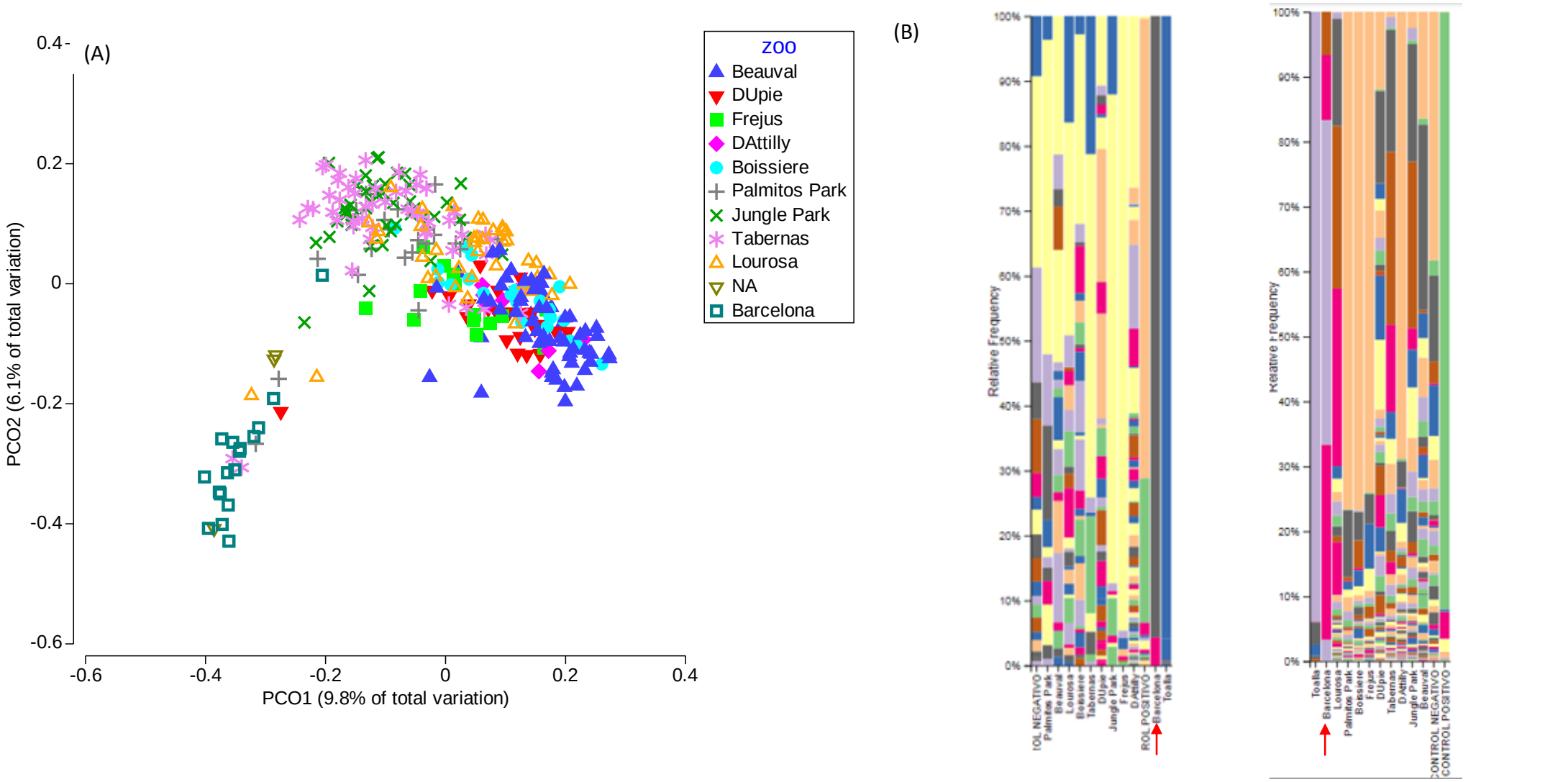


Fig. 5. (A) PCoA graph showing the differences among samples in theirbacteriome composition based on Unweighted Unifrac distances after rarefaction to 1,100 sequences. Samples obtained from different zoos are depicted with different combinations of colors and point shapes (see legend). Points labelled with NA include a positive control, a negative control and a sample taken from a towel used to cover a hornbill during capture. (B) Comparison of the taxa dominating communities in different zoos and control samples. Bars of different colors are single sOTUs and their height represents the median ceiling of each feature across samples of the zoo. The graph on the left shows profiles after decontamination and rarefaction to 1,100 sequences, the graph on the right side shows the same before decontamination and after rarefaction to 2,302 sequences. Red arrows mark the Barcelona profiles.

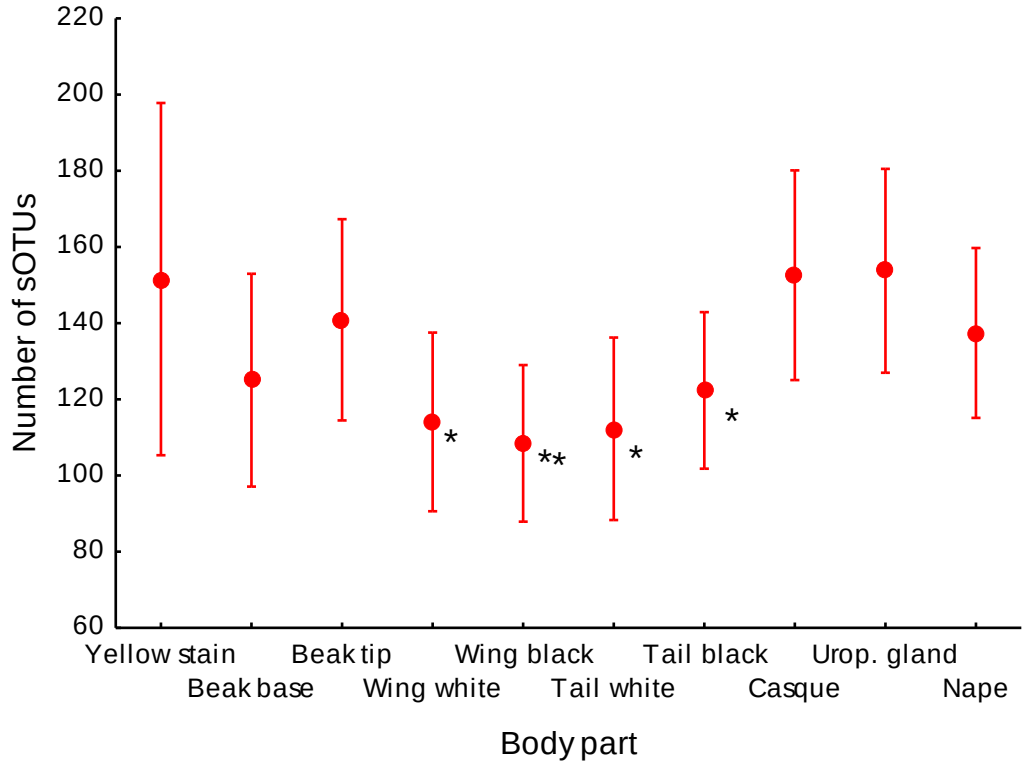


Fig. 6. Differences in the number of sOTUs present in the samples taken from different hornbill body parts**.** The whiskers represent weighted means(95% confidence interval) for the fixed effect in a model with individual identity as random factor nested within species identity. Whole model: R^2 = 0.642, F(129,163) = 2.27, p < 0.0001; body part effect F(9,163) = 3.43, p < 0.001. Single asterisks mark body parts differing from Casque and Uropygial gland, double asterisks mark those differing additionally from Beak tip in post hoc Tukey tests.

Factors affecting bacteriome composition

The zoo from which samples came was a significant predictor of the composition of bacteriomes even when those from Barcelona were not considered (Permanova, Unweighted Unifrac distances PseudoF(8,284) = 6.95, pPerm < 0.0001, see distribution of samples per zoo in Fig. 5a). Part of these differences could be due to variation among zoos in the hornbill species hosted, however, a more complex model including individual as a random factor nested within hornbill species and zoo, together with body parts also found a significant effect

of zoo (Table 1). Moreover, when we performed the comparison among zoos only for the single species which is more common in these installations: *Bucorvus leadbeateri*, differences persisted (Fig. 7, Table 1), suggesting there are bacteriome components typical of different zoos.

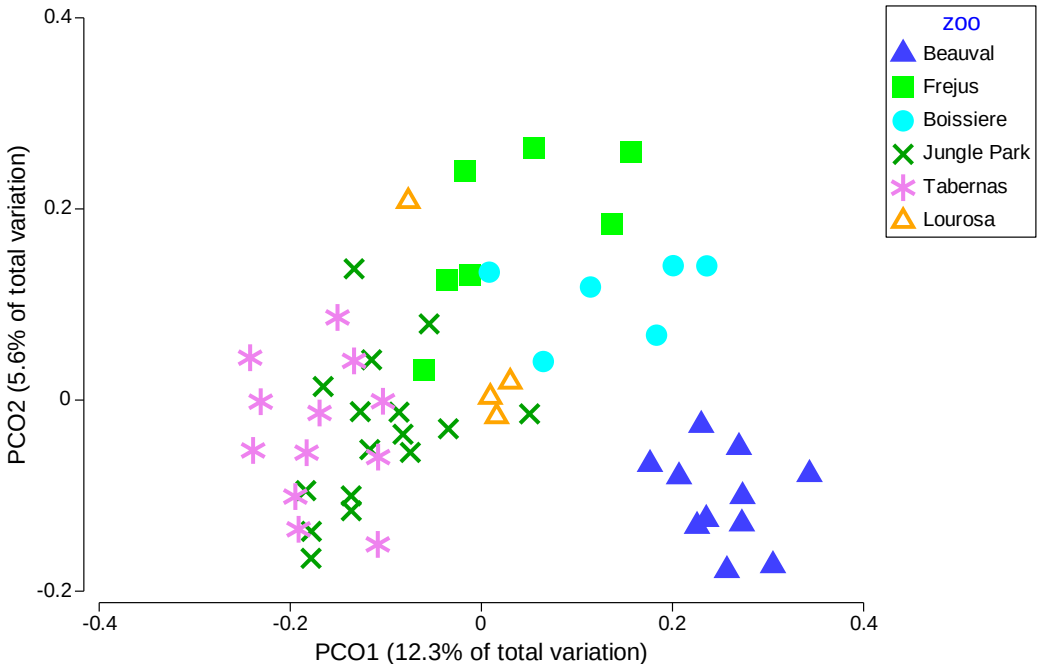


Fig. 7.PCoA graph showing the differences among samples of the single species *Bucorvus leadbeateri* in theirbacteriome composition based on Unweighted Unifrac distances after rarefaction to 1,100 sequences. Samples obtained from different zoos are depicted with different combinations of colors and point shapes (see legend).

Table 1. Results of Permanova analyses testing the effects of zoo and body part on differences in composition of microbiome of samples taken from hornbills. Based on Unweighted Unifrac distances for decontamined samples rarefied to 1,100 sequences. (A) For all species, with individual as random factor nested within species and zoo. (B) Only for *Bucorvus leadbeateri* with individual as random factor nested within zoo.

	Effect	df	Pseudo-F	P(perm)	perms
A) All species					
1. Zoo	Fixed	8	3.26	**<0.001**	9866
2. Hornbill species	Fixed	11	1.50	**0.014**	9813
1x2**	Fixed	8	1.37	**0.018**	9813
Individual(1x2)	random	17	1.40	**<0.001**	9204
Body part	Fixed	9	1.42	**<0.001**	9341
Error		239			
B) *Bucorvus leadbeateri*					
Zoo	fixed	5	2.48	**<0.001**	8885
Individual(Zoo)	random	3	1.32	**<0.001**	9720
Body part	fixed	7	1.18	**<0.004**	9593
Error		40			

**Term has one or more empty cells

The Permanova analyses summarized in Table 1 also highlights a clear effect of body part in the composition of hornbill microbiomes after controlling for hornbill species and zoo. As with alpha-diversity, there seems to be a general trend for some types of samples to have a more similar composition than others. In this case, pair-wise analyses show that beak tip has the most clearly separated community, differing significantly from all other samples except

beak base. Wing and tail black and white areas do not differ, and casque and gland do not differ, but show different combinations of differences with other body parts; and nape only differs from beak samples (results not shown). This analysis can only show effects of parts when trends are the same for the whole set of species, although our hypothesis is that different species may have specialized communities. Moreover, Permanovas test for differences in distances among samples, but do not distinguish whether distances are caused by the same particular sOTUs. To gain deeper insight in the differences in composition, identifying the sOTUs that are responsible of differences among samples, we have used GNEISS analyses.

Tables 2 and 3 show the results of two GNEISS analyses (one for all species, the other only for *Bucorvus leadbeateri*), testing for differences among zoos in the main balances between groups of OTUs that tend to co-occur in the samples (identified in Table 3, Fig. 3 in Supplementary Material provides additional information useful for understanding the tables). Several combinations of zoos differ in their values of the balances tested, and the same trend is observed for some balances when considering all species or only *Bucorvus leadbeateri*. For example, in the balance y_0, the same two sOTUs of the genus *Rhodoplanes* (Family *Hyphomicrobiaceae*, O. *Rhizobiales*, P. *Proteobacteria*) and another unknown genus of the O. *Rhizobiales*, are especially abundant in relation to all the remaining sOTUs of the whole set, for the zoos: Boissiere, Jungle Park, Lourosa, Palmitos Park and Tabernas, both when considering all species or only *Bucorvus leadbeateri*. Also, several *Corynebacteriaceae*, *Dermacoccacea*e, *Deinococcaceae* in combination with different other sOTUs from other families, mark differences of Beauval zoo for all species (balance $y_{2)}$ and also for *Bucorvusleadbeateri* (balance y_5). On the other hand, particular taxa that are important in some balances when considering all hornbill species together, disappear in the analysis for *Bucorvusleadbeateri* suggesting that they are more typical of particular species than of the

zoo. For example, the 15 sOTUs of the family *Rhodobacteraceae* (O. *Rhodobacterales*, P. *Proteobacteria*) heading the y_2 balance as typical of Beauval zoo with all species considered, are not present in the balances typical of this zoo for *Bucorvus leadbeateri* (Tables 2 and 3). Given that most individuals sampled in this zoo are of the species *Buceros rhinoceros*, probably the *Rhodobacteraceae* group is particularly present in this hornbill species. Indeed, when we analyzed the significant levels of the effect *species identity* on the balances for all samples together (Fig. 4 in Supplementary Material), balance y_2 was significant only for *Buceros rhinoceros*, and an additional balance (y_{21}) with 17 sOTUs of the families *Rhodobacteraceae*, *Corynebacteriaceae*, *Deinococcaceae*, *Staphylococcaceae* and *Moraxelaceae* resulted significant for this hornbill species. The only other significant effects of *species identity* on balances for the whole set of samples (Fig. 4 in Supplementary Material) that can be tentatively assigned from this analysis to a particular hornbill species or genus, are y_5 (Table 2) and y_{10} (*Dermacoccaceae*, *Micrococcaceae*, *Moraxelaceae*) again for *Buceros rhinoceros* and y_{13} (*Clostridiaceae*, *Staphylococcaceae*, *Aerococcaceae*, *Aeromonadaceae*, *Deinococcaceae*) to the two species of the genus *Bucorvus*. Additionally, y_{28} includes a significant effect of relative abundance of a *Rubrobacteraceae* (*Rubrobacter sp*., P. *Actinobacteria*) in relation to a group of 40 taxa including 10 *Rhodobacteraceae*, differentiating *Buceros rhinoceros* and *Bycanistes buccinator* from other species. Nevertheless, even in these cases, the different abundances of species among zoos still can be responsible of erroneous assignation of bacterial taxa from balance analysis to particular hornbill species.

Since all except one *Buceros rhinoceros* individuals were from Beauval, it is impossible to distinguish the effect of zoo and species. In this special case, of interest for the hypothesis of the study, since it is an Asian species with coloured uropygial secretion and

cosmetic coloration in several body parts, we have checked whether the 15 *Rhodobacteraceae* sOTUs are present in other species and/or zoos (Table 4). Four of these features were only present in Beauval Zoo and only in *Buceros rhinoceros* individuals, while the remaining 11 were also in other zoos and species (Table 4). However, the prevalences of 13 out of the 15 *Rhodobacteraceae* sOTUs were significantly higher in *Buceros rhinoceros* than in the other species considered together (chi-square tests, asterisks in the fifth column in Table 4). If we compare prevalence only for the individuals hosted in Beauval, this was higher for *Buceros rhinoceros* individuals than for those of the other species together (N: 2 *Bucorvus abyssinicus* + 2 *Buorvus leadbeateri* + 2 *Tockus deckeni* = 6) for 12 out of 15 sOTUs (Binomial test, two-tailed $p = 0.035$). Low sample sizes make the interpretation unreliable of a 2x2 table for each sOTU in this subsample, but still, two-tailed Fisher exact tests were significant ($p < 0.05$) for sOTUs num9 and num14, and had $p < 0.10$ for num1, num4 and num15. All this evidence shows that this group of bacteria can be considered more typical for *Buceros rhinoceros* than for the other species.

Table 2. Summary of the main balances in GNEISS analyses presenting significant differences among zoos for the samples obtained from different hornbill body parts. Results of an analysis using all species together and one only with the samples of *Bucorvus leadbeateri* are presented in parallel for comparisons. The batch in which samples of different zoos were sequenced are indicated. The taxa involved in the balances are shown in Table 3.

Zoo	All available samples Balances: $y_n = Ln(y_{n\ Numerator}/ y_{n\ Denominator})$									Sequencing batch	Only Bucorvus leadbeateri samples Balances: $y_n = Ln(y_{n\ Numerator}/ y_{n\ Denominator})$									
	y_0	y_1	y_2	y_3	y_5	y_6	y_7	y_8	y_9		y_0	y_1	y_2	y_3	y_5	y_6	y_7	y_8	y_9	y_{12}
Beauval	0	0	*	0	*	*	**	*	0	2	0		(*)	0	**	(+)		*	0	*
Boissiere	*	*	0	0	+	*	0	**	0	1	*		(+)	*	*	0		**	0	**
D´Atilly	0	0	0	0	(+)	*	**	**	*	1										
D´Upie	0	0	0	0	(+)	*	(+)	*	*	2										
Frejus	0	0	0	0	*	**	0	**	*	1	0		0	0	*	0		*	0	0
Jungle Park	*	*	0	(+)	*	**	*	**	0	2	*		(*)	(*)	*	*		*	+	*
Lourosa	*	*	0	+	*	**	0	*	0	2	*		(+)	0	*	0		*	0	*
Palmitos Park	*	*	0	+	*	**	**	*	0	1										
Tabernas	*	*	0	+	*	**	0	**	0	2	*		0	*	*	0		*	0	0

* Balances are negative (denominator>numerator), and there are other positive or zero balances differing significantly ($p<0.05$ after FDR correction)

**Balances are negative (denominator>numerator), and there are other negative balances closer to zero differing significantly

0 Balances are approximately zero and there are other positive or negative balances differing significantly

+ Balances are positive (denominator<numerator) and there are other negative or zero balances differing significantly

( ) 0.05 <p-values < 0.1

Table 3. Taxa (sOTUs) involved in the main balances from the two GNEISS analyses summarized in Table 2 (one for all species of hornbill together and another only for *Bucorvus leadbeateri*). The taxa included in the denominator and denominator are indicated for each balance. The denominator taxa present a significant higher abundance in relation to those of the numerator for the effect levels with a significant coefficient after FDR correction in Table 2. Taxa are named at the family level, but when the genus or species identification is known, they are presented for families with low number of sOTUs selected. Alphanumeric chains identify sOTUs individually. Numbers in parenthesis in the denominator indicate the total number of sOTUs when not all are indicated with their taxonomic affiliation. Taxa of different Phyla are written in different colors (see legend at Table foot).

Balance	**All available samples**		**Only *Bucorvus leadbeateri* samples**	
	Taxa (denominator)	Taxa (numerator)	Taxa (denominator)	Taxa (numerator)
y_0	2 Hyphomicrobiaceae (G. Rhodoplanes) 99994b12131e74ec824ddb8008c66699 37d2e61deecc2c4ae3e586d25f1fcb13 1 Rhizobiales 2df7309fb0b0e6c04976feac4c4e867e 1 Rhodospirillaceae 2abdf7d5372805ba22bb91e8cafbbe92	4,450 sOTUs	2 Hyphomicrobiaceae (G. Rhodoplanes) 99994b12131e74ec824ddb8008c66699 37d2e61deecc2c4ae3e586d25f1fcb13 1 Rhizobiales 2df7309fb0b0e6c04976feac4c4e867e	2,227 sOTUs
y_1	1 Hyphomicrobiaceae (G. Rhodoplanes) 99994b12131e74ec824ddb8008c66699 1 Rhizobiales 2df7309fb0b0e6c04976feac4c4e867e	1 Hyphomicrobiaceae (G. Rhodoplanes) 37d2e61deecc2c4ae3e586d25f1fcb13 1 Rhodospirillaceae 2abdf7d5372805ba22bb91e8cafbbe92		

y_2	15 Rhodobacteraceae 5 Intrasporangiaceae 4 Dermabacteraceae 4 Dermacoccaceae 4 Corynebacteriaceae 4 Deinococcaceae 4 Weeksellaceae, … (67)	4,383 sOTUs	3 Corynebacteriaceae 8ee130e16717012abf5a59dc6f477354 e3859130f5b777ac285056dd82d8218a 7ccb19e3489f96b584a78386c98fab5f 1 Propionibacteriaceae (G. Propionibacterum) 4ef58fef4fb0ff6ef2b249619cd8abe6	2,223 sOTUs
y_3	1 Hyphomicrobiaceae (G. Rhodoplanes) 99994b12131e74ec824ddb8008c66699	1 Rhizobiales 2df7309fb0b0e6c04976feac4c4e867e	1 Hyphomicrobiaceae (G. Rhodoplanes) 37d2e61deecc2c4ae3e586d25f1fcb13	1 Hyphomicrobiaceae (G. Rhodoplanes)99994b12131e74ec824ddb8 008c66699
y_5	1 Staphylococcaceae(G. Staphylococcus) 10eab44d7b9f6b36684fb802361bb7c0	66 sOTUs	3 Micrococcaceae (1 G. Kocuria, 1 Kocuria rhizophila) cd871d357f428044923be36bc25798a7 6a6f4586c7871ce4c3109af7d87eed22 c62b2bb480bcc17b7449ca28408ed3b9 1 Corynebacteraceae (G. Corynebacterium) f45a9cf83335baf202289b511707d8c8 1 Dermacoccaceae (G. Dermacoccus) 77a6cb87bdd52a2cf600d9f96d58be9c 2 Deinococcaceae (G. Deinococcus) 728e2911b225d5c580b19656af3dfa41 508dd23e62cd3385389499ff8299d66c	2,207 sOTUs

			2 Moraxelaceae (Acinetobacter johnsonii, G. Enhydrobacter) 1fc3a0c640ad2c319e53d343d00ad739 78330633222b06d693cdde05108c8bbb 2 Aeromonadaceae 3bb8e412123e67c1630d9c8746377a1c 81449da837debdb7c81d1e1fb71f0c11 1 Flavobacteriaceae 8b40ac5f3c607725e8a6d425440cc835 1 Clostridiaceae e354f7c40cca3fff105dcfabc771aa55 1 Aerococcaceae 5c0b630029fe064b2b6d8c6f5b551092 1 Peptostreptococcaceae 6005b8862abf1ac2af852c375606a344 1 Staphylococcaceae (Staphylococcus sciuri) aae1d0621d90f1ffc9e8b641304a4aa0	
y_6	1 Vibrionaceae (Vibrio metschnikovii) 12c43e8595bb712b4131f4f85841e287 1 Alcaligenaceae (G. Denitrobacter) 76924f6079dbccb4288eff590633c6bb 1 Streptococcaceae (G. Streptococcus)	4,380 sOTUs	1 Corynebacteriaceae (G. Corynebacterium) 7ccb19e3489f96b584a78386c98fab5f	2 Corynebacteriaceae (G. Corynebacterium) 8ee130e16717012abf5a59dc6f477354 e3859130f5b777ac285056dd82d8218a

	2f56969ccc377592f6ed1d5de13557c3			
y_7	1 Micrococcaceae(Kocuria palustris) 0c1b656a4a178cb06e19ab0fff07d336	65 sOTUs		
y_8	1 Streptococcaceae 2f56969ccc377592f6ed1d5de13557c3	1 Vibrionaceae 1 Alcaligenaceae	1 Corynebacteriaceae (Corynebacterium kroppenstedtii) 1 Aerococcaceae (G. Alloiococcus)	2,205 sOTUs
y_9	6 Flavobacteriaceae 6 Planococcaceae (G. Planomicrobium) 5 Dermabacteraceae(G. Brachybacterium) 5 Micrococcaceae(1 G. Arthrobacter, 2 Arthrobacter crystallopoietes,1 Kocuria palustris) 2 Mycobacteraceae (G. Mycobacterium) 2 Brevibacteriaceae (G. Brevibacterium) 2 Nocardioidaceae 2 Bacillaceae (G. Bacillus), … (37)	4,343 sOTUs	1 Corynebacteriaceae (G. Corynebacterium) e3859130f5b777ac285056dd82d8218a	1 Corynebacteriaceae (G. Corynebacterium)8ee130e16717012abf5a5 9dc6f477354
y_{14}			1 Peptostreptococcaceae6005b8862abf1ac 2af852c375606a344	2 Aeromonadaceae 3bb8e412123e67c1630d9c8746377a1c 81449da837debdb7c81d1e1fb71f0c11 1 Deinococcaceae(G. Deinococcus) 508dd23e62cd3385389499ff8299d66c 1 Flavobacteriaceae 8b40ac5f3c607725e8a6d425440cc835

			1 Aerococcaceae 5c0b630029fe064b2b6d8c6f5b551092 1 Clostridiaceae e354f7c40cca3fff105dcfabc771aa55

Blue- P. Proteobacteria
Green- P. Actinobacteria
Violet- P. Thermi
Brown- P. Bacteroidetes
Red- P. Firmicutes

Table 4. Summary of the occurrence of sOTUs of the Family*Rhodobacteraceae* (O. *Rhodobacterales,* P. *Proteobacteria*) in the zoos, hornbill species, individuals and body parts from which samples were taken. The number of zoos and species in which each of the 15 sOTUs appeared is indicated. Number of individuals in which they were found are separated for the species *Buceros rhinoceros* (out of a total of 5 individuals sampled) and the remaining species together (out of 44 individuals sampled). The relative abundance of each sOTU (% of sequences corresponding to that OTU) is calculated only for the samples in which it was found, and is presented only for species other than *Buceros rhinoceros* (all together). See Fig. 8 for the relative abundances of the same sOTUs for *Buceros rhinoceros* samples. The means in bold indicate the kind of sample in which each sOTU was most abundant, while the N in bold indicate in which type was most prevalent for the total of species different of *Buceros rhinoceros*.

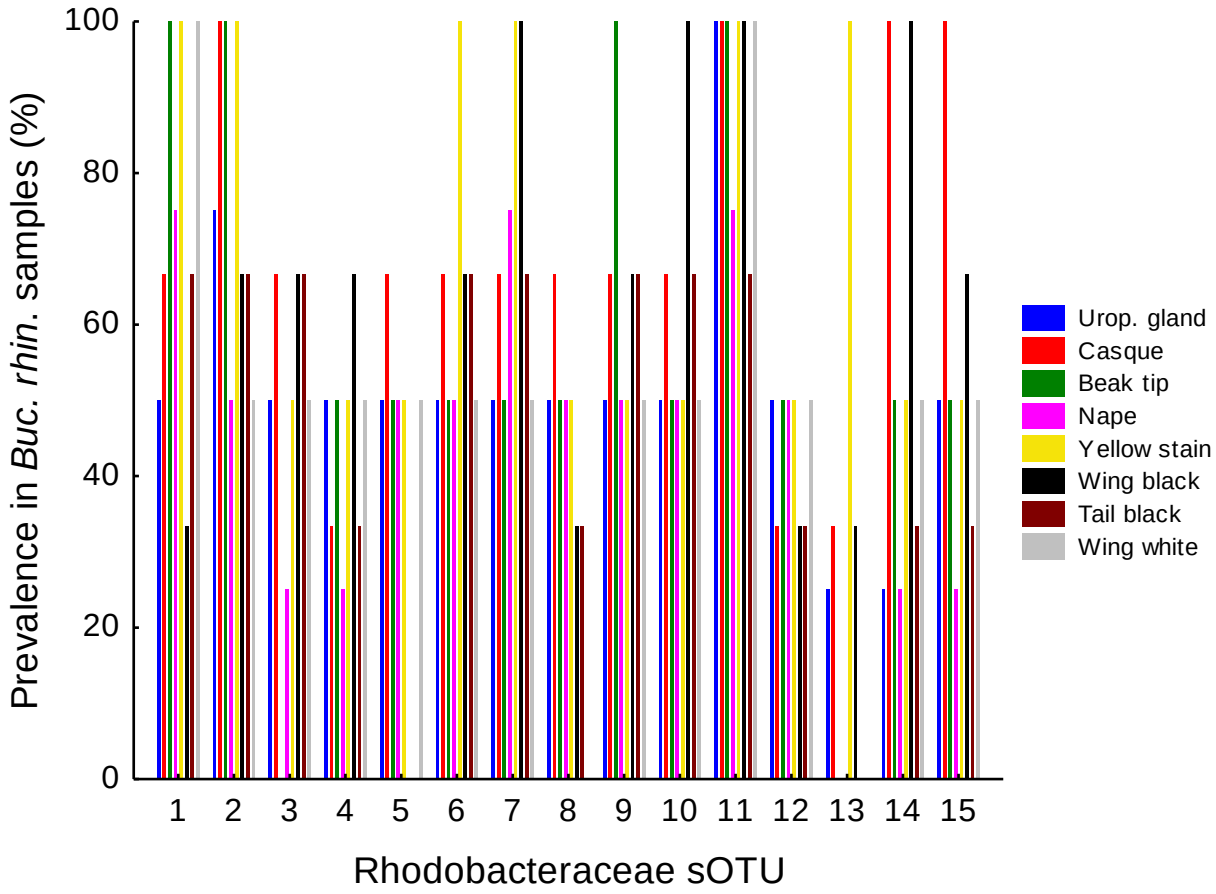
sOTU	Taxa	No. zoos	No. species	No. ind.		Relative abundance (%) in body parts of other species when present (mean(n))									
				Buc rhin Total = 5[1]	Other Total = 44	Urop. gland	Casque	Beak tip	Beak base	Tail black	Tail white	Wing black	Wing white	Nape	Yellow
7845a7198d9e3c16e6a68bb91fca08c3	Unknown sOTU1	2	4	4*	3	**1.22**(1)	-	0.81(1)	-	0.40**(2)**	0.57(1)	0.22(1)	0.57(1)	-	-
740ce45b67368281994a2e6fdfc1a1e4	Unknown sOTU2	3	6	4*	5	0.01(1)	0.05(1)	-	-	**1.86**(1)	0.54(2)	0.18(1)	0.08(1)	0.19**(3)**	-
425ce5c2b800c6bd9d9dedce4ad32127	Unknown sOTU3	2	5	2	5	0.15(2)	-	0.21**(3)**	-	0.26(1)	-	-	-	**0.50**(1)	-
5b6bbcdf61f9c519f127453b0379638f	Unknown sOTU4	2	4	4*	3	-	-	**0.95**(1)	-	-	0.50(1)	-	0.22**(2)**	-	-
9c152832ac2332777efbde870adb7d7d	*Amaricoccus sp.*sOTU5	1	1	2*	0	-	-	-	-	-	-	-	-	-	-
59854fc75f8ebe7f58902d539bfff8f7	*Paracoccus sp.* sOTU6	4	9	2	11	0.25(3)	**1.33**(2)	0.66(2)	-	0.67**(4)**	0.16(1)	0.82(1)	0.12(1)	**1.29**(2)	-
839c72cc0252feeec9ded0258fdeb617	*Paracoccus sp.*sOTU7	3	7	4*	8	0.11(1)	**1.74**(2)	0.62(3)	-	-	0.50(2)	0.46**(4)**	-	0.57(3)	-
305a7a8d194c5654fd6836b882e35531	*P. aminovorans*sOTU8	1	1	2*	0	-	-	-	-	-	-	-	-	-	-

4d984bf6468d8cdd88410b15da52750a	*Paracoccus sp.* sOTU9	1	1	3*	0	-	-	-	-	-	-	-	-	-	-
c40c8a47b6a678d1185fabb7f3602468	*Paracoccus sp.* sOTU10	3	4	3*	3	0.14(1)	-	0.04(1)	-	-	-	**1.39**(1)	-	0.46(1)	-
c2987611e725f7c87433ed9842587b03	*Paracoccus sp.* sOTU11	6	10	4*	12	**1.07**(2)	0.33(2)	0.67**(5)**	0.07(1)	**1.52**(2)	0.67(1)	0.21(3)	-	0.38(2)	0.02(1)
6aa734a90ab12e57f82dd34784ca16a8	*P. aminovorans* sOTU12	3	3	2*	2	**0.67**(1)	0.07(1)	-	0.48(1)	-	-	-	-	-	-
d217f32218d5c320c94072fb730adc3c	*Paracoccus sp.* sOTU13	1	3	2*	2	-	0.06(1)	-	-	-	-	-	0.07(1)	-	-
14d47bf1dc158de586c2a21b3734d249	*P. aminovorans* sOTU14	1	1	4*	0	-	-	-	-	-	-	-	-	-	-
f8d0c0facd84ec6457d8f7ea7257b1bf	*Rubellimicrobium sp.*sOTU15	2	4	4*	4	0.06(1)	-	-	-	-	-	-	-	**0.34(2)**	-

(1) The individual hosted in Lourosa zoo did not have any of the sOTUs therefore all individuals counted below are from the four found in Beauval.

* Significant differences in prevalence for the sOTU between *Buceros rhinoceros* individuals and all other individuals considered together (chi-square tests, $p < 0.05$).

Regarding body location of this group of bacteria, the uropygial gland was the type of sample with more sOTUs in hornbill species other than *Buceros rhinoceros* (9 out of 15, Table 4) and also registered the highest relative abundances for three of them (Table 4), however, all were found in several types of samples (Table 4). In the case of *Buceros rhinoceros*, 11 out of the 15 sOTUs were found in all kinds of samples, except num3 (absent in beak), num5 (absent in tail), num8 (not in wing white) and num13 which was absent in four types of samples and showed the clearest association with yellowstains in tail, in comparison with the other body locations where it was present (Fig. 8a). Yellow stain was the only type of sample for which all 15 sOTUs had at least 50% prevalence (Fig. 8a), and also that with more cases (six) of 100% prevalence in a sOTU (Fig. 8a). These stains, together with casque and uropygial gland, were the only type of samples harboring all 15 sOTUs (Fig. 8a). Among them, casque presented the highest prevalence for seven sOTUs and the second highest for four more. However, when present, most sOTUs reached their higher relative abundances in black areas of tail or wing (seven cases) with the exception of num7 for beak tip and num6 for casque (Fig. 8b). Although, all these features showed clear peaks of abundance in yellow stains (Fig. 8b). The opposite trend between prevalence and abundance for the sample types probably results from these bacteria reaching higher relative abundances where other taxa are scarce. Black plumage areas are probably the worst environment for bacteria, while white areas are more used by keratinolytic bacteria, and other body surfaces provide other kinds of nutrients different from feather keratine. Given that casque and feather yellow stained surfaces are actively and preferentially covered with uropygial secretion in this species (Poonswad et al., 2013), this pattern of prevalence and abundance is compatible with *Rhodobacteraceae* coming with or depending on the bird uropygial secretion for its presence.


Prevalence in *Buc. rhin.* samples (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Rhodobacteraceae sOTU
Urop. gland
Casque
Beak tip
Nape
Yellow stain
Wing black
Tail black
Wing white

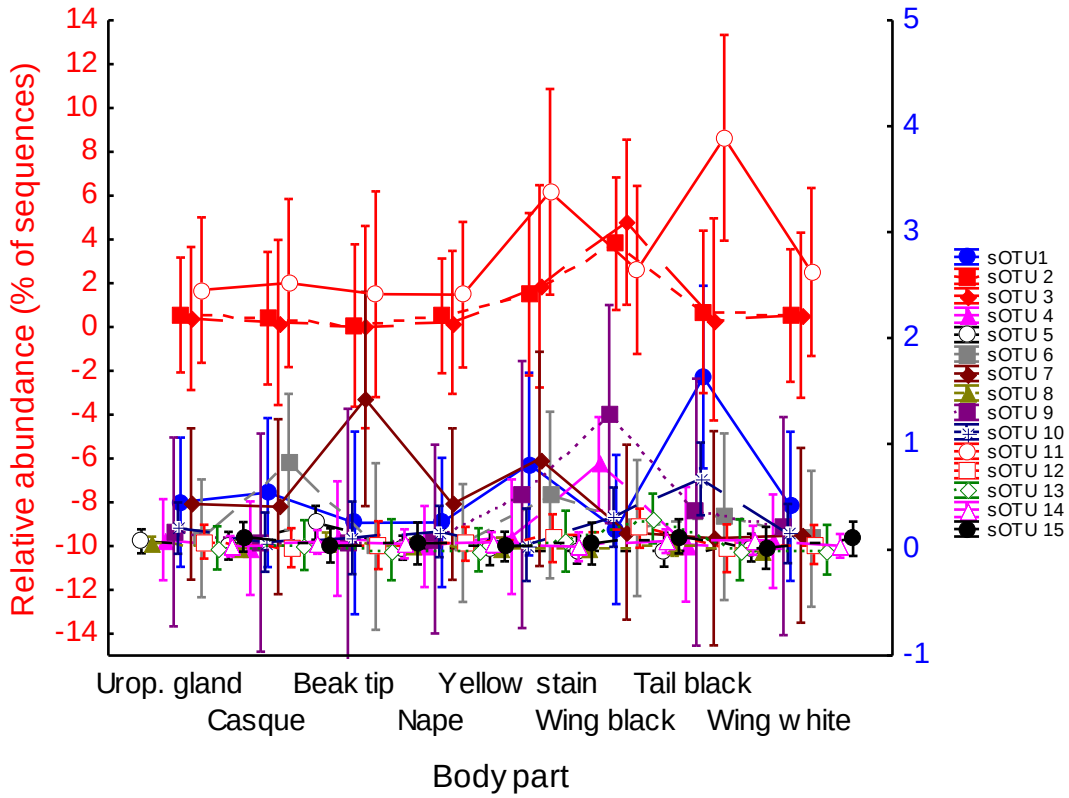

Relative abundance (% of sequences)
14
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
5
4
3
2
1
0
-1
Urop. gland
Casque
Beak tip
Nape
Yellow stain
Wing black
Tail black
Wing white
Body part
sOTU1
sOTU 2
sOTU 3
sOTU 4
sOTU 5
sOTU 6
sOTU 7
sOTU 8
sOTU 9
sOTU 10
sOTU 11
sOTU 12
sOTU 13
sOTU 14
sOTU 15

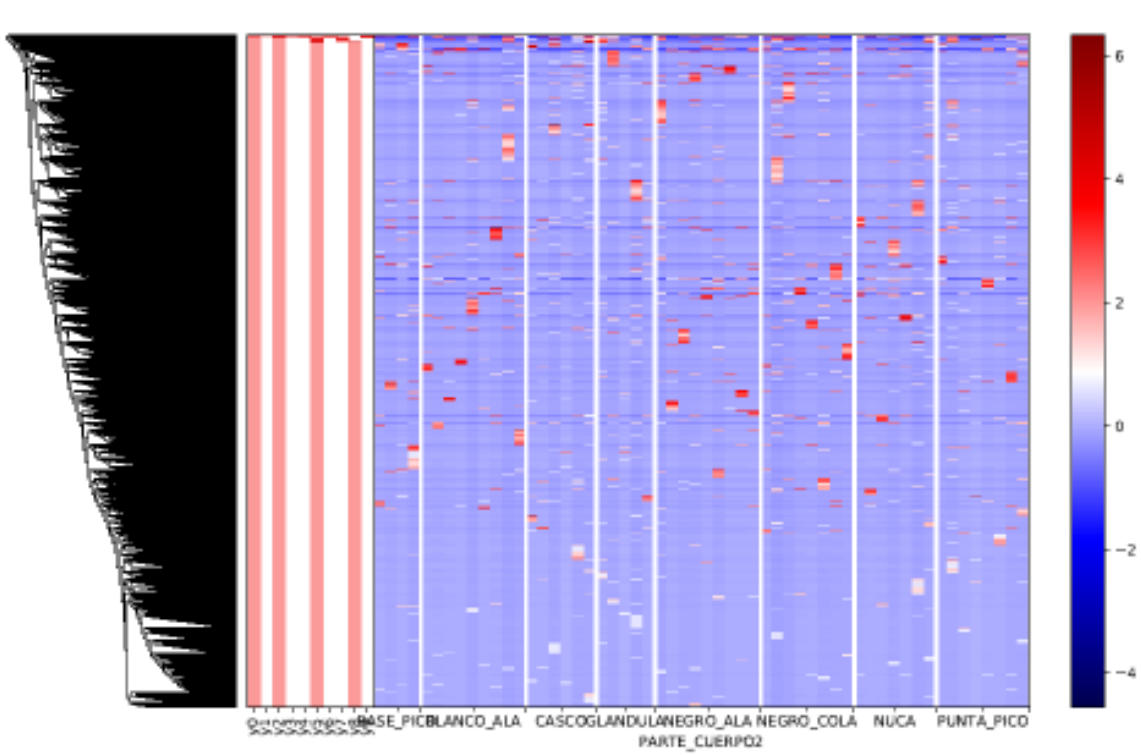
Fig. 8. Prevalences (a) and relative abundances (b) of the 15 *Rhodobacteraceae* sOTUs from Table 4, for the different types of samples obtained from *Buceros rhinoceros* individuals of the Beauval Zoo. Relative abundances include mean and confidence intervals only for the samples in which the sOTU was present. The left side Y axis scale is for the sOTUs 2, 3 and 11, the scale for the remaining sOTUs is on the right Y axis.

To detect other possible associations between uropygial gland and bacteria, we performed comparisons among body parts with GNEISS analyses for the whole set of species, single species with sufficiently many individuals, or groups of related species to have larger sample sizes.

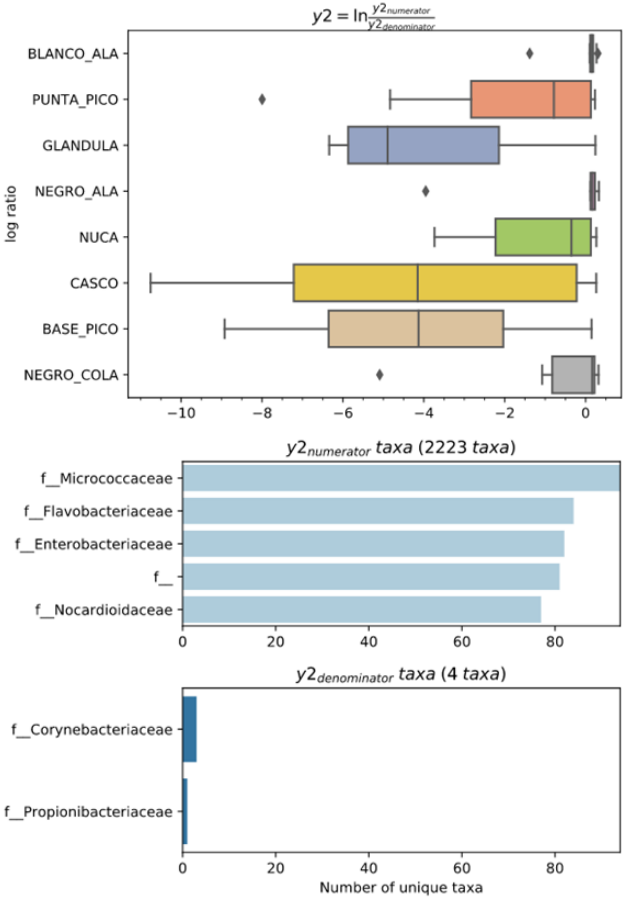
The hornbill species with more individualssampled: *Bucorvus leadbeateri* presented six significant comparisons of values of balances among types of samples (y2, y4, y8, y13, y15 and y21). All referred to similarities among beak base, casque and uropygial gland differing from the remaining samples, and differences among these three types of samples (Fig. 9). The main effects were caused by the relative abundances of three *Corynebacteraceae* and one *Propionibacteraceae* (P. *Actinobacteria*) in relation to other bacteria. These four *Actinobacteria* sOTUs were significantly more abundant in the three body parts referred to above (y2, Fig. 9), but the *Propionibacteracea* (*Propionibacterium sp.*) was significantly more abundant than the other three sOTUs in the uropygial gland (y4, Fig. 9). Another *Corynebacteraceae* and one *Aerococcaceae* (*Alloiococcus* sp, P. *Firmicutes*) again marked differences by the higher abundance in the same three type of samples (y8, Fig. 9), and another *Corynebacteriaceae* was differentially more abundant in Casque and Beak base than in the remaining samples (y15, Fig. 9). Finally, an unidentified *Cardiobacteriaceae* (P. *Proteobacteria*) was differentially more abundant in casque and uropygial gland (y21, Fig. 9).

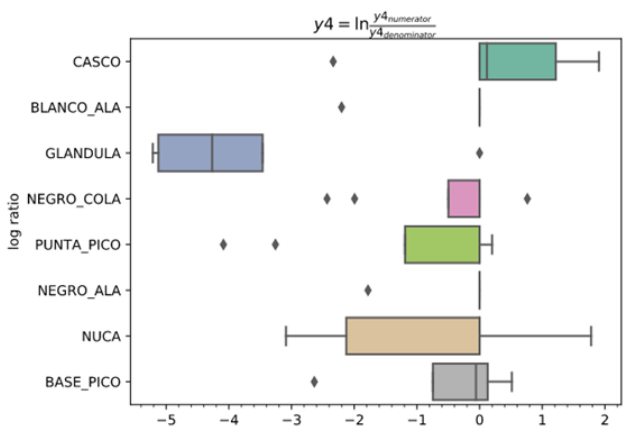
Similar results were obtained for all samples of the two species of the genus *Bucorvus* considered together (results not shown).

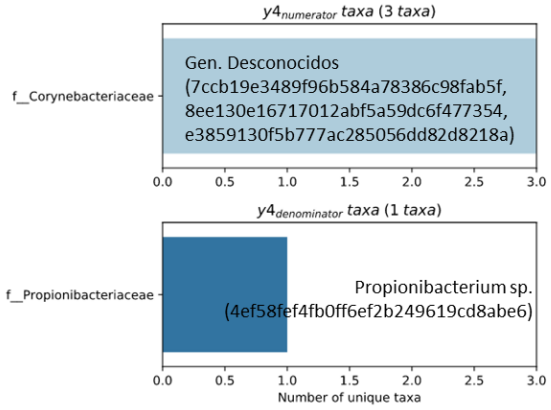
A similar analysis for the African hornbill genus *Bycanistes* (including three species in our data set, see Table 1 in Supplementary Material) only found a significant balance characterizing the uropygial gland. In this case, an *Enterococcaceae* (*Enterococcus casseliflavus*, P. *Firmicutes*) was differentially more abundant in relation to one *Turicibacteraceae* and one *Bifidobacteraceae* in the uropygial gland and nape than in other types of samples (y29, results not shown).

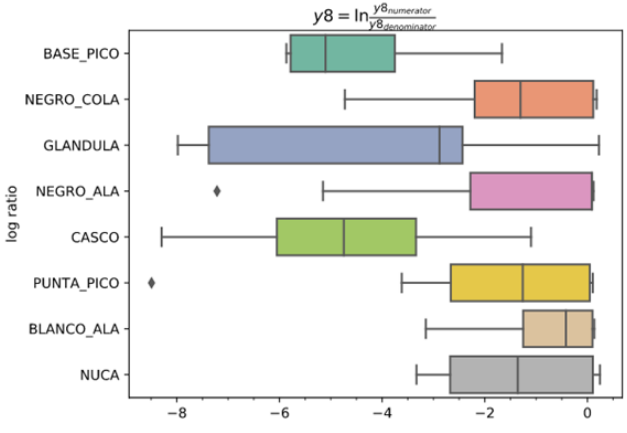


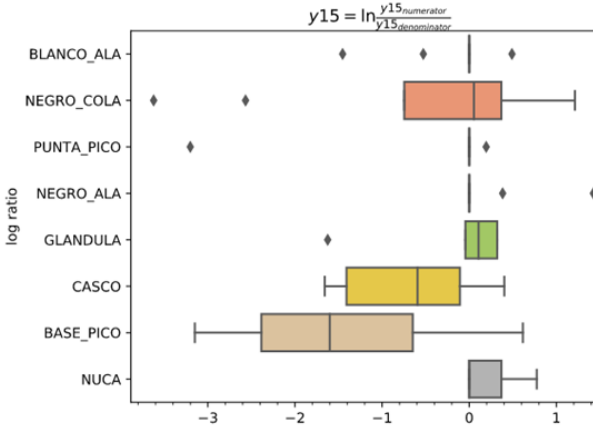
Bucorvus leadbeateri

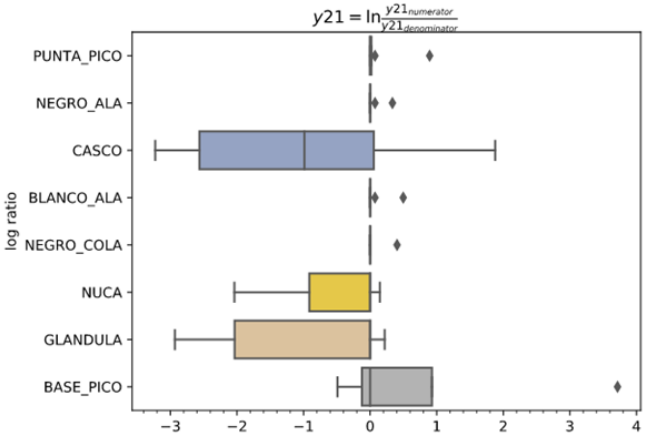



$y4 = \ln \frac{y4_{numerator}}{y4_{denominator}}$
CASCO
BLANCO_ALA
GLANDULA
NEGRO_COLA
PUNTA_PICO
NEGRO_ALA
NUCA
BASE_PICO
log ratio
−5
−4
−3
−2
−1
0
1
2


$y4_{numerator}$ taxa (3 taxa)
f__Corynebacteriaceae
Gen. Desconocidos
(7ccb19e3489f96b584a78386c98fab5f,
8ee130e16717012abf5a59dc6f477354,
e3859130f5b777ac285056dd82d8218a)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
$y4_{denominator}$ taxa (1 taxa)
f__Propionibacteriaceae
Propionibacterium sp.
(4ef58fef4fb0ff6ef2b249619cd8abe6)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Number of unique taxa


$y8 = \ln \frac{y8_{numerator}}{y8_{denominator}}$
BASE_PICO
NEGRO_COLA
GLANDULA
NEGRO_ALA
CASCO
PUNTA_PICO
BLANCO_ALA
NUCA
log ratio
−8
−6
−4
−2
0


$y15 = \ln \frac{y15_{numerator}}{y15_{denominator}}$
BLANCO_ALA
NEGRO_COLA
PUNTA_PICO
NEGRO_ALA
GLANDULA
CASCO
BASE_PICO
NUCA
log ratio
−3
−2
−1
0
1


$y21 = \ln \frac{y21_{numerator}}{y21_{denominator}}$
PUNTA_PICO
NEGRO_ALA
CASCO
BLANCO_ALA
NEGRO_COLA
NUCA
GLANDULA
BASE_PICO
log ratio
−3
−2
−1
0
1
2
3
4

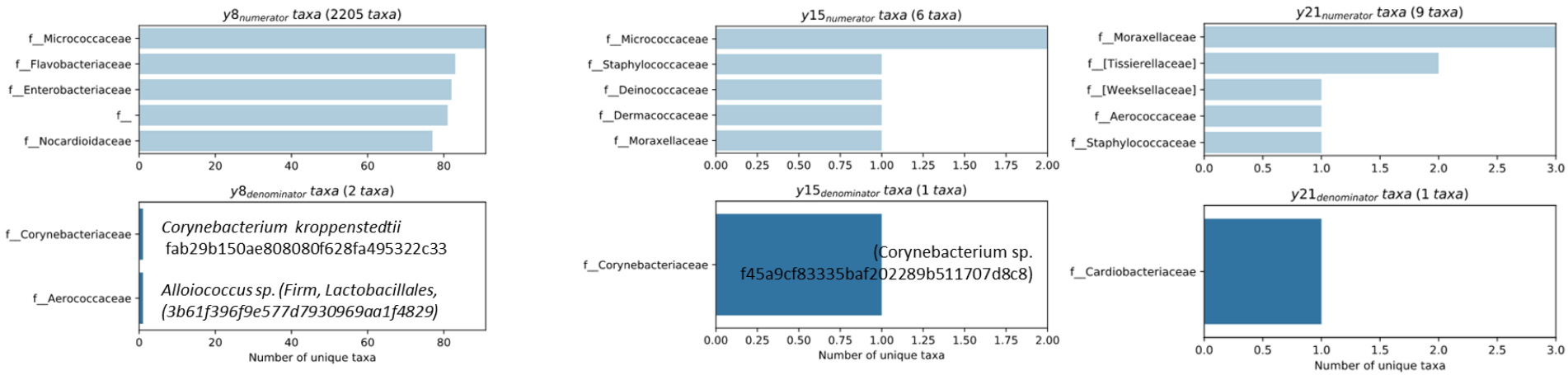


Fig. 9. Heatmap showing the results of the Gneiss analysis on the available *Bucorvus leadbeateri* samples. The tree shows the clustering of sOTUs by their co-occurrence in samples. Bars y0 to y9 indicate the sOTUs included in the first nine balances (red denominator, pink numerator) and increasing relative abundances of each sOTU per sample are depicted in color tones from dark blue to dark red. Bars diagrams show the values for the most relevant balances for differences among body parts.

DISCUSSION

We have found clear evidence of the presence of bacterial symbionts associated with the uropygial glands of hornbills. For several species of the African lineage of this group of birds, the microscopy study of secretion extensions showed that bacteria can be very abundant and occupy the whole secretion, suggesting that they may live within the uropygial gland. For other species, culture and microscopic images suggest that this may also be the case, although evidence is not clear. The analysis of the composition of microbiomes associated with the tuft and surroundings of the uropygial gland indicate that some taxa are typical of this environment for particular hornbill species or groups, and, therefore, may be specialized symbionts that live around or even within the uropygial glands. All these results agree with our hypothesis that the order *Bucerotiformes*, sister group of the order *Upupiformes* in which a tight association between the uropygial glands of the birds and mutualistic bacteria living within that have widely been demonstrated, may show similar associations. Below we discuss in detail the strength of our findings and the implications of the associations with particular taxa for understanding the evolutionary ecology of hornbill-bacteria relationships.

A main limitation of our study is the low sample size obtained for some species and body parts. This probably has made it impossible to detect associations with particular taxa in the hornbill species with the smaller samples size, and this will require further research when more samples are available. However, we have taken all the precautions to be sure that the relationships detected are well supported, and, therefore, we have restricted some analyses to species, genera or lineages with sufficient samples available.

An important methodological question to highlight is that this study only has the objective of testing the presence of symbiotic bacteria in uropygial glands of this group of birds, and the taxonomic range of such relationships. Since we are not testing any evolutionary hypothesis, we have not used comparative methods controlling for the phylogenetic relationships among hornbills species used.

Our main result is that several species of hornbills have abundant bacteria in the secretions extracted from within the gland. This result is based both on culture and visualization of secretions in a microscope, and, therefore, it has similar support as that for the single bird species in which this kind of relationship has more thoroughly been studied: the hoopoe (Soler et al., 2008; Rodríguez-Ruano et al., 2018). Other recent studies have obtained bacterial growth from uropygial secretion samples of different groups of birds, but have not examined secretions in a microscope, and, therefore, the extent to which such bacteria occupy the secretions has not been elucidated (Braun et al., 2016; Braun et al., 2018b; Braun et al., 2018c; Braun et al., 2019a; Braun et al., 2019b). To the best of our knowledge, the uropygial glands of hornbills do not have enlarged ampullae used to store secretions (Jacob and Ziswiler, 1982), as is the case in the hoopoe, where the secretions accumulate and the symbiotic bacteria proliferate (Fig. 1.l, Soler et al., 2008). In hoopoes, the bacteria do not reach the internal ducts of secretory lobes (unpublished data), but at this moment we cannot be sure of their particular location in the glands of hornbills, which needs an anatomical approach using fixed glands of dead specimens to stain bacteria within their natural position.

The two species with stronger evidence of abundant bacteria living within the secretions are *Bucorvus leadbeateri* and *Bycanistes brevis*, both with more than 50% of secretions producing bacterial growth and two out of three microscopic preparations

showing abundant bacterial cells stained throughout the secretions. Indeed, growth was prevalent in all species of the African lineage except *Ceratogymna atrata*, which also presented low densities of bacteria in microscopic images. Despite several of these species samples sizes being low and microscopic images unavailable, the general pattern is for African species showing more frequent evidence of presence of bacteria in secretions than Asian species. This result should be taken with caution given the smaller sample sizes for Asian species, although for three out of four species of this lineage there was no growth when culturing secretions, a fact that never occurred in the African species. This result is contrary to that expected by our hypothesis of colored secretions of Asian hornbills depending on bacteria hosted in their glands. Nevertheless, many bacteria are uncultivable in the conditions used (Baumann and Moran, 1997) and other approaches, specially sequencing of bacterial DNA present in secretions, are needed to have a complete knowledge of the composition of the entire microbiomes present in these environments. For example, the use of ARISA and high throughput sequencing methods (Pyrosequencing and Illumina) has shown that the secretions of hoopoes, in which only *enterococci* are detected by culture methods (Soler et al., 2008; Ruiz-Rodríguez et al., 2012), is instead occupied by a complex community of many different taxa (Rodríguez-Ruano et al., 2015; Martínez-García et al., 2016; Martín-Vivaldi et al., 2017; Rodríguez-Ruano et al., 2018).

Interestingly, when we applied sequencing methods to the samples taken from different body parts in hornbills, we found that in the two species/genera with stronger evidence for the presence of bacteria inside the uropygial gland, the GNEISS analysis identified particular taxa associated with samples of this organ. In *Bucorvus leadbeateri* (as well as in the whole genus *Bucorvus*), four *Actinobacteria* sOTUs were significantly

associated with the beak base, casque and uropygial gland in comparison to the remaining body parts, and among them, a *Propionibacterium* (*Propionibacteraceae*) was especially abundant in comparison with the other three unidentified *Corynebacteraceae* in the uropygial gland. An unidentified *Cardiobacteriaceae* (P.*Proteobacteria*) was also typical of the uropygial gland and casque. It is interesting that most of these sOTUs associated with the uropygial gland and casque are *Corynebacteriaceae*, and, therefore, of the same family (we do not know whether of the same genus) of the symbionts found in the uropygial glands of turkeys and Egyptian geese (Braun et al., 2016; Braun et al., 2018b). These bacteria are *Actynomycetales*, a group well known for their ability to produce antibiotics (Goodfellow and Cross, 1984) and which are usually involved in protective associations with animals such as fungus growing ants (Barke et al., 2010; Cafaro et al., 2011) and other groups (Piel, 2004; Florez et al., 2015). *Corynebacterium* species was previously isolated from cloacae and other body parts in several bird species (reviewed in Braun et al., 2016) and several unidentified *Actinomycetales* have also been detected in the community present within hoopoe uropygial glands (Rodríguez-Ruano et al., 2015; Rodríguez-Ruano et al., 2018). Cardiobacteraceae are a group of facultatively anaerobic *Gammaproteobacteria* with several species involved in pericardium and pulmonary diseases in man and other animals including birds, and apparently uncommon in non-clinical samples (Moore et al., 2014) and, therefore, we cannot evaluate the explanation for its association with glands in this species.

In the case of the hornbill genus *Bycanistes*, the bacterial taxa associated with the uropygial gland was *Enterococcus casseliflavus*. *Enterococci* are the more studied mutualists present in *Upupiformes* uropygial glands (Law-Brown and Meyers, 2003; Martin-Platero et al., 2006; Soler et al., 2008; Ruiz-Rodríguez et al., 2012; Rodríguez-

Ruano et al., 2018). Moreover they have known ability to produce antimicrobials (Martin-Platero et al., 2006; Ruiz-Rodríguez et al., 2012; Ruiz-Rodríguez et al., 2013) and demonstrated beneficial effects on hosts (Soler et al., 2008; Ruiz-Rodríguez et al., 2009; Martín-Vivaldi et al., 2014). Therefore, it is also a prime candidate to maintain a similar relationship in hornbill glands. We cannot be sure of its role with the evidence available, but undoubtedly is a case that deserves further research.

Finally, the glands of *Buceros rhinoceros* were clearly associated with a group of *Rhodobacteraceae* and other taxa in comparison with other species and body parts. Despite such associations occurring with most individuals of the species sampled, it was the case only for those housed in Beauval Zoo. Although this could indicate an effect of zoo, we have made special analytic efforts to discern this possibility, and can assert that, at least in Beauval, such taxa are clearly linked to uropygial glands of this species and body parts stained with its coloured uropygial secretion. This is an interesting result for two reasons. First, *Buceros rhinoceros* is an Asian species with cosmetic coloration caused by yellow-orange uropygial secretions, and is also the Asian hornbill species in which we have found higher prevalence (three out of three) of bacterial growth from inoculated secretions. All this evidence suggests that there are special bacteria linked to colored uropygial secretions in this species, as we hypothesized for Asian hornbills. *Rhodobacteraceae* is one of the more diverse family of the domain Bacteria, with a variety of ecologies and physiological capabilities, but with the particularity of frequent yellow-orange-pink pigmentation, which occurs not only inphotosynthetic members (that present Bchl a and carotenoids of spheroidene class), but also in non-phototrophic members (Pujalte et al., 2014). Of the three genera found in hornbills, both *Paracoccus* (nine sOTUs detected) and *Rubellimicrobium* (one sOTU) are known producers of carotenoids

(Pujalte et al., 2014). Although most *Rhodobacteraceae* inhabit marine environments, there are known cases of symbioses with marine invertebrates and rhizosphere of plants, and, therefore, they may have evolved a relationship with hornbills. This relationship is very suggestive, since these yellow-red bacteria were found in yellow-orange secretions and stains. At this point we can only speculate about the possibility that such bacteria are involved in the production of the secretion color which cannot be discarded. We have no evidence that birds in this zoological parkare provided with colored soil or special carotenoid enriched food sources or other artificial ways by which such colored bacteria may have been incorporated by birds, but the particular location suggests is closely interrelated with the uropygial secretion.

In summary, hornbills maintain an association with bacteria in their uropygial glands, which contain several taxa of bacteria which may provide birds with advantages in their fight against parasites-pathogens, or even provide substances used for their particular cosmetic ornaments. This is only a preliminary survey, but the results obtained are very important because they show that this system is a promising field of study for better understanding the evolutionary ecology of bird-bacteria symbioses.

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References

Amat, J. A., M. A. Rendón, J. Garrido-Fernández, A. Garrido, M. Rendón-Martos, and A. Pérez-Gálvez. 2011. Greater flamingos Phoenicopterus roseus use uropygial secretions as make-up. Behavioral Ecology and Sociobiology 65(4):665-673. doi: 10.1007/s00265-010-1068-z

Amir, A., D. McDonald, J. A. Navas-Molina, E. Kopylova, J. T. Morton, Z. Zech Xu, E. P. Kightley, L. R. Thompson, E. R. Hyde, A. Gonzalez, and R. Knight. 2017. Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. mSystems 2(2):e00191-00116. doi: 10.1128/mSystems.00191-16

Barbieri, E., K. Barry, A. Child, and N. Wainwright. 1997. Antimicrobial activity in the microbial community of the accessory nidamental gland and egg cases of Loligo pealei (Cephalopoda: Loliginidae). Biological Bulletin 193(2):275-276.

Barke, J., R. Seipke, S. Gruschow, D. Heavens, N. Drou, M. Bibb, R. Goss, D. Yu, and M. Hutchings. 2010. A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant Acromyrmex octospinosus. Bmc Biology 8(1):109.

Baumann, P., and N. A. Moran. 1997. Non-cultivable microorganisms from symbiotic associations of insects and other hosts. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 72(1):39-48.

Bolyen, E., J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet, G. A. Al-Ghalith, H. Alexander, E. J. Alm, M. Arumugam, F. Asnicar, Y. Bai, J. E. Bisanz, K. Bittinger, A. Brejnrod, C. J. Brislawn, C. T. Brown, B. J. Callahan, A. M. Caraballo-Rodríguez, J. Chase, E. K. Cope, R. Da Silva, C. Diener, P. C. Dorrestein, G. M. Douglas, D. M. Durall, C. Duvallet, C. F. Edwardson, M. Ernst, M. Estaki, J. Fouquier, J. M. Gauglitz, S. M. Gibbons, D. L. Gibson, A. Gonzalez, K. Gorlick, J. Guo, B. Hillmann, S. Holmes, H. Holste, C. Huttenhower, G. A. Huttley, S. Janssen, A. K. Jarmusch, L. Jiang, B. D. Kaehler, K. B. Kang, C. R. Keefe, P. Keim, S. T. Kelley, D. Knights, I. Koester, T. Kosciolek, J. Kreps, M. G. I. Langille, J. Lee, R. Ley, Y.-X. Liu, E. Loftfield, C. Lozupone, M. Maher, C. Marotz, B. D. Martin, D. McDonald, L. J. McIver, A. V. Melnik, J. L. Metcalf, S. C. Morgan, J. T. Morton, A. T. Naimey, J. A. Navas-Molina, L. F. Nothias, S. B. Orchanian, T. Pearson, S. L. Peoples, D. Petras, M. L. Preuss, E. Pruesse, L. B. Rasmussen, A. Rivers, M. S. Robeson, P. Rosenthal, N. Segata, M. Shaffer, A. Shiffer, R. Sinha, S. J. Song, J. R. Spear, A. D. Swafford, L. R. Thompson, P. J. Torres, P. Trinh, A. Tripathi, P. J. Turnbaugh, S. Ul-Hasan, J. J. J. van der Hooft, F. Vargas, Y. Vázquez-Baeza, E. Vogtmann, M. von Hippel, W. Walters, Y. Wan, M. Wang, J. Warren, K. C. Weber, C. H. D. Williamson, A. D. Willis, Z. Z. Xu, J. R. Zaneveld, Y. Zhang, Q. Zhu, R. Knight, and J. G. Caporaso. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 37(8):852-857. doi: 10.1038/s41587-019-0209-9

Braun, M., F. Sporer, S. Zimmermann, and M. Wink. 2018a. Birds, feather-degrading bacteria and preen glands: The antimicrobial activity of preen gland secretions from turkeys (Meleagris gallopavo) is amplified by keratinase. FEMS microbiology ecology 94doi: 10.1093/femsec/fiy117

Braun, M., E. Wang, S. Zimmermann, and M. Wink. 2018b. Corynebacterium heidelbergense sp. nov., isolated from the preen glands of Egyptian geese (Alopochen aegyptiacus). Systematic and Applied Microbiology 41doi: 10.1016/j.syapm.2018.06.002

Braun, M. S., E. Wang, S. Zimmermann, S. Boutin, H. Wagner, and M. Wink. 2019a. Kocuria tytonicola, new bacteria from the preen glands of American barn owls (Tyto furcata). Systematic and Applied Microbiology 42(2):198-204. doi: https://doi.org/10.1016/j.syapm.2018.11.004

Braun, M. S., E. Wang, S. Zimmermann, S. Boutin, and M. Wink. 2018c. Kocuria uropygioeca sp. nov. and Kocuria uropygialis sp. nov., isolated from the preen glands of Great Spotted Woodpeckers (Dendrocopos major). Systematic and Applied Microbiology 41(1):38-43. doi: https://doi.org/10.1016/j.syapm.2017.09.005

Braun, M. S., E. Wang, S. Zimmermann, H. Wagner, and M. Wink. 2019b. Kocuria tytonis sp. nov., isolated from the uropygial gland of an American barn owl (Tyto furcata). International Journal of Systematic and Evolutionary Microbiology 69(2):447-451. doi: https://doi.org/10.1099/ijsem.0.003170

Braun, M. S., S. Zimmermann, M. Danner, H. o. Rashid, and M. Wink. 2016. *Corynebacterium uropygiale* sp. nov., isolated from the preen gland of Turkeys (*Meleagris gallopavo*). Systematic and Applied Microbiology 39(2):88-92.

Burger, B. V., B. Reiter, O. Borzyk, and M. A. Du Plessis. 2004. Avian exocrine secretions. I. Chemical characterization of the volatile fraction of the uropygial secretion of the green woodhoopoe, *Phoeniculus purpureus*. Journal of Chemical Ecology 30(8):1603-1611.

Cafaro, M. J., M. Poulsen, A. E. F. Little, S. L. Price, N. M. Gerardo, B. Wong, A. E. Stuart, B. Larget, P. Abbot, and C. R. Currie. 2011. Specificity in the symbiotic association between fungus-growing ants and protective Pseudonocardia bacteria. Proceedings of the Royal Society B-Biological Sciences 278(1713):1814-1822.

Caruso, V., X. Song, M. Asquith, and L. Karstens. 2019. Performance of Microbiome Sequence Inference Methods in Environments with Varying Biomass. mSystems 4(1):e00163-00118. doi: 10.1128/mSystems.00163-18

Chau, R., J. A. Kalaitzis, and B. A. Neilan. 2011. On the origins and biosynthesis of tetrodotoxin. Aquatic Toxicology 104(1):61-72. doi: https://doi.org/10.1016/j.aquatox.2011.04.001

Currie, C. R., M. Poulsen, J. Mendenhall, J. J. Boomsma, and J. Billen. 2006. Coevolved Crypts and Exocrine Glands Support Mutualistic Bacteria in Fungus-Growing Ants. Science 311:81-83.

Currie, C. R., J. A. Scott, R. C. Summerbell, and D. Malloch. 1999. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398:701-704.

Davis, N. M., D. M. Proctor, S. P. Holmes, D. A. Relman, and B. J. Callahan. 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 6(1):226. doi: 10.1186/s40168-018-0605-2

de Goffau, M. C., S. Lager, S. J. Salter, J. Wagner, A. Kronbichler, D. S. Charnock-Jones, S. J. Peacock, G. C. S. Smith, and J. Parkhill. 2018. Recognizing the reagent microbiome. Nature Microbiology 3(8):851-853. doi: 10.1038/s41564-018-0202-y

Delhey, K., A. Peters, and B. Kempenaers. 2007. Cosmetic Coloration in Birds: Occurrence, Function, and Evolution. The American Naturalist 169(S1):S145-S158.

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Applied and Environmental Microbiology 72(7):5069-5072.

Dunlap, P. V., J. C. Ast, S. Kimura, A. Fukui, T. Yoshino, and H. Endo. 2007. Phylogenetic analysis of host-symbiont specificity and codivergence in bioluminescent symbioses. Cladistics 23(5):507-532.

Edgar, R. C. 2016. UNCROSS: Filtering of high-frequency cross-talk in 16S amplicon reads. bioRxiv:088666. doi: 10.1101/088666

Eisenhofer, R., J. J. Minich, C. Marotz, A. Cooper, R. Knight, and L. S. Weyrich. 2019. Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. Trends in Microbiology 27(2):105-117. doi: https://doi.org/10.1016/j.tim.2018.11.003

Ezenwa, V. O., and A. E. Williams. 2014. Microbes and animal olfactory communication: Where do we go from here? BioEssays 36(9):847-854. doi: 10.1002/bies.201400016

Florez, L. V., P. H. W. Biedermann, T. Engl, and M. Kaltenpoth. 2015. Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. Natural Product Reports 32(7):904-936.

Gamble, K. C. 2012. Chapter 37 - Squamous Cell Carcinoma in Buceros Hornbills. In: R. E. Miller and M. Fowler, editors, Fowler's Zoo and Wild Animal Medicine. W.B. Saunders, Saint Louis. p. 281-285.

Gonzalez, J. C. T., B. C. Sheldon, N. J. Collar, and J. A. Tobias. 2013. A comprehensive molecular phylogeny for the hornbills (Aves: Bucerotidae) (vol 67, pg 468, 2013). Molecular phylogenetics and evolution 68(3):715-715.

Goodfellow, M., and T. Cross. 1984. The Biology of Actinomycetes. Academic Press, London.

Hackett, S. J., R. T. Kimball, S. Reddy, R. C. K. Bowie, E. L. Braun, M. J. Braun, J. L. Chojnowski, W. A. Cox, K. L. Han, J. Harshman, C. J. Huddleston, B. D. Marks, K. J. Miglia, W. S. Moore, F. H. Sheldon, D. W. Steadman, C. C. Witt, and T. Yuri. 2008. A Phylogenomic Study of Birds Reveals Their Evolutionary History. Science 320(5884):1763-1768.

Herring, P. J. 2007. Sex with the lights on? A review of bioluminescent sexual dimorphism in the sea. Journal of the Marine Biological Association of the United Kingdom 87(4):829-842.

Hornung, B. V. H., R. D. Zwittink, and E. J. Kuijper. 2019. Issues and current standards of controls in microbiome research. FEMS Microbiology Ecology 95(5)doi: 10.1093/femsec/fiz045

Hwang, D. F., O. Arakawa, T. Saito, T. Noguchi, U. Simidu, K. Tsukamoto, Y. Shida, and K. Hashimoto. 1989. Tetrodotoxin-producing bacteria from the blue-ringed octopus Octopus maculosus. Marine Biology 100(3):327-332. doi: 10.1007/BF00391147

Jacob, J., and V. Ziswiler. 1982. The uropygial gland, Avian Biology No. VI. Academic Press, London. p. 199-324.

Janssen, S., D. McDonald, A. Gonzalez, J. A. Navas-Molina, L. Jiang, Z. Z. Xu, K. Winker, D. M. Kado, E. Orwoll, M. Manary, S. Mirarab, and R. Knight. 2018. Phylogenetic Placement of Exact Amplicon Sequences Improves Associations with Clinical Information. mSystems 3(3):e00021-00018. doi: 10.1128/mSystems.00021-18

Jervis-Bardy, J., L. E. Leong, S. Marri, R. J. Smith, J. M. Choo, H. C. Smith-Vaughan, E. Nosworthy, P. S. Morris, S. O'Leary, and G. B. Rogers. 2015. Deriving accurate microbiota profiles from human samples with low bacterial content through post-

sequencing processing of Illumina MiSeq data. Microbiome 3doi: 10.1186/s40168-015-0083-8

Kaltenpoth, M., W. Gottler, G. Herzner, and E. Strohm. 2005. Symbiotic bacteria protect wasp larvae from fungal infestation. Current Biology 15(5):475-479.

Kaltenpoth, M., and E. Strohm. 2007. Life within insect antennae: Symbiotic bacteria protect wasp larvae against fungal infestation. Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology 146(4):S66-S67.

Karstens, L., M. Asquith, S. Davin, D. Fair, W. T. Gregory, A. J. Wolfe, J. Braun, and S. McWeeney. 2019. Controlling for Contaminants in Low-Biomass 16S rRNA Gene Sequencing Experiments. mSystems 4(4):e00290-00219. doi: 10.1128/mSystems.00290-19

Kemp, A. C. 2001. Family Bucerotidae (hornbills). In: J. del Hoyo, A. Elliot and J. Sargatal, editors, Handbook of the birds of the world. Vol 6. Mousebirds to hornbills. Lynx Edicions, Barcelona. p. 436-523.

Kimbell, J. R., and M. J. McFall-Ngai. 2003. The squid-vibrio symbioses: From demes to genes. Integrative and Comparative Biology 43(2):254-260.

Lago, J., L. P. Rodríguez, L. Blanco, J. M. Vieites, and A. G. Cabado. 2015. Tetrodotoxin, an Extremely Potent Marine Neurotoxin: Distribution, Toxicity, Origin and Therapeutical Uses. Mar Drugs 13(10):6384-6406. doi: 10.3390/md13106384

Larsson, A. J. M., Stanley, G., Sinha, R., Weissman, I. L. and Sandberg, R. 2018. Computational correction of index switching in multiplexed sequencing libraries. - Nat. Methods 15: 305–307.

Law-Brown, J. 2001. Chemical defence in the red-billed woodhoopoe, *Phoeniculus purpureus*, University of Cape Town.

Law-Brown, J., and P. R. Meyers. 2003. *Enterococcus phoeniculicola* sp nov., a novel member of the *enterococci* isolated from the uropygial gland of the Red-billed Woodhoopoe, *Phoeniculus purpureus* International Journal of Systematic and Evolutionary Microbiology 53:683-685.

Leclaire, S., S. Jacob, L. K. Greene, G. R. Dubay, and C. M. Drea. 2017. Social odours covary with bacterial community in the anal secretions of wild meerkats. Scientific Reports 7(1):3240. doi: 10.1038/s41598-017-03356-x

Little, A. E. F., T. Murakami, U. G. Mueller, and C. R. Currie. 2006. Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. Biology Letters 2(1):12-16.

Lozupone, C., and R. Knight. 2005. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. Applied and Environmental Microbiology 71(12):8228-8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Martin-Platero, A. M., E. Valdivia, M. Ruiz-Rodriguez, J. J. Soler, M. Martin-Vivaldi, M. Maqueda, and M. Martinez-Bueno. 2006. Characterization of antimicrobial substances produced by *Enterococcus faecalis* MRR 10-3, isolated from the uropygial gland of the hoopoe (*Upupa epops*). Applied and Environmental Microbiology 72(6):4245-4249.

Martín-Vivaldi, M., A. Peña, J. M. Peralta-Sánchez, L. Sánchez, S. Ananou, M. Ruiz-Rodríguez, and J. J. Soler. 2010. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. Proceedings of the Royal Society of London, Series B Biological Sciences 277(1678):123-130.

Martín-Vivaldi, M., M. Ruiz-Rodríguez, J. J. Soler, J. M. Peralta-Sánchez, M. Méndez, E. Valdivia, A. M. Martín-Platero, and M. Martínez-Bueno. 2009. Seasonal, sexual and developmental differences in hoopoe preen gland morphology and secretions. Evidence for a role of bacteria. Journal of Avian Biology 40:191-205.

Martín-Vivaldi, M., J. J. Soler, Á. Martínez-García, L. Arco, N. Juárez-García-Pelayo, M. Ruiz-Rodríguez, and M. Martínez-Bueno. 2018. Acquisition of Uropygial Gland Microbiome by Hoopoe Nestlings. Microbial Ecology doi: 10.1007/s00248-017-1125-5

Martín-Vivaldi, M., J. J. Soler, J. M. Peralta-Sánchez, L. Arco, A. M. Martín-Platero, M. Martínez-Bueno, M. Ruiz-Rodríguez, and E. Valdivia. 2014. Special structures of hoopoe eggshells enhance the adhesion of symbiont-carrying uropygial secretion that increase hatching success. Journal of Animal Ecology 83(6):1289-1301.

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200

Martínez-García, A., M. Martín-Vivaldi, M. Ruiz-Rodríguez, M. Martínez-Bueno, L. Arco, S. Rodríguez-Ruano, J. M. Peralta-Sánchez, and J. J. Soler. 2016. The

microbiome of the uropygial secretion in hoopoes is shaped along the nesting phase. Microbial Ecology:1-10.

McFall-Ngai, M., M. G. Hadfield, T. C. G. Bosch, H. V. Carey, T. Domazet-Lošo, A. E. Douglas, N. Dubilier, G. Eberl, T. Fukami, S. F. Gilbert, U. Hentschel, N. King, S. Kjelleberg, A. H. Knoll, N. Kremer, S. K. Mazmanian, J. L. Metcalf, K. Nealson, N. E. Pierce, J. F. Rawls, A. Reid, E. G. Ruby, M. Rumpho, J. G. Sanders, D. Tautz, and J. J. Wernegreen. 2013. Animals in a bacterial world, a new imperative for the life sciences. Proceedings of the National Academy of Sciences 110(9):3229. (10.1073/pnas.1218525110)

McKnight, D. T., R. Huerlimann, D. S. Bower, L. Schwarzkopf, R. A. Alford, and K. R. Zenger. 2019. microDecon: A highly accurate read-subtraction tool for the post-sequencing removal of contamination in metabarcoding studies. Environmental DNA 1(1):14-25. doi: 10.1002/edn3.11

Minich, J. J., Q. Zhu, S. Janssen, R. Hendrickson, A. Amir, R. Vetter, J. Hyde, M. M. Doty, K. Stillwell, J. Benardini, J. H. Kim, E. E. Allen, K. Venkateswaran, and R. Knight. 2018. KatharoSeq Enables High-Throughput Microbiome Analysis from Low-Biomass Samples. mSystems 3(3):e00218-00217. doi: 10.1128/mSystems.00218-17

Moore, E. R. B., S. A. Mihaylova, and M. Gomila. 2014. The Family Cardiobacteriaceae. In: E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt and F. Thompson, editors, The Prokaryotes: Gammaproteobacteria. Springer Berlin Heidelberg, Berlin, Heidelberg. p. 135-146.

Morton, J. T., J. Sanders, R. A. Quinn, D. McDonald, A. Gonzalez, Y. Vázquez-Baeza, J. A. Navas-Molina, S. J. Song, J. L. Metcalf, E. R. Hyde, M. Lladser, P. C. Dorrestein, and R. Knight. 2017. Balance Trees Reveal Microbial Niche Differentiation. mSystems 2(1):e00162-00116. doi: 10.1128/mSystems.00162-16

Nyholm, S. V., and M. J. McFall-Ngai. 1998. Sampling the light-organ microenvironment of Euprymna scolopes: Description of a population of host cells in association with the bacterial symbiont Vibrio fischeri. Biological Bulletin 195(2):89-97.

Piel, J. 2004. Metabolites from symbiotic bacteria. Natural Product Reports 21(4):519-538.

Poonswad, P., A. Kemp, and M. Strange. 2013. Hornbills of the world: A photographic guide. Draco Publishing.

Pujalte, M. J., T. Lucena, M. A. Ruvira, D. R. Arahal, and M. C. Macián. 2014. The Family Rhodobacteraceae. In: E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt and F. Thompson, editors, The Prokaryotes: Alphaproteobacteria and Betaproteobacteria. Springer Berlin Heidelberg, Berlin, Heidelberg. p. 439-512.

Rodríguez-Ruano, S. M., M. Martín-Vivaldi, A. M. Martín-Platero, J. P. López-López, J. M. Peralta-Sánchez, M. Ruiz-Rodríguez, J. J. Soler, E. Valdivia, and M. Martínez-Bueno. 2015. The hoopoe's uropygial gland hosts a bacterial community influenced by the living conditions of the bird. Plos One 10(10)

Rodríguez-Ruano, S. M., M. Martín-Vivaldi, J. M. Peralta-Sánchez, A. B. García-Martín, Á. Martínez-García, J. J. Soler, E. Valdivia, and M. Martínez-Bueno. 2018. Seasonal and Sexual Differences in the Microbiota of the Hoopoe Uropygial Secretion. Genes 9(8):407. doi: 10.3390/genes9080407

Rognes, T., T. Flouri, B. Nichols, C. Quince, and F. Mahé. 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4:e2584.

Ruiz-Rodríguez, M., M. Martínez-Bueno, M. Martín-Vivaldi, E. Valdivia, and J. J. Soler. 2013. Bacteriocins with a broader antimicrobial spectrum prevail in enterococcal symbionts isolated from the hoopoe's uropygial gland. Fems Microbiology Ecology 85(3):495-502.

Ruiz-Rodríguez, M., E. Valdivia, M. Martín-Vivaldi, A. M. Martín-Platero, M. Martínez-Bueno, M. Méndez, J. M. Peralta-Sánchez, and J. J. Soler. 2012. Antimicrobial activity and genetic profile of enteroccoci isolated from hoopoes uropygial gland. Plos One 7 e41843, 41841-41846.

Ruiz-Rodríguez, M., E. Valdivia, J. J. Soler, M. Martín-Vivaldi, A. M. Martín-Platero, and M. Martínez-Bueno. 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. Journal of Experimental Biology 212(22):3621-3626.

Schwartzman, J. A., E. Koch, E. A. C. Heath-Heckman, L. Zhou, N. Kremer, M. J. McFall-Ngai, and E. G. Ruby. 2015. The chemistry of negotiation: Rhythmic, glycan-driven acidification in a symbiotic conversation. Proceedings of the National Academy of Sciences 112(2):566-571.

Sibley, C. G., and J. E. Ahlquist. 1990. Phylogeny and classification of birds. Yale University Press, New Haven.

Soini, H. A., D. J. Whittaker, D. Wiesler, E. D. Ketterson, and M. V. Novotny. 2013. Chemosignaling diversity in songbirds: Chromatographic profiling of preen oil volatiles in different species. Journal of Chromatography A 1317:186-192.

Soler, J. J., M. Martín-Vivaldi, J. M. Peralta-Sánchez, L. Arco, and N. Juárez-García-Pelayo. 2014. Hoopoes color their eggs with antimicrobial uropygial secretions. Naturwissenschaften 101(9):697-705.

Soler, J. J., M. Martín-Vivaldi, J. M. Peralta-Sánchez, and M. Ruiz-Rodríguez. 2010. Antibiotic-producing bacteria as a possible defence of birds against pathogenic microorganisms. Open Ornithology Journal 3:93-100.

Soler, J. J., M. Martín-Vivaldi, M. Ruiz-Rodríguez, E. Valdivia, A. M. Martín-Platero, M. Martínez-Bueno, J. M. Peralta-Sánchez, and M. Méndez. 2008. Symbiotic association between hoopoes and antibiotic-producing bacteria that live in their uropygial gland. Functional Ecology 22(5):864-871.

Sorgo, A., F. Gaill, J. P. Lechaire, C. Arndt, and M. Bright. 2002. Glycogen storage in the *Riftia pachyptila* trophosome: contribution of host and symbionts. Marine Ecology-Progress Series 231:115-120.

Theis, K. R., A. Venkataraman, J. A. Dycus, K. D. Koonter, E. N. Schmitt-Matzen, A. P. Wagner, K. E. Holekamp, and T. M. Schmidt. 2013. Symbiotic bacteria appear to mediate hyena social odors. Proceedings of the National Academy of Sciences 110(49):19832. doi: 10.1073/pnas.1306477110

Wang, B., L. Wan, A. Wang, and L. M. Li. 2017. An adaptive decorrelation method removes Illumina DNA base-calling errors caused by crosstalk between adjacent clusters. Scientific Reports 7(1):41348. doi: 10.1038/srep41348

Whittaker, D. J., K. M. Richmond, A. K. Miller, R. Kiley, C. B. Burns, J. W. Atwell, and E. D. Ketterson. 2011. Intraspecific preen oil odor preferences in dark-eyed juncos (*Junco hyemalis*). Behavioral ecology 22(6):1256-1263.

Whittaker, D. J., S. P. Slowinski, J. M. Greenberg, O. Alian, A. D. Winters, M. M. Ahmad, M. J. E. Burrell, H. A. Soini, M. V. Novotny, E. D. Ketterson, and K. R. Theis. 2019. Experimental evidence that symbiotic bacteria produce chemical cues in a songbird. The Journal of Experimental Biology 222(20):jeb202978. doi: 10.1242/jeb.202978

Whittaker, D. J., H. A. Soini, J. W. Atwell, C. Hollars, M. V. Novotny, and E. D. Ketterson. 2010. Songbird chemosignals: volatile compounds in preen gland

secretions vary among individuals, sexes, and populations. Behavioral ecology 21(3):608-614.

Whittaker, D. J., and K. R. Theis. 2016. Bacterial Communities Associated with Junco Preen Glands: Preliminary Ramifications for Chemical Signaling. In: A. B. Schulte, E. T. Goodwin and H. M. Ferkin, editors, Chemical Signals in Vertebrates 13. Springer International Publishing, Cham. p. 105-117.

Widder, E. A. 2010. Bioluminescence in the Ocean: Origins of Biological, Chemical, and Ecological Diversity. Science 328(5979):704. doi: 10.1126/science.1174269

Wright, E. S., and K. H. Vetsigian. 2016. Quality filtering of Illumina index reads mitigates sample cross-talk. BMC Genomics 17(1):876. doi: 10.1186/s12864-016-3217-x

Zinter, M. S., M. Y. Mayday, K. K. Ryckman, L. L. Jelliffe-Pawlowski, and J. L. DeRisi. 2019. Towards precision quantification of contamination in metagenomic sequencing experiments. Microbiome 7(1):62. doi: 10.1186/s40168-019-0678-6

CAPÍTULO V

CAPÍTULO V

Colouration and bacterial load of uropygial secretion and hornbills' teguments

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Abstract

Ornamental colourations have frequently evolved as signals of individual quality. Different modes of producing colours transfer different messages. For instance, carotenoid-based pigments indicate access to limiting resources obtained in the diet, while easily degraded white feathered areas are handicaps, revealing resistance to feather degrading bacteria. Cosmetic colours may also indicate individual quality when the product staining the body reflects properties or abilities of the user. It might be the case of uropygial secretions of some hornbills (*Bucerotiformes*) species, which harbour symbiotic bacteria, are of flamboyant coloration, and are used to stain feathers and the beak, including the striking voluminous casque. Most species also present a contrasted design of white and black areas in their plumage, suggesting the evolution of multiple signals transferring messages related to their interaction with bacteria. We tested, through comparative methods, whether the white patches, and body areas coloured with carotenoids or/and cosmetic colouration may have evolved as signals of individual quality related to ability to cope with pathogenic bacteria in hornbills. To this end, we sampled cultivable bacteria present in the uropigial secretion and tegument (beak, casque and tuft of the uropygial gland) of 13 species of hornbills kept in captivity in different zoos across Europe and tested for associations between presence and extent of different kinds of colour signals, and presence and abundance of several types of

cultivable bacteria. Our main findings are that the degree of exaggeration of two ornaments: white plumage areas and yellow-orange-red beak surface colouration, are positively correlated across the phylogeny of the hornbills, and that variation in ornamentation among species is related to bacterial loads in different body parts. Moreover, the prevalence in the uropygial gland of a particular group of bacteria known for their production of bacteriocins (*enterococci*) was related to abundance of potential pathogenic (keratynolitic) bacteria on the casque. The results suggest that bacteria have played an important role in the evolution of the complex colour designs found in hornbills. Further studies with a longer list of species covering a wider phylogenetic range, as well as more individuals per species and in natural populations are needed for reaching strong conclusions.

INTRODUCTION

Sexual signals have evolved due to preferences by individuals of the choosy sex for individuals with the most elaborate ornaments because such signals reliably reveal the quality of the holder (Andersson 1994). In birds, songs and colouration are the most frequent sexual signals (Andersson 1994). Ornamental coloration can be obtained structurally or by the incorporation of pigments, such as melanins and carotenoids, into the tissue (Fox and Vevers 1960). Some pigments, such as melanins, can be synthesized by birds from basic biological precursors (Hill 2006). Conversely, carotenoids cannot be synthesized by animals and need to be incorporated through the diet (Olson and Owens 1998). Carotenoids are responsible for red, yellow and orange colours (Hill 2002). Several studies have supported the hypothesis that the access to carotenoids in the diet is reliably reflected by the expression of colour signals in

some species of vertebrates (reviewed in Hill 2006). That is because investment in carotenoid dependent colouration has physiological costs, since these compounds are a limiting and scarce resource in nature (Grether et al. 1999) and they are important for several biological functions (Olson and Owens 1998, Hill 2000, Amat et al. 2011). In this way, poorer colouration can reflect lower availability of carotenoids (Hill 2006). Thus, carotenoids based colouration can signal the access and ability to obtain nutritive resources, but also other quality traits, such as body condition, parental abilities (Massaro et al. 2003), antioxidant capacity (Olson and Owens 1998, Hill 2002, McGraw and Ardia 2003, Searcy and Nowicki 2005), parasite load (Hill 2002, reviewed in Hill 2006) and their capacity to fight against microbial infections (Hamilton and Zuk 1982, Hill et al. 2004, Ruiz-Rodríguez et al. 2020 under review). Therefore, only high quality individuals would be able to display more colourful structures (Zahavi and Zahavi 1997). Thus, differences in carotenoid based colouration between species could indicate fundamental differences in their efficiency of carotenoids use, and if they are metabolized or directly deposited in the tegument since carotenoid absorption, transport and deposition requires prime nutritional condition (Hill 2006). In addition to carotenoids, white plumage patches may play an important role in sexual signalling, being condition-dependent and a predictor of phenotypic quality (Doucet et al. 2004, reviewed in Prum 2006). That is because the presence of white feathers in the plumage can reflect conspecifics the handicap of having white feathers in good physical condition since they resist bacterial degradation worse than melanised ones (Goldstein et al. 2004, Azcárate-García et al. 2020 in press). Moreover, feather degradability is negatively related to individual quality (Ruiz-De-Castañeda et al. 2015).

The mode of deposition of pigments in body structures influences the message being transferred. Thus, carotenoids included in the construction of feather, would be a signal of the

availability of these nutrients at the time of feather construction, but not later. On the other hand, in species able to change colouration by staining some body parts with substances secreted daily (Mcgraw and Hill 2004), the signal would be more dynamic allowing updating information. That is the case for the application of uropygial gland secretions on feathers and other body structures (Uchida 1970, Montgomerie 2006, Delhey et al. 2007, Surmacki and Nowakowski 2007, Piault et al. 2008, López-Rull et al. 2010, Amat et al. 2011). This cosmetic colouration of uropygial secretions can be based on the presence of carotenoid pigments (Delhey et al. 2007) or symbiotic bacteria living within the uropygial gland (Soler et al. 2014). Bacteria living in the uropygial secretion might also produce antibiotics and, thus, when using that secretion as cosmetic, colouration might also inform of the antimicrobial producing capacity of symbionts. Indeed, some of the symbionts living in the uropigial glands of bird families of the order *Upupiformes* are direct producers of antimicrobial substances (Law-Brown and Meyers 2003, Martín-Platero et al. 2006, Ruiz-Rodríguez et al. 2013) and this property of the secretion could be signalled by their colour (Soler et al. 2014).

The order *Upupiformes* is the sister clade of the order *Bucerotiformes*, a group of 61 species of hornbills (Gonzalez et al. 2013). Both clades have colourful uropygial secretions that they use to pigment parts of their body (Kemp 2001, Poonswad et al. 2013, Martín-Vivaldi et al. 2014b, Naish 2015), which are possibly related to social signalling (Kemp 2001, Poonswad et al. 2013). Hornbills have a remarkable design, including wide white areas of the plumage contrasting against a general black colour, crests, large bills and specially a unique ornamental anatomical keratinized structure on the dorsal maxillary beak, the casque (Gamble 2007). In each species, the casque has a different shape, colour and size that progressively becomes enlarged with sexual maturity and age (Kemp 2001). It also develops differently in each sex, being generally larger and more elaborate in males. Thus, it can act as an indicator of maturity and it could have evolved by sexual selection (Naish 2015). Still, its function

remains unknown. Some hypothesis include structural beak reinforcement for pounding behaviour, vocalization enhancement through resonance, and visual cue of quality, social status or sexual maturity (Kemp 2001, Poonswad et al. 2013). However, different functions depending on the species cannot be ruled out (Gamble 2007). This structure is a common location for injuries, environmental damage, and diseases (Miller et al. 1985, Suedmeyer et al. 2001, Gamble 2007). Beak and casque colouration is highly variable among hornbill species. Many species have yellow, orange and red colours on beaks and casques as well as yellow-stained white patches on tail and wing feathers, which apparently could be linked to an ornamental role of uropygial secretions coloured by carotenoids. The advantage of cosmetically staining other body parts besides the feathers, such as the beak and the casque, is that they do not moult it so they can signal their quality during the whole year, and especially during the breeding season. Conversely, plumage colour changes at moult that occurs several months before the breeding season (Montgomerie 2006). Since the beak and the casque covers are made of keratin, their physical damage and colouration could signal individual quality related to ability to fight against keratinolytic bacteria (Gunderson 2008, Shawkey et al. 2009, Ruiz-de-Castañeda et al. 2012).

The colour of the uropygial secretion or stained plumage of hornbills may be due to carotenoids (Delhey *et al*. 2007) or other kinds of pigments contained in the uropygial gland; which may come directly from the frugivorous diet of some species (Viseshakul *et al.* 2011). There is evidence that hornbills can also have bacteria in their uropygial glands, and, therefore, these colours could also be dependent on these bacteria as in hoopoes (Chapter IV). Moreover, they are known for their "self-incarceration" strategy in which females remain inside the nest hole for long periods of time sealing the entrance and leaving only a small slit. Since hole nests may be old and hence accumulate microbes over years (Møller and Erritzoe

1996), hornbills could have selected for antimicrobial defences to protect themselves against parasites from the nest. Moreover, given the extreme dependence of females on male help during the breeding season, we should expect extremely demanding criteria for mate choice in females, which is paramount for their own survival (Kemp 2001; Poonswad et al. 2013).

More than a third of the species of hornbills are considered of interest for conservation worldwide, including 62% of Asian species, some of them are in danger of extinction (Gonzalez et al. 2013). For this reason, studies of the ecology and the conservation biology these species have increased recently (Gonzalez et al., 2013). However, more studies about their ecology and behaviour and their phylogeny and distribution are needed (Naish, 2015). Interestingly, captive breeding success is low for these species (Crofoot et al. 2003) maybe because of demanding criteria for mate choice in females. Since there are many differences between hornbill species in their ornamental characters, a comparative approach could reflect how these signals could have evolved inter-specifically.

Here, we tested whether the white/black colour designs, casque size and body areas yellow-orange-red coloured may have evolved as signals of individual quality related to ability to cope with pathogens in hornbills. To this end, we performed a comparative study of the possible evolution of these signals in hornbills linked to their interaction with bacteria. We used 13 hornbill species with available information on morphological and colour variables and bacterial load of different body parts. We predicted that (1) a positive relationship between colour surface (yellow-orange-red) of different body parts or casque size and extent of white plumage. (2) A positive relationship between the presence of coloured secretions and the surface area of red-orange-yellow on different body structures, if they stain their body with it. (3) A relationship between bacterial load of different body parts and number of yellow-orange-red coloured structures (body colouration) as a possible quality signal related

to ability to cope with pathogens. (4) Since some hornbill species have coloured uropygial secretions, we tested if, as in hoopoes, there is a relationship between coloured secretions and bacterial load and the feathers around the uropygial gland tuft. Finally, (5) we predicted a relationship between density of bacteria of uropygial gland tuft and bacteria of different body structures since they use these for staining their body. Both a positive relationship (because there are more bacteria in the gland there) and a negative one (if it eliminates other bacteria) is expected depending on the groups of bacteria.

MATERIAL AND METHODS

Study Species

Hornbills include 61 species of 15 genera, distributed throughout tropical Asia (32 species) and Sub-Saharan Africa (29 species) (Gonzalez et al. 2013). Little is known about them since they are elusive, making it difficult to study (Naish 2015). They are known for the cooperative breeding system in some species and for their "self-incarceration" strategy of all but the *Bucorvus* species, in which females remain in a cavity during the entire breeding period (between two and five months depending on the species) (Moreau 1934, Kemp 2001). Males seal the nest cavity entrance leaving only a small slit through which they feeds females until the nestlings have the appropriate size for fledging (Moreau 1934, Kemp 2001).

Asian hornbills, predominantly frugivorous, are considered key species in long-distance seed dispersal (Kemp 2001, Kinnaird and O'Brien 2007, Trail 2007). African hornbills, unlike Asian, are predominantly carnivorous. Due to their need for extensive foraging areas, they are especially sensitive to habitat loss and fragmentation, which together

with hunting and international trade (Trail 2007), makes them one of the most endangered group of species of tropical ecosystems (Kinnaird and O'Brien 2007).

Study Area

The study was carried out during 2015-2017 with 53 individuals of 13 species of hornbills from different zoological parks in Spain, Portugal and France. Table 1 shows species and number of individual of the species sampled in different zoos.

Sampling Procedure

To obtain samples of microbial communities from different body parts (see below), sterile cotton swabs (EUROTUBO® DeltaLab) previously moistened with sterile sodium phosphate buffer (PBS, 0.2 M; pH = 7.08) were used to gently swab a square of about 2x2 cm. Afterwards, the swab was introduced into a sterile microcentrifuge tube with phosphate buffer and transported to the laboratory in a portable refrigerator at 4–6°C. Samples were stored at 4°C until being processed within the next three days.

Each individual was sampled at the following different sites: (1) Uropygial gland tuft (in this case usually there was less than 4 cm^2 available, and, therefore, we sampled the whole tuft); (2) beak and (3) casque (on one of their sides at the middle of its length). In addition, whenever possible, we took samples of uropygial secretion in sterile capillary tubes. For this, we cleaned the tuft and surroundings of the gland pores with a cotton swab soaked in 96º ethanol, and gently pressed the gland ducts towards the openings taking the secretion with sterile capillary tubes. To evaluate the colour of secretions and possible body parts stained with it, i.e. whether the colour was cosmetic, all microcentrifuge tubes sampled per individual were photographed together over a gray card with a colour table (Fig. 1). We used disposable

latex gloves previously washed with ethanol 70% to prevent bacterial contamination among individuals.

Microbiological study

We evaluated bacterial loads of different body surface using culturing methods. To facilitate the transmission of bacteria from swabs to the phosphate buffer as well as its homogenization, the microcentrifuge tubes were gently vortexed. Afterwards, 100 µl of sample was spread onto different solid culture media (Scharlau Chemie S.A., Barcelona) depending on the body part (see below): Tryptone Soya Agar (TSA), a broadly used general medium to grow heterotrophic bacteria; Feather Medium Agar (FMA), a selective medium for keratinolytic bacteria; and Kenner Fecal Agar (KF), a selective medium for *Enterococcus*, and two specific media for potentially pathogenic bacteria: *Enterobacteriaceae* (Hektoen Enteric Agar, HK) and *Staphylococcus* (Vogel Johnson Agar, VJ). Uropygial gland tuft samples were spread onto all media, while beak samples onto TSA, KF, HK and VJ, and casque samples onto TSA and FMA media. That is because we expected that the casque was a quality signal (against degrading bacteria) and the beak an exposure area of all bacteria. The presence of cultivable bacteria in secretions was tested by inoculating plates with a TSA medium, by directly sinking or streaking the extreme of the capillary tubes used to take the secretion. In this case, growth was measured qualitatively as a dichotomic (presence/absence) variable reflecting whether there were bacterial colonies after incubation in aerobic conditions for 24 h at 37ºC.

Serial tenfold dilutions were used to reach the concentration in which colonies grew while spread sufficiently to be countable. However, the number of dilutions to perform was decided from previous experience, and in some cases was insufficient to obtain isolated colonies. In these cases, we assigned conservatively the highest value of CFU/ml (number of

colony forming units * $10^{\text{dilution factor}}$) / ml spread) of this type of sample and medium for the same dilution or the lowest value of CFU/ml of the following dilution of this sample and medium when it was available for other samples. CFU/ml averages and prevalence per species, sample and medium, were calculated.

Morphological and colour variables

The variables used in the analysis were (1) the proportion of coloured surface of the beak, (2) the proportion of coloured surface of the casque, (3) the sum of the proportions of coloured surfaces of the beak and the casque. Proportions of coloured surfaces were estimated between 0 and 1 for beak and casque based on the fraction of the total surface of the structure that was coloured. 0 when there were no yellow, orange and/or red colours on these structures and, 1 when it was completely coloured with some of these colours. (4) Casque size. Relative casque size estimates (in length and width) between 0 and 1 were taken in comparison to the size of the beak and the result of both variables multiplied. (5) Proportion of white feathers on the body with the wing extended. For this, the body was divided into 5 parts of approximately the same length, from the head to the tip of the tail: head and neck, upper wings and breast, lower wings and abdomen, wing base, and wing tip. The proportion of white surface of each part was added and the result was divided by 5. Thus, the score was 0 when there were no white feathers in their entire plumage and 1 when it was completely white. (6) Number of yellow-orange-red coloured surfaces (*total body colouration*). It referred to the number of structures (beak, casque, uropygial gland tuft and/or naked skin) coloured red, orange or yellow. When we had clear evidence that the uropygial secretion was coloured (the species has cosmetic colouration in the body) we took into account the uropygial gland as a pigmented ornamental trait summed as the previous variable. and (7) cosmetic uropygial colouration (yes or not). We considered it was cosmetic if the swab was coloured after taking

the simple (See pictures of microcentrifugue tubes (Fig. 1)). The information was extracted from pictures of Poonswad et al. (2013) and Kemp (2001). When some of these variables differed between sexes, we only took the most ornamented sex into account.

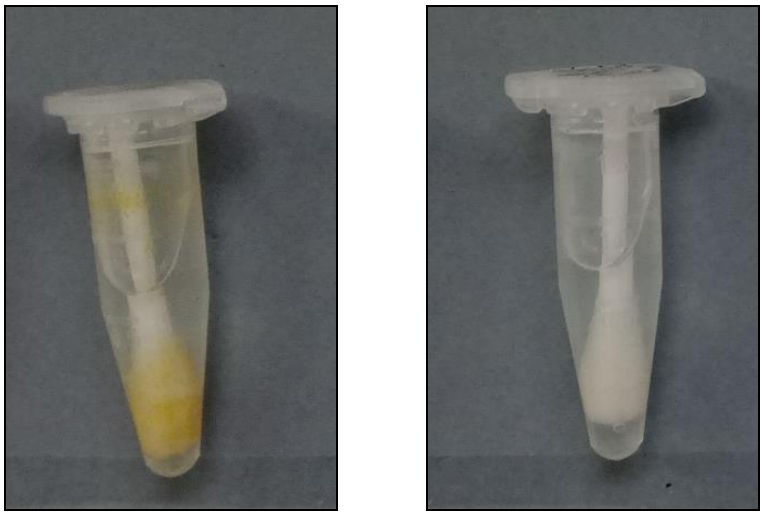

Figure 15. Pictures of two microcentrifugue tubes with the swab sampled inside. Example of a cosmetic coloured sample on the left (uropygial gland tuft sample from *Buceros rhinoceros.* Zoo Beauval) and one not coloured on the right (uropygial gland tuft sample from *Tockus deckeni.* Zoo Beauval).

Phylogenetic analysis

Bacterial load variables (CFU/ml) were log10 (x + 0.1) transformed except for KF, HK and VJ media in which, due to abundance of zeroes, prevalence for each species was used.

We used mean values of bacterial counts as species-specific character, since we found interspecific differences. For comparative analysis, we calculated the mean of each variable per species since we did not find differences between sexes in bacterial load (General Linear mixed model (GLMM), species as random. Casque, FMA medium: Sex: $F(1,17) = 1.65$, $P = 0.215$; TSA medium: Sex: $F(1,19) = 0.01$, $P = 0.943$. Beak, TSA medium: Sex: $F(1,35) = 0.30$, $P = 0.588$. Uropygial gland tuft: KF medium: Sex: $F(1,23) = 0.46$, $P = 0.505$).

Values of morphological and colour variables assigned to different species of birds, or bacterial load estimates of different body parts cannot be considered statistically independent information because species that are phylogenetically closely related due to common ancestry also have more similar traits (Harvey and Pagel 1991). Therefore, to control for possible effects of common ancestry, we performed phylogenetic general lineal models (PGLM) (Pagel 1997, 1999). PGLM is a linear regression model that incorporates phylogenetic information into the error term, controlling for the shared evolutionary history among species.

The best phylogenetic subset for tree constructions was obtained through Bird Tree tool (http://www.birdtree.org) (Jetz et al. 2012, Rubolini et al. 2015). It combines relaxed molecular clock trees of well-supported avian clades with a fossil calibrated backbone with representatives from each clade. 10,000 trees were downloaded with the 13 species of hornbills sampled in this study (Table 1). Then, a consensus tree was created through MESQUITE version 3.6 (Maddison and Maddison 2018) (Fig. 2).

Differences in sampling effort can be sources of bias because different estimates are not estimated with similar precision (Garamszegi and Møller 2010, 2011). Therefore, when the dependent variable had different sample size depending on the species, weighted PGLM models were analyzed in addition to unweighted ones, taking into account the number of individuals sampled by species. To select the best model between weighted and unweighted models we used the lower Akaike information corrected criterion values (AICc). Because all samples were not taking all species into account and were not processed in all media, sample size is different depending on the model considered.

To explore the possible covariation among ornaments, we performed analyses for all combinations of pairs of ornaments (total body colouration, colour of beak, size and colour of casque and proportion of white feathers on the whole body). The presence of coloured

secretions was used as a predictor when analysing its effect on the surface of red-orange-yellow on casque and/or beak (three different PGLM analyses).

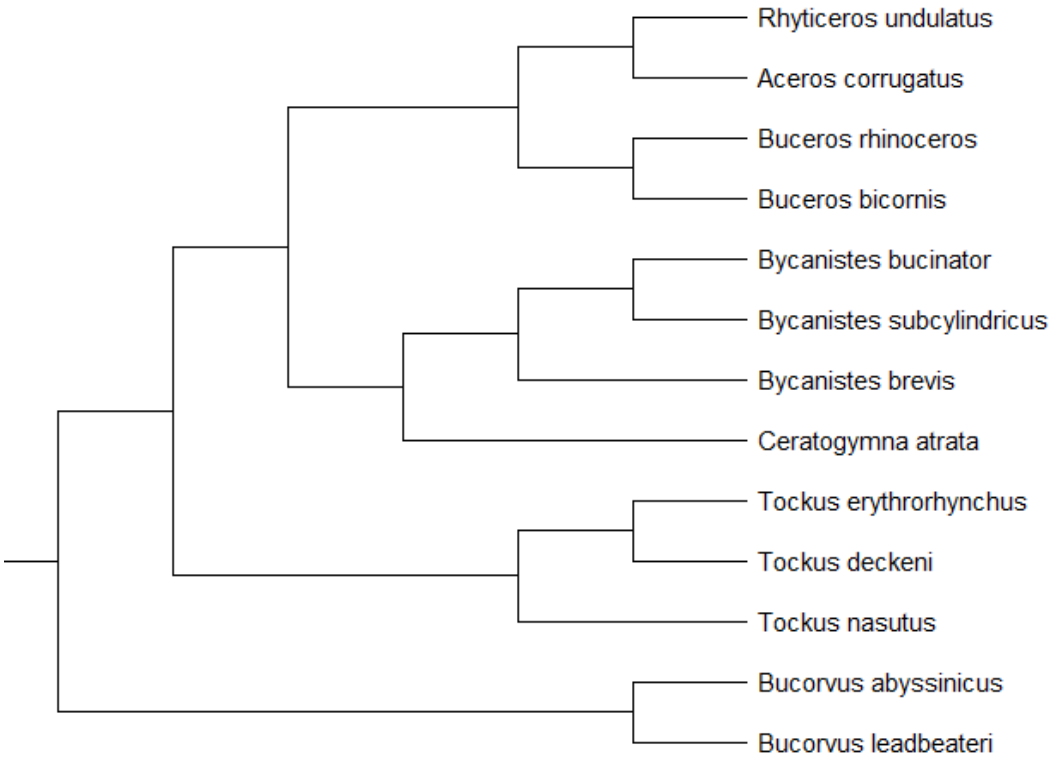

Figure 16. Consensus tree obtained with the 13 hornbill species used in this study.

To analyse the possible influence of bacteria on the evolution of ornaments, the best PGLM model for each ornament as dependent variable was selected based in the AIC criterion, among all possible models considering all estimates of bacterial growth in different body parts, as good as all the remaining ornaments.

To explore the possible relationship between bacterial load in the uropygial gland tuft and that of other body parts, we performed PGLM analyses with bacterial load in the gland in the different media as dependent variable and growing in all the remaining body parts in

different media as predictors. We show only significant results with the best model according to AICc.

Phylogenetic methods do not allow the graphical presentation of the models. Thus, our figures are based on raw species data. All statistical analyses were conducted in the R version 3.5.3 (R Core Team 2019) with "ape" add-on package and the pglm function (Paradis et al. 2004). Figures were performed using Statistica 7 software (Statsoft 2006).

Table 10. List of species used in this study with the information of the number of individuals sampled, their sex and the Zoo in which they were sampled, per species.

Species	N	Sex	Zoos
Aceros corrugatus	1	♂	Palmitos Park (1 ♂)
Rhyticeros undulatus	1	♀	Boissière du Doré (1♀)
Buceros bicornis	4	*2* ♀ + *2* ♂	Lourosa (2 ♂ + 1 ♀); Upie (1 ♂)
Buceros rhinoceros	5	*2* ♀ + *3* ♂	Beauval (2 ♂ + 2 ♀); Lourosa (1 ♂)
Bucorvus abyssinicus	4	*1* ♀ + *3* ♂	Boissière du Doré (2 ♂); Beauval (1 ♂ + 1 ♀)
Bucorvus leadbeateri	12	*6* ♀ + *6* ♂	Boissière du Doré (1 ♂); Beauval (1 ♂ + 1 ♀); Frejus (1 ♀); Barcelona (1 ♂ + 1 ♀); Jungle Park (1 ♂ + 1 ♀); Lourosa (1 ♂ + 1 ♀); Tabernas (1 ♂ + 1 ♀)
Bycanistes brevis	6	*3* ♀+ *2* ♂+ *1*	Barcelona (1♀); Jungle Park (1 ♂ + 1 ♀); Palmitos Park (1 ?); Tabernas (1 ♂ + 1 ♀)
Bycanistes bucinator	3	*1* ♀ + *1* ♂ + *1*	Upie (1 ♂ + 1 ♀); Palmitos Park (1 ?)
Bycanistes subcylindricus	2	*1* ♂ + *1*	Tabernas (1?); Attilly (1 ♂)
Ceratogymna atrata	6	*2* ♀ + *4* ♂	Frejus (1 ♂); Lourosa (2 ♂ + 1 ♀); Tabernas (1 ♂ + 1 ♀)
Tockus deckeni	5	*2* ♀ + *3* ♂	Upie (1 ♂ + 1 ♀); Beauval (1 ♂ + 1 ♀); Lourosa (1 ♂)
Tockus erythrorhynchus	2	*1* ♀ + *1* ♂	Barcelona (1 ♂ + 1 ♀)
Tockus nasutus	2	*2* ♀	Jungle Park (2 ♀)

RESULTS

Relationships among ornaments

Species with a greater proportion of white feathers in their body surface were also those with greater beak surface coloured in red-orange-yellow (PGLM: Proportion of white feathers: Estimate = 1.20, t = 2.83, P = 0.018. N=12. Figure 3). On the other hand, the coloured surface of the casque or its size was not significantly related to any of the other ornaments except the coloured surface of the casque with the one of the beak (PGLM: Coloured surface of casque: Estimate = 0.83, t = 7.42, P < 0.001. N=13). Moreover, the the total surface of red-orange-yellow on casque and beak together was related to the presence of coloured secretions (Coloured secretion: Estimate = 1.39, t = 2.66, P = 0.022. N = 13). This relationship was maintained when considering colour of the casque alone (Coloured secretion: Estimate = 0.77, t = 2.93, P = 0.014. N = 13), although it only had a non-significant trend for beak colouration alone (Coloured secretion: Estimate = 0.61, t = 2.19, P = 0.051. N = 13)

Ornaments and bacterial loads

The proportion of the beak surface that was coloured was negatively related to bacterial load of mesophilic bacteria (growing in TSA medium) of the beak, after controlling for the proportion of white feathers in the body (PGLM: log transformed variable CFU/ml TSA: Estimate = -0.26, t = 4.44, P = 0.002, white the percentage of the feathers that were white showed a positive relationship: Estimate = 1.64, t = 6.17, P < 0.001. N = 12. Figure 4). When considering the total number of coloured structures based on carotenoids in the body, we found a trend for a similar negative relationship with mesophilic bacterial load, but in this case for the casque (PGLM: log-transformed variable CFU/ml TSA: Estimate = -0.43, t = 2.22, P = 0.050. N = 12. Figure 5). When controlling for keratinolytic bacterial load of the casque, this relationship was significant (PGLM: log CFU/ml TSA casque: Estimate = -0.93, t = 2.50, P = 0.034; log CFU/ml FMA casque: Estimate = 0.34, t = 1.53, P = 0.159. N = 12.

Figure 6). The proportion of white feathers, casque coloured surface or casque size were not related to any estimate of bacterial load.

Uropygial gland bacteria

We found bacterial growth after inoculating uropygial secretions for all the species except for *Aceros corrugatus, Aceros undulatus and Buceros bicornis* (Chapter IV). In the case of swabs obtained from the uropygial gland tuft, there was bacterial growth in TSA medium for all species, in six species in KF medium, in eight species in VJ medium, in all the nine species sampled in FMA medium and in five of the 12 species sampled in HK medium (Table 2).

Cosmetic uropygial colouration was not related to bacterial load in the uropygial gland tuft in any medium except for KF medium, where we found that species with uropygial cosmetic colouration tended to have higher prevalence of *enterococci* in the uropygial gland tuft (PGLM. Cosmetic colouration: Estimate = 0.39, t = 2.04, P = 0.066. N = 13).

In addition, we found that species with higher prevalence of *enterococci* in the uropygial gland tuft had higher bacterial load in the casque in FMA medium (log CFU/ml casque: PGLM: Estimate = 0.10, t = 2.31, P = 0.043. N = 12. Figure 7).

All models present here are unweighted since no differences were found between weighted and unweighted models (results not shown).

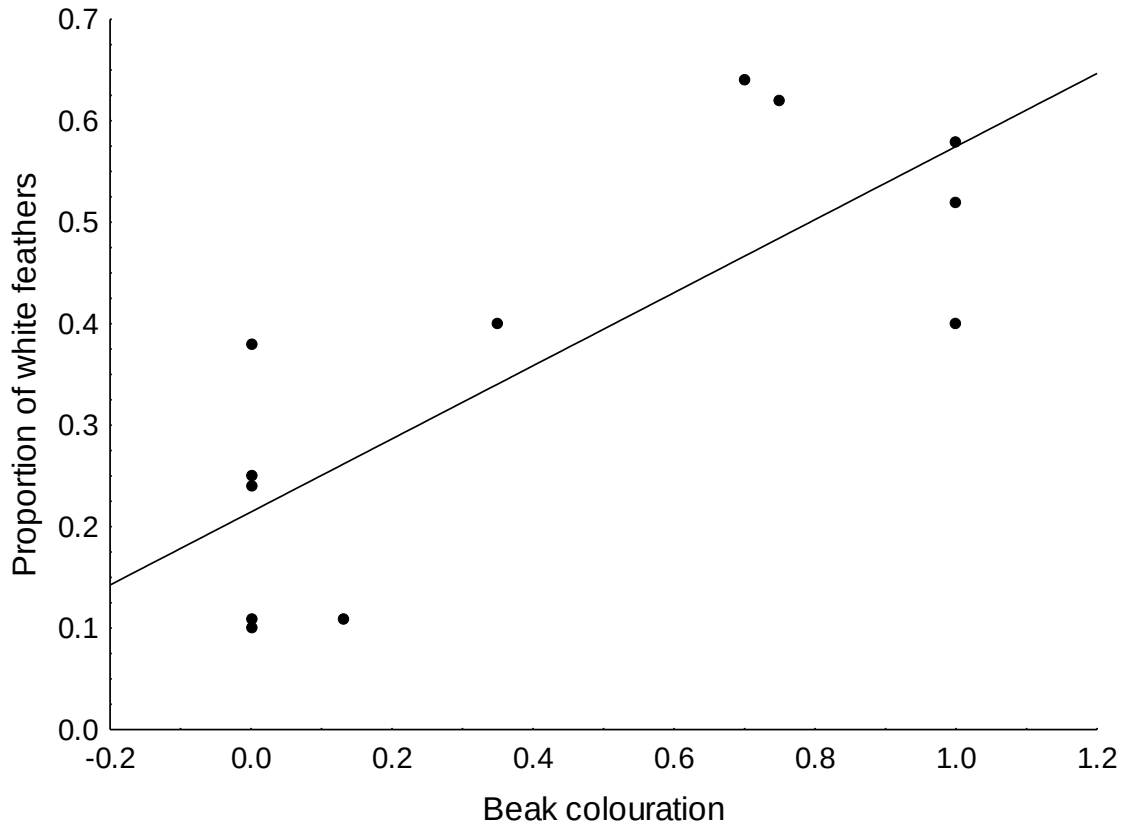

Figure 17. Relationship between beak colouration and proportion of white in feather surface of the body for 12 hornbill species. The line is the linear regression line.

Table 11. Bacterial growth (Log CFU/ml or prevalence) in the uropygial gland tuft in each medium and prevalence of bacterial growth after inoculating uropygial secretions. N indicates the number of individuals per species sampled in each medium (TSA, FMA, KF, VJ and HK).

	log CFU/ml TSA	N TSA	log CFU/ml FMA	N FMA	Prevalence KF	N KF	Prevalence VJ	N VJ	Prevalence HK	N HK	Prevalence secretion	N Secretions
Bucorvus leadbeateri	3.522	10	2.198	4	0.38	8	0.38	8	0	8	0.60	10
Bucorvus abyssinicus	3.543	4	3.739	4	0.25	4	0.50	4	0.25	4	1.00	3
Tockus nasutus	1.004	1	-	0	0	1	0	1	1	1	-	0
Tockus erythrorhynchus	3.290	1	-	0	0	1	1	1	0	1	-	0
Tockus deckeni	3.861	4	3.858	4	0.25	4	0.75	4	0	4	0.60	5
Aceros corrugatus	1.004	4	-	0	0	1	0	1	0	1	-	0
Rhyticeros undulatus	1.904	1	1.004	1	0	1	1	1	-	0	0.00	1
Buceros rhinoceros	3.612	5	3.620	4	1	5	1	5	0.40	5	0.67	3
Buceros bicornis	3.214	4	3.049	1	0.75	4	0.25	4	0.25	4	0.00	3
Ceratogymna atrata	2.080	2	1.479	1	0	3	0	2	0	2	0.33	6
Bycanistes bucinator	2.308	3	2.022	2	0	3	0	3	0	3	1.00	4
Bycanistes brevis	2.080	3	-	0	0	3	0	2	0	2	-	0
Bycanistes subcylindricus	3.275	2	3.265	1	0.50	2	0.50	2	0	2	0.33	3

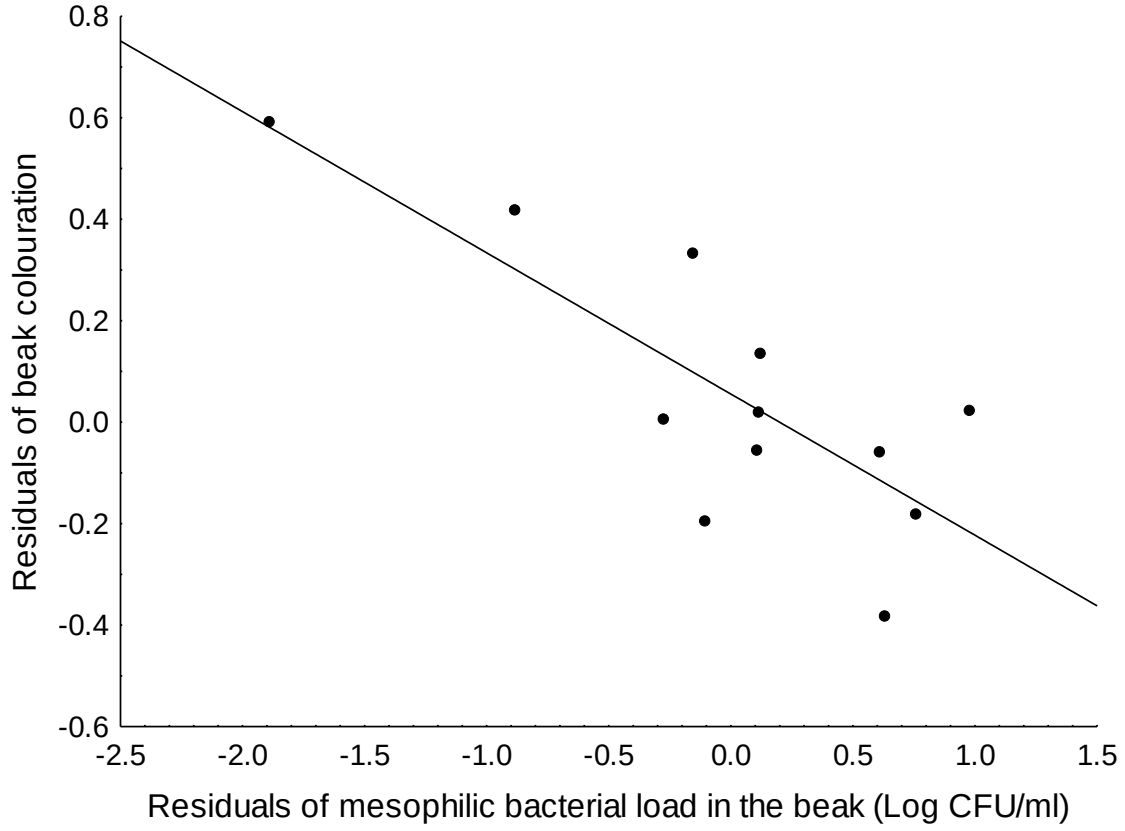

Figure 18. Relationship between proportion of beak surface coloured and bacterial load of mesophilic bacteria (grown in TSA medium) in the beak. The residuals after correcting for proportion of white feathers are presented. The line is the linear regression line.

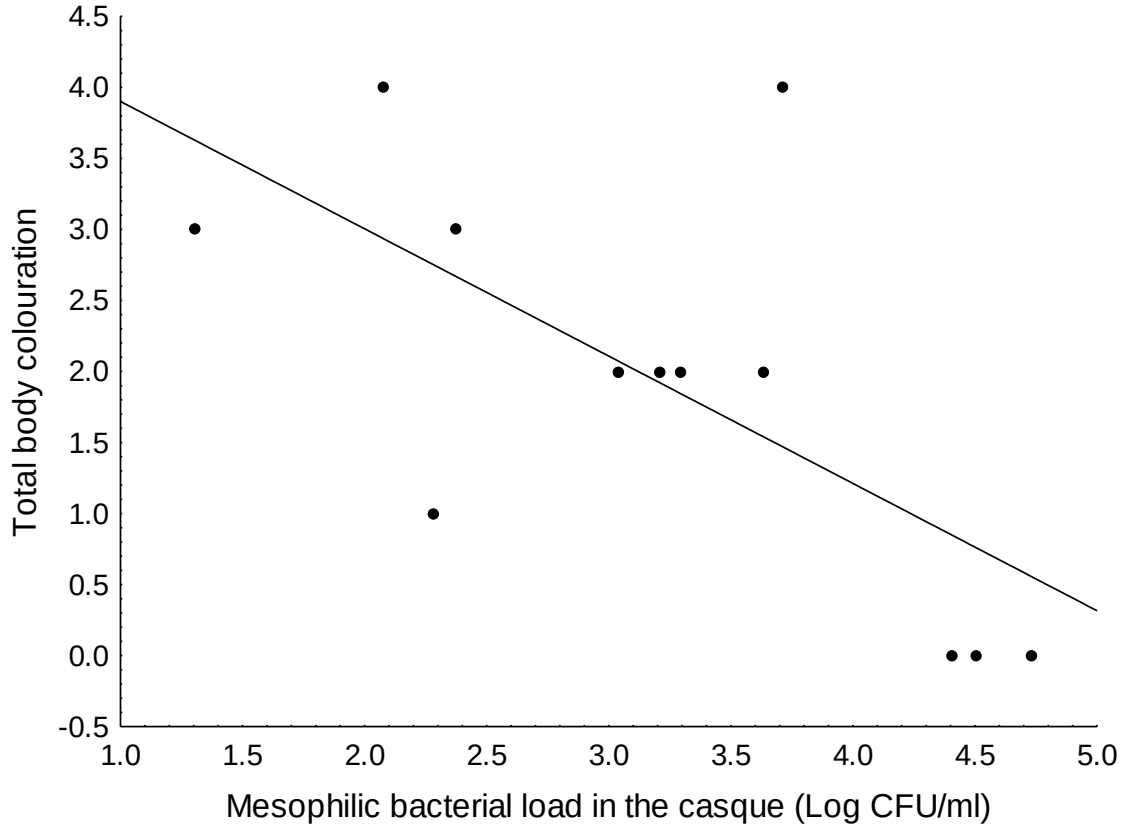

Figure 19. Relationship between the number of coloured structures (red, orange, yellow) and the bacterial load of mesophilic bacteria in the casque of different species of hornbills. The line is the linear regression line.

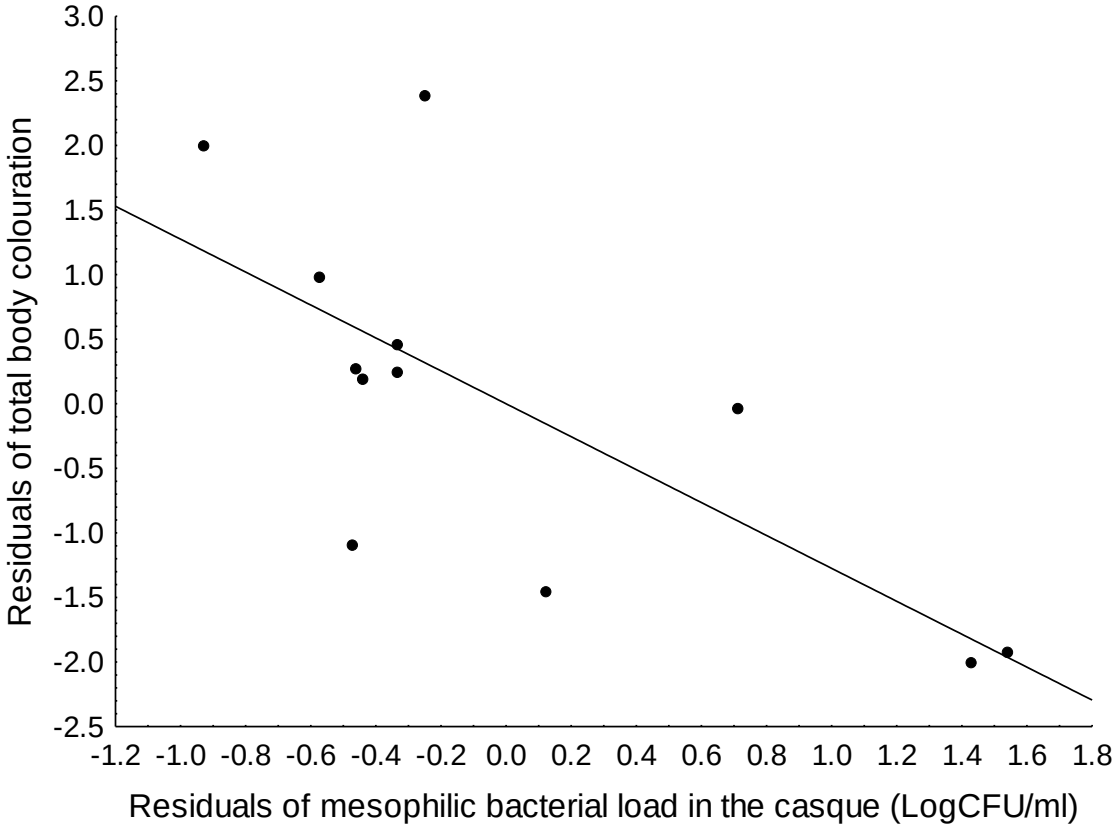

Figure 20. Relationship between the number of coloured structures (red, orange, yellow) and the bacterial load of mesophilic bacteria in the casque. The residuals after correcting for keratynolitic bacteria in the casque are presented. The line is the linear regression line.

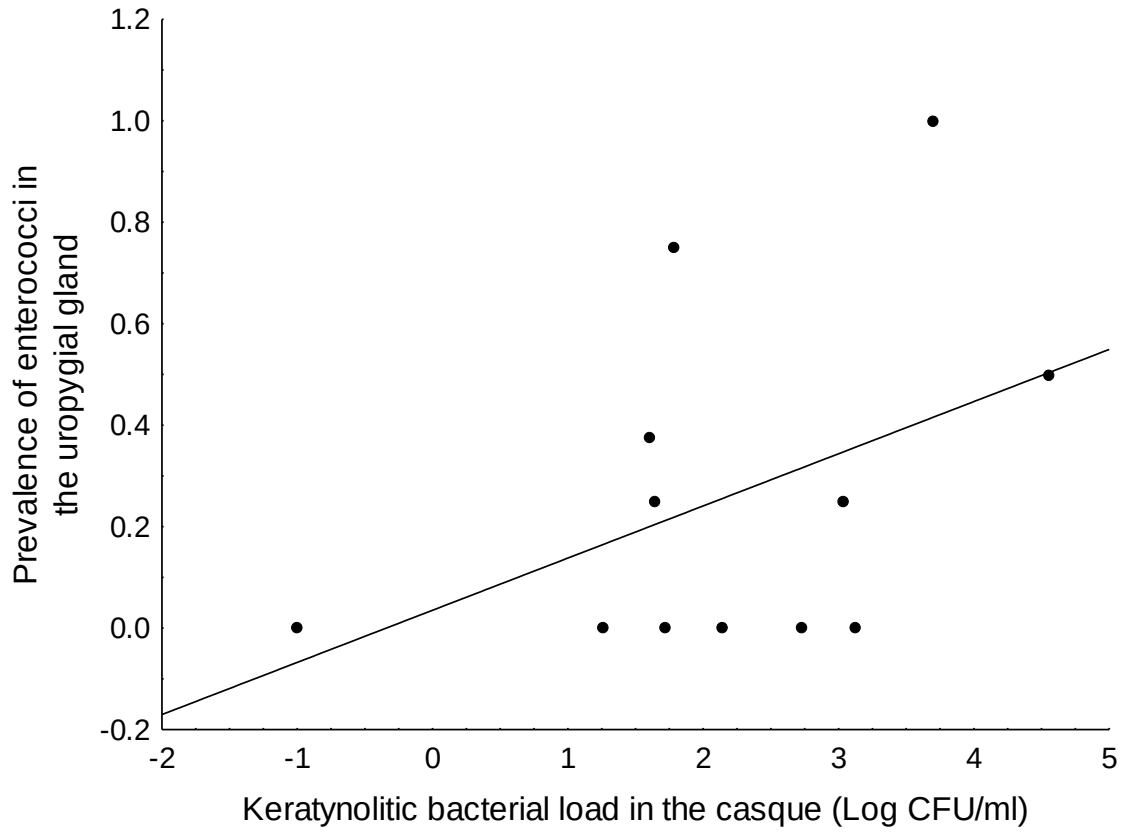

Figure 21. Relationship between prevalence of *enterococci* in the tuft of the uropygial gland (prevalence in KF medium) and keratynolitic bacterial load in the casque (CFU/ml in FMA medium) in different species of hornbills. The line is the linear regression line.

DISCUSSION

Our main findings are that the degree of exaggeration of two ornaments: white plumage areas and beak coloured surface, are positively correlated across hornbill phylogeny, and that variation in ornamentation among species is related to differences in bacterial loads in different body parts. Moreover, the prevalence in the uropygial gland of a particular group of bacteria known for their production of bacteriocins (*enterococci*) was related to abundance of potential pathogenic (keratynolitic) bacteria in the distinctive ornament of hornbills: the

casque. Below, we first discuss the possible limitations of our data and how they could affect these results, and afterwards the importance of the results found and the improvements that can be made in future studies for reaching more robust conclusions.

A main criticism that can be made of our study is that samples come from captive individuals, from different zoos, and that they are taken in different seasons. We have not sampled wild individuals so we cannot be sure that microbial loads found in captive individuals reflect that of wild ones. Indeed, some studies have shown how microbiomes of captive and wild individuals differ (Hird 2017). One example of this occurs in hoopoes, whose uropygial gland microbiome is slightly larger in wild individuals, compared to captive ones, but only in a small fraction of the microbiome bacterial strains, with very similar core microbiomes (Martínez-García 2015, Rodríguez-Ruano 2015). A main reason for the use of captive individuals is the great difficulty in obtaining samples from the wild especially in large Asian hornbill species. Although we cannot completely rule out the effect of captivity on our results, all individuals sampled were captive, the species with known colourful secretions also had these in captivity, and from our experience with hoopoes we know that the typical constituents of uropygial microbiomes, and the particular properties of uropygial secretions are maintained in captivity. We expect that the same can be applied to hornbills, where we have also found particular bacterial species associated with uropygial glands (Chapter IV). Even if captivity may have affected our conclusions, we can still draw the conclusions that the patterns in captive and wild specimens must show similar relationships for coloration and the size of the microbiome. Moreover, our results indicated that variation due to zoos is relatively low when we tested bacterial loads of *Bucorvus leadbeateri* in different Zoos (we have more individuals of this species from different zoos). It suggests that

most of the variance explained by zoos should be due to the different pool of species located in different Zoos.

It is clear from our analysis of whole microbiomes (Chapter IV) that the zoo of origin affects the presence of particular bacteria. It is an expected result, since there is geographic variation in the presence of particular bacterial strains (Dequiedt et al. 2009). Probably the different managements in the parks, including food sources, affect the bacterial communities present in the environment where these hornbills live. However, it is expected that in their particular environment, animals are colonized by a particular assemblage of bacteria typical for or able to live in each particular body part, and thus they should be ecologically equivalent. First, some components of the microbiomes may be vertically transmitted and therefore maintained throughout generations (Darby and Douglas 2003, Sachs et al. 2011, Martín-Vivaldi et al. 2018). Second, part of (Martín-Vivaldi et al. (2018)) or the entire assemblages can be obtained from the environment causing the species or strains filling a particular niche within the microbiome, causing the microbiome in a specific environment (either captive or wild), reflect local effects caused by natural patterns of microbial colonization. Even if these effects may vary among zoos in a different way from those caused by dispersal in the wild, given that for most species we have samples from different zoos, the zoo of origin probably may have diluted rather than caused effects. Since we have performed the analyses both with weighted and unweighted approaches, the results are also controlled by the possible effects of species with fewer individuals derived from a smaller number of zoos.

Regarding variation in the season of sampling different individuals, it is known that, at least the microbiome composition of the uropygial secretion and its signalling function can vary depending on season and on the sex in several species (Tuttle et al. 2014, Rodríguez-Ruano et al. 2018). The hornbill samples used in the present study have been collected in

different seasons (see Methods), and, therefore, we cannot rule out the possibility that the differences in bacterial load between species could be influenced by the season in which they were sampled. We can state that all samples were obtained during the breeding season, given that caregivers prefer not to disturb animals during breeding, and, therefore, in some sense, all samples were taken under similar conditions. In any case, different species may differ in their relationship with bacteria among seasons, as is the case for hoopoes (dark secretions and complex microbiomes only during the breeding season, (Martín-Vivaldi et al. 2009, Martínez-García et al. 2016b) and woodhoopoes (dark secretions and specialized bacteria living in gland throughout the year (Law-Brown and Meyers 2003, Burger et al. 2004). Again, we consider that if season has any influence on the variables measured, it should not cause effects but dilute them. Probably it should also be indicated that our results may be valid only outside the breeding season. We cannot be sure whether some aspects may disappear while others become evident during the breeding season. Finally, as for the sex, we have tested but did not find differences in bacterial load among them, and, therefore, this should not have biased our results. Taking into account all these considerations, and accepting that probably we are able to detect only very evident effects that are present at least outside the breeding season, we discuss below the implications of the main relationships detected.

Species with coloured secretions had a larger surface of red-orange-yellow on the casque and on the beak suggesting that cosmetic colouration is the main way of increasing these colours by species with colorful secretions. The fact that the casque explained this relationship more than did the beak, suggests that this unique structure in hornbills could have partially evolved to show the colour of their uropygial secretions.

Regarding colouration and bacterial load, species with more coloured beaks had lower bacterial loads on their teguments. This result can be interpreted in different ways. First, it

may be a consequence of pigmented secretions covering the beak and by their antimicrobial properties preventing the survival of bacteria on the surface. However, the inclusion of the variable cosmetic coloration in the model did not change the results, suggesting it was not the result of direct antimicrobial effects of coloured secretions. Alternatively, the relationship may indicate that species investing in this kind of ornament are more able to cope with and fight bacteria through evolved defences, or the contrary, that species less exposed to bacteria have been able to evolve more exaggerated carotenoid dependent signals. The first scenario is compatible with studies on other bird species in which beak colouration has been found to reflect inter-individual differences in antimicrobial defences (spotless starling (*Sturnus unicolor*) (Ruiz-Rodríguez et al. 2020, under review). However, we cannot directly test these alternatives, since we have not estimated immunocompetence of the different hornbill species. In any case, the last interpretation seems more plausible, since our estimate of beak bacterial load is probably the most reliable sample reflecting global exposure to bacteria from the surrounding ambient environment, but also food. The fact that we have found this relationship only with a general medium (TSA) also supports this view.

Moreover, species with more coloured surface of the beaks had more white plumage surface on their body. This may be due to the existence of multiple signals. First, beak colouration can signal access to carotenoids and, hence their individual quality. Second, the proportion of white feathers on the body can signal individual quality through degradation (Ruiz-De-Castañeda et al. 2015) since they are more easily degradable than melanised feathers (Goldstein et al. 2004; Azcárate-García et al. 2019 under review). Several studies in other species have shown how white plumage patches are a predictor of phenotypic quality, being condition-dependence and influencing reproductive success (Doucet et al. 2004; reviewed in Prum 2006). Moreover, white areas can be used for staining by cosmetics, also playing an important role in sexual signalling.

Figura 22. *Buceros bicornis* with its white patches stained yellow by its uropygial secretion. Photo acknowledgment: Kajornyot Krunkitsatien

The casque is a unique exaggerated and sexually dimorphic structure in hornbill species, so we predicted it would have evolved to signal individual quality. The size of this structure is highly variable among species and in some (e. g. genus Tockus) it is small and difficult to distinguish from the beak (Kemp 2001, Poonswad et al. 2013). Therefore, different functions of the casque depending on the species cannot be ruled out (Gamble 2007). However, we did not find a relationship between casque coloration or casque size and bacterial load. This could be due to the small number of species included in our study. However, when we considered total body coloration, we found that it was negatively related to load of mesophylic bacteria on the casque. This result could be interpreted in the same alternative ways as the negative relationship between beak colour and mesophilic bacteria on it, since colour of the beak was related to colour of the casque. A more remarkable result for the casque is the positive relationship between prevalence of *enterococci* in the uropygial gland and load of keratynolitic bacteria on the casque. This result seems to reflect the association between the evolution of uropygial secretions able to host *enterococci*, with selection caused by

keratynolitic bacteria, which can deteriorate the appearance of an exaggerated keratine structure such as the casque. Uropygial secretions of birds can have antimycotic (Bandyopadhyay and Bhattacharyya 1996) and antibacterial properties per se (Shawkey et al. 2003), but in hoopoes and woodhoopoes this ability is mediated by symbiotic *enterococci* (Martín-Platero et al. 2006, Ruiz-Rodríguez et al. 2013). Since we have also found bacteria in the secretions of hornbills (Chapter IV), it may be that some of these bacteria are *enterococci* or other producers of antimicrobials, and that the symbiotic relationship has evolved as a means to protect the casque. This could explain why many hornbill species stain the casque with uropygial secretions (Kemp 2001, Poonswad et al. 2013, Naish 2015). Thus, this behaviour could have evolved to protect the casque from keratynolitic bacteria and from diseases like carcinoma, common in the casque of *Buceros bicornis* (Miller et al. 1985, Suedmeyer et al. 2001). The fact that we have found a positive relationship between coloured surface of the casque and cosmetic colouration also supports this view.

In summary, our results suggest an evolutionary relationship between colouration and bacterial load in hornbills. We hypothesize that application of knowledge about the colour signaling structures of hornbills may be used for conservation and captive breeding of these endangered species. Understanding possible relationships with bacterial load in *Bucerotiformes* would open new insights into the study of coevolution and sexual selection. It would for instance be interesting to know whether bacteria present in secretions facilitate the birds to combat pathogens (i.e. using antimicrobials and/or carotenoids from bacterial metabolism). More research is needed to determine whether ornamental coloration is a sexually selected trait in hornbills. A robust analysis from a longer list of species covering a wider phylogenetic range, as well as more individuals per species in natural populations

would be needed to reach strong conclusions. Experimental manipulation of carotenoids based colours would be particularly revealing.

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REFERENCES

Alberdi, A., Aizpurua, O., Bohmann, K., Zepeda-Mendoza, M. L. and Gilbert, M. T. P. 2016. Do Vertebrate Gut Metagenomes Confer Rapid Ecological Adaptation? - Trends Ecol. Evol. 31: 689–699.

Alexander, G. D., Houston, D. C. and Campbell, M. 1994. A possible acoustic function for the casque structure in hornbills (Aves: Bucerotidae). - J. Zool. 233: 57–67.

Amat, J. A., Rendón, M. A., Garrido-Fernández, J., Garrido, A., Rendón-martos, M. and Pérez-gálvez, A. 2011. Greater flamingos Phoenicopterus roseus use uropygial secretions as make-up. - Behav. Ecol. 65: 665–673.

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Zech Xu, Z., Kightley, E. P., Thompson, L. R., Hyde, E. R., Gonzalez, A. and Knight, R. 2017. Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. - mSystems 2: e00191-16.

Amundsen, T. 2000. Why are female birds ornamented? - Trends Ecol. Evol. 15: 149–155.

Amundsen, T. 2018. Sex roles and sexual selection: Lessons from a dynamic model system. - Curr. Zool. 64: 363–392.

Andersson, M. 1994. Sexual selection (Princeton Univ. Press, Ed.).

Andersson, M. and Simmons, L. W. 2006. Sexual selection and mate choice. - Trends Ecol. Evol. 21: 296–302.

Archie, E. A. and Theis, K. R. 2011. Animal behaviour meets microbial ecology. - Anim. Behav. 82: 425–436.

Arlettaz, R., Schaub, M., Fournier, J., Reichlin, T. S., Sierro, A., Watson, J. E. M. and Braunisch, V. 2010. From Publications to Public Actions: When Conservation Biologists Bridge the Gap between Research and Implementation. - Bioscience 60: 835–842.

Aronesty, E. 2011. ea-utils: Command-line tools for processing biological sequencing data.: https://github.com/ExpressionAnalysis/ea-utils.

Avilés, J. M. and Soler, J. J. 2009. Nestling colouration is adjusted to parent visual performance in altricial birds. - J. Evol. Biol. 22: 376–386.

Avilés, J. M., Soler, J. J. and Pérez-Contreras, T. 2006. Dark nests and egg colour in birds: a possible functional role of ultraviolet reflectance in egg detectability. - Proc. Biol. Sci. 273: 2821–2829.

Avilés, J. M., Pérez-Contreras, T., Navarro, C. and Soler, J. J. 2009. Male spotless starlings adjust feeding effort based on egg spots revealing ectoparasite load. - Anim. Behav. 78: 993–999.

Avilés, J. M., Vikan, J. R., Fossøy, F., Antonov, A., Moksnes, A., Røskaft, E. and Stokke, B. G. 2010. Avian colour perception predicts behavioural responses to experimental brood parasitism in chaffinches. - J Evol Biol 23: 293–301.

Avilés, J. M., Soler, J. J. and Hart, N. S. 2011. Sexual selection based on egg colour: Physiological models and egg discrimination experiments in a cavity-nesting bird. - Behav. Ecol. Sociobiol. 65: 1721–1730.

Azcárate-García, M., González-Braojos, S., Díaz-Lora, S., Ruiz-Rodríguez, M., Martin-Vivaldi, M., Martínez-Bueno, M., Moreno, J. and Soler, J. J. 2020. Interspecific variation in deterioration and degradability of avian feathers: The evolutionary role of the microbiome. - J. Avian Biol.: in press.

Baker, L. J., Freed, L. L., Easson, C. G., Lopez, J. V, Fenolio, D., Sutton, T. T., Nyholm, S. V and Hendry, T. A. 2019. Diverse deep-sea anglerfishes share a genetically reduced luminous symbiont that is acquired from the environment. - Elife 8: e47606.

Bandyopadhyay, A. and Bhattacharyya, S. P. 1996. Influence of fowl uropygial gland and its secretory lipid components on growth of skin surface bacteria of fowl. - Indian J. Exp. Biol. 34: 48–52.

Banning, J. L., Weddle, A. L., Wahl III, G. W., Simon, M. A., Lauer, A., Walters, R. L. and Harris, R. N. 2008. Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, Hemidactylium scutatum. - Oecologia 156: 423–429.

Barr, J. J., Auro, R., Furlan, M., Whiteson, K. L., Erb, M. L., Pogliano, J., Stotland, A., Wolkowicz, R., Cutting, A. S., Doran, K. S., Salamon, P., Youle, M. and Rohwer, F. 2013. Bacteriophage adhering to mucus provide a non–host-derived immunity. - Proc. Natl. Acad. Sci. 110: 10771–10776.

Beltran-Bech, S. and Richard, F.-J. 2014. Impact of infection on mate choice. - Anim. Behav. 90: 159–170.

Bennett, G. M. and Moran, N. A. 2015. Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. - Proc. Natl. Acad. Sci. 112: 10169–10176.

Bentley, D. R., Balasubramanian, S., Swerdlow, H. P., Smith, G. P., Milton, J., Brown, C. G., Hall, K. P., Evers, D. J., Barnes, C. L., Bignell, H. R., Boutell, J. M., Bryant, J., Carter, R. J., Keira Cheetham, R., Cox, A. J., Ellis, D. J., Flatbush, M. R., Gormley, N. A., Humphray, S. J., Irving, L. J., Karbelashvili, M. S., Kirk, S. M., Li, H., Liu, X., Maisinger, K. S., Murray, L. J., Obradovic, B., Ost, T., Parkinson, M. L., Pratt, M. R., Rasolonjatovo, I. M. J., Reed, M. T., Rigatti, R., Rodighiero, C., Ross, M. T., Sabot, A., Sankar, S. V., Scally, A., Schroth, G. P., Smith, M. E., Smith, V. P., Spiridou, A., Torrance, P. E., Tzonev, S. S., Vermaas, E. H., Walter, K., Wu, X., Zhang, L., Alam, M. D., Anastasi, C., Aniebo, I. C., Bailey, D. M. D., Bancarz, I. R., Banerjee, S., Barbour, S. G., Baybayan, P. A., Benoit, V. A., Benson, K. F., Bevis, C., Black, P. J., Boodhun, A., Brennan, J. S., Bridgham, J. A., Brown, R. C., Brown, A. A., Buermann, D. H., Bundu, A. A., Burrows, J. C., Carter, N. P., Castillo, N., Catenazzi, M. C. E., Chang, S., Neil Cooley, R., Crake, N. R., Dada, O. O., Diakoumakos, K. D., Dominguez-Fernandez, B., Earnshaw, D. J., Egbujor, U. C., Elmore, D. W., Etchin, S. S., Ewan, M. R., Fedurco, M., Fraser, L. J., Fuentes Fajardo, K. V., Scott Furey, W., George, D., Gietzen, K. J., Goddard, C. P., Golda, G. S., Granieri, P. A., Green, D. E., Gustafson, D. L., Hansen, N. F., Harnish, K., Haudenschild, C. D., Heyer, N. I., Hims, M. M., et al. 2008. Accurate whole human genome sequencing using reversible terminator chemistry. - Nature 456: 53–59.

Boisbunon, A., Canu, S., Fourdrinier, D., Strawderman, W. and Wells, M. T. 2013. AIC , Cp and estimators of loss for elliptically symmetric distributions. - hal-00851206

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., Mills, D. A. and Caporaso, J. G. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. - Nat. Methods 10: 57–59.

Brandl, H. B., van Dongen, W. F. D., Darolová, A., Kristofík, J., Majtan, J. and Hoi, H. 2014. Composition of Bacterial Assemblages in Different Components of Reed Warbler Nests and a Possible Role of Egg Incubation in Pathogen Regulation. - PLoS One 9(12): 1–19.

Braun, M. S., Zimmermann, S., Danner, M., Rashid, H. and Wink, M. 2016. Corynebacterium

uropygiale sp. nov., isolated from the preen gland of Turkeys (Meleagris gallopavo). - Syst. Appl. Microbiol. 39: 88–92.

Braun, M. S., Wang, E., Zimmermann, S. and Wink, M. 2018a. Corynebacterium heidelbergense sp. nov., isolated from the preen glands of Egyptian geese (Alopochen aegyptiacus). - Syst. Appl. Microbiol. 41: 564–569.

Braun, M. S., Wang, E., Zimmermann, S., Boutin, S. and Wink, M. 2018b. Kocuria uropygioeca sp. nov. and Kocuria uropygialis sp. nov., isolated from the preen glands of Great Spotted Woodpeckers (Dendrocopos major). - Syst. Appl. Microbiol. 41: 38–43.

Braun, M. S., Sporer, F., Zimmermann, S. and Wink, M. 2018c. Birds, feather-degrading bacteria and preen glands: The antimicrobial activity of preen gland secretions from turkeys (Meleagris gallopavo) is amplified by keratinase. - FEMS Microbiol. Ecol. 94: fiy117.

Braun, M. S., Wang, E., Zimmermann, S., Boutin, S., Wagner, H. and Wink, M. 2019a. Kocuria tytonicola, new bacteria from the preen glands of American barn owls (Tyto furcata). - Syst. Appl. Microbiol. 42: 198–204.

Braun, M. S., Wang, E., Zimmermann, S., Wagner, H. and Wink, M. 2019b. Kocuria tytonis sp. nov., isolated from the uropygial gland of an American barn owl (Tyto furcata). - Int. J. Syst. Evol. Microbiol. 69: 447–451.

Bright, M. and Bulgheresi, S. 2010. A complex journey: transmission of microbial symbionts. - Nat. Rev. Microbiol. 8: 218–230.

Brulez, K., Pike, T. W. and Reynolds, S. J. 2015. Egg signalling: The use of visual, auditory, and chemical stimuli. - In: Deeming, D. C. and Reynolds, S. J. (eds), Nest, Egg, and Incubation. New ideas about avian reproduction. Oxford University Press, pp. 127–141.

Brunetti, A. E., Lyra, M. L., Melo, W. G. P., Andrade, L. E., Palacios-Rodríguez, P., Prado, B. M., Haddad, C. F. B., Pupo, M. T. and Lopes, N. P. 2019. Symbiotic skin bacteria as a source for sex-specific scents in frogs. - Proc. Natl. Acad. Sci. 116: 2124–2129.

Bulla, M., Šálek, M. and Gosler, A. G. 2012. Eggshell spotting does not predict male incubation but marks thinner areas of a shorebird's shells. - Auk 129: 26–35.

Burger, B. V, Reiter, B., Borzyk, O. and du Plessis, M. A. 2004. Avian Exocrine Secretions. I. Chemical Characterization of the Volatile Fraction of the Uropygial Secretion of the Green Woodhoopoe, Phoeniculus purpureus. - J. Chem. Ecol. 30: 1603–1611.

Burley, N. 1986. Sexual Selection for Aesthetic Traits in Species with Biparental Care. - Am. Nat. 127: 415–445.

Burley, N. 1988. The Differential Allocation hypothesis. An experimental test. - Am. Nat. 132: 611–628.

Callahan, B. J., McMurdie, P. J. and Holmes, S. P. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. - ISME J. 11: 2639–2643.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., González Peña, A., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., Mcdonald, D., Muegge, B., Pirrung, M., Reeder, J., Sevinsky, J., Turnbaugh, P., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J. and Knight, R. 2010. QIIME allows analysis of high-throughput community sequencing data. - Nat Methods. 7: 335–336.

Caruso, V., Song, X., Asquith, M. and Karstens, L. 2019. Performance of Microbiome Sequence Inference Methods in Environments with Varying Biomass (SM Gibbons, Ed.). - mSystems 4: e00163-18.

Chapman, T., Arnqvist, G., Bangham, J. and Rowe, L. 2003. Sexual conflict. - Trends Ecol. Evol. 18: 41–47.

Cherry, M. I. and Gosler, A. G. 2010. Avian eggshell coloration: New perspectives on adaptive explanations. - Biol. J. Linn. Soc. 100: 753–762.

Chiarello, M., Villéger, S., Bouvier, C., Auguet, J. C. and Bouvier, T. 2017. Captive bottlenose dolphins and killer whales harbor a species-specific skin microbiota that varies among individuals. - Sci. Rep. 7: 15269.

Clayton, D. H. 1991. The influence of parasites on host sexual selection. - Parasitol. Today 7: 329–334.

Clutton-Brock, T. H. 1991. The evolution of parental care (Princeton University Press, Ed.).

Clutton-Brock, T. 2007. Sexual Selection in Males and Females. - Science (80-.). 318: 1882–1885.

Clutton-Brock, T. 2009. Sexual selection in females. - Anim. Behav. 77: 3–11.

Cook, M. I., Beissinger, S. R., Toranzos, G. A. and Arendt, W. J. 2005. Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection. - Ecol. Lett. 8: 532–537.

Corti, M., Romano, A., Costanzo, A., Bentz, A. B., Navara, K. J., Parolini, M., Saino, N. and Rubolini, D. 2018. Protoporphyrin-based eggshell pigmentation predicts hatching success and offspring sex ratio in the barn swallow. - J. Avian Biol. 49: 1–17.

Cramp, S. 1998. The complete birds of the western Palearctic on CD-ROM. - Oxford University Press.

Cunningham, E. J. A. and Russell, A. F. 2000. Egg investment is influenced by male attractiveness in the mallard. - Nature 404: 74–77.

Currie, C. R., Scott, J. A., Summerbell, R. C. and Malloch, D. 1999. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. - Nature 398: 701–704.

D'Alba, L. and Shawkey, M. D. 2015. Mechanisms of antimicrobial defense in avian eggs. - J. Ornithol. 156: S399–S408.

Darby, A. C. and Douglas, A. E. 2003. Elucidation of the Transmission Patterns of an Insect-Borne Bacterium. - Appl. Environ. Microbiol. 69: 4403 LP – 4407.

Darwin, C. 1871. The descent of man and selection in relation to sex (J Murray, Ed.).

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A. and Callahan, B. J. 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. - Microbiome 6: 226.

de Goffau, M. C., Lager, S., Salter, S. J., Wagner, J., Kronbichler, A., Charnock-Jones, D. S., Peacock, S. J., Smith, G. C. S. and Parkhill, J. 2018. Recognizing the reagent microbiome. - Nat. Microbiol. 3: 851–853.

Delhey, K., Peters, A. and Kempenaers, B. 2007. Cosmetic Coloration in Birds: Occurrence, Function, and Evolution. - Am. Nat. 169: S145–S158.

Dequiedt, S., Thioulouse, J., Jolivet, C., Saby, N. P. A., Lelievre, M., Maron, P.-A., Martin, M. P., Prévost-Bouré, N. C., Toutain, B., Arrouays, D., Lemanceau, P. and Ranjard, L. 2009. Biogeographical patterns of soil bacterial communities. - Environ. Microbiol. Rep. 1: 251–255.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P. and Andersen, G. L. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. - Appl. Environ. Microbiol. 72: 5069–5072.

Devaynes, A., Antunes, A., Bedford, A. and Ashton, P. 2018. Progression in the bacterial load during the breeding season in nest boxes occupied by the Blue Tit and its potential impact on hatching or fledging success. - J. Ornithol. 159: 1009–1017.

Díaz-Lora, S., Pérez-Contreras, T., Azcárate-García, M., Peralta-Sánchez, J. M., Martínez-Bueno, M., Soler, J. J. and Martín-Vivaldi, M. Cosmetic coloration of cross-fostered eggs affects male parental investment in the Hoopoe (Upupa epops). - Under Rev. in press.

Díaz-Lora, S., Pérez-Contreras, T., Azcárate-García, M., Martínez-Bueno, M., Soler, J. J. and Martín-Vivaldi, M. Hoopoe (Upupa epops) male feeding effort is related to female cosmetic egg colouration. - Under Rev. in press.

Díaz-Lora, S., Martín-Vivaldi, M., Juárez-García-Pelayo, N., Azcárate-García, M., Rodríguez-Ruano, S., M., Martínez-Bueno, M. and Soler, J. J. 2019. Data from: experimental old nest material predicts hoopoe Upupa epops eggshell and uropygial gland microbiota. − Dryad Digital Repository, <http://dx.doi. org/10.5061/dryad.8574ft0>.

Dillon, R. J., Vennard, C. T., Buckling, A. and Charnley, A. K. 2005. Diversity of locust gut bacteria protects against pathogen invasion. - Ecol. Lett. 8: 1291–1298.

Douglas, A. E. 1998. Nutritional Interactions in Insect-Microbial Symbioses: Aphids and Their Symbiotic Bacteria Buchnera. - Annu. Rev. Entomol. 43: 17–37.

Dumbrell, A. J., Ferguson, R. M. W. and Clark, D. R. 2017. Microbial Community Analysis by Single-Amplicon High-Throughput Next Generation Sequencing: Data Analysis – From Raw Output to Ecology. - In: McGenity, T. J. et al. (eds), Springer. Hydrocarbon and Lipid Microbiology Protocols: Microbial Quantitation, Community Profiling and Array Approaches, pp. 155–206.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. - Bioinformatics 26: 2460–2461.

Edgar, R. C. 2017. UNCROSS: Filtering of high-frequency cross-talk in 16S amplicon reads. - PeerJ 5: e3889.

Edward, D. A. and Chapman, T. 2011. The evolution and significance of male mate choice. - Trends Ecol. Evol. 26: 647–654.

Eisenhofer, R., Minich, J. J., Marotz, C., Cooper, A., Knight, R. and Weyrich, L. S. 2019. Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. - Trends Microbiol. 27: 105–117.

Ellegaard, K. M. and Engel, P. 2016. Beyond 16S rRNA Community Profiling: Intra-Species Diversity in the Gut Microbiota . - Front. Microbiol. 7: 1475.

Endler, J. A. 1990. On the measurement and classification of color in studies of animal color patterns. - Biol. J. Linn. Soc. 41: 315–352.

Endler, J. A. and Mielke, P. W. 2005. Comparing color patterns as birds see them. - Biol. J. Linn. Soc. 86: 405–431.

English, P. A. and Montgomerie, R. 2011. Robin's egg blue: Does egg color influence male parental care? - Behav. Ecol. Sociobiol. 65: 1029–1036.

Erckmann, W., Beletsky, L., Orians, G., Johnsen, T., Sharbaugh, S. and D´Antonio, C. 1990. Old nests as cues for nest-site selection: an experimental test with Red-winged Blackbirds. - Condor 92: 113–117.

Ezenwa, V. O. and Williams, A. E. 2014. Microbes and animal olfactory communication: Where do we go from here? - BioEssays 36: 847–854.

Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M. and Xavier, J. B. 2012. Animal Behavior and the Microbiome. - Science (80-.). 338: 198–199.

Fitzpatrick, C. L. and Servedio, M. R. 2018. The evolution of male mate choice and female ornamentation: A review of mathematical models. - Curr. Zool. 64: 323–333.

Flórez, L. V. and Kaltenpoth, M. 2017. Symbiont dynamics and strain diversity in the defensive mutualism between *Lagria* beetles and *Burkholderia*. - Environ. Microbiol. 19: 3674–3688.

Flórez, L. V, Biedermann, P. H. W., Engl, T. and Kaltenpoth, M. 2015. Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. - Nat. Prod. Rep. 32: 904–936.

Folstad, I. and Karter, A. J. 1992. Parasites, bright males, and the immunocompetence handicap. - Am. Nat. 139: 603–622.

Fons, M., Gomez, A. and Karjalainen, T. 2000. Mechanisms of Colonisation and Colonisation Resistance of the Digestive Tract Part 2: Bacteria/Bacteria Interactions. - Microb. Ecol. Health Dis. 12: 240–246.

Fontaine, J. J. and Martin, T. E. 2006. Parent birds assess nest predation risk and adjust their reproductive strategies. - Ecol. Lett. 9: 428–434.

Fox, H. M. and Vevers, G. 1960. The nature of animal colors.

Friedman, J. and Alm, E. J. 2012. Inferring Correlation Networks from Genomic Survey Data. - PLoS Comput. Biol. 8: e1002687.

Fronstin, R. B., Doucet, S. M. and Christians, J. K. 2016. Haematocrit, eggshell colouration and sexual signaling in the European starling (Sturnus vulgaris). - BMC Ecol. in press.

Gamble, K. C. 2007. Internal Anatomy of the Hornbill Casque Described by Radiography , Contrast Radiography , and Computed Tomography. - J. Avian Med. Surg. 21: 38–49.

Gamble, K. C. 2012. Chapter 37. Squamous Cell Carcinoma in Buceros Hornbills. - In: Miller, R. E. and Fowler, M. (eds), Fowler's Zoo and Wild Animal Medicine. pp. 281–285.

Garamszegi, Z. and Møller, A. P. 2010. Effects of sample size and intraspecific variation in phylogenetic comparative studies : a meta-analytic review. - Biol. Rev. 85: 797–805.

Garamszegi, L. Z. and Møller, A. P. 2011. Nonrandom Variation in Within-Species Sample Size and Missing Data in Phylogenetic Comparative Studies. - Syst. Biol. 60: 876–880.

Gaston, A. J., De Forest, L. N. and G.Noble, D. 1993. Egg Recognition and Egg Stealing in Murres (Uria spp.). - Anim. Behav. 45: 301–306.

Gawryszewski, F. M. 2018. Color vision models: Some simulations, a general n-dimensional model, and the colourvision R package. - Ecol. Evol.: 8159–8170.

Giard, J.-C., Laplace, J.-M., Rincé, A., Pichereau, V., Benachour, A., Leboeuf, C., Flahaut, S., Auffray, Y. and Hartke, A. 2001. The stress proteome of *Enterococcus faecalis*. - Electrophoresis 22: 2947–2954.

Gil-Turnes, M. S. and Fenical, W. 1992. Embryos of Homarus americanus are Protected by Epibiotic Bacteria. - Biol. Bull. 182: 105–108.

Giordano, M., Costantini, D., Pick, J. L. and Tschirren, B. 2015. Female oxidative status, egg antioxidant protection and eggshell pigmentation: a supplemental feeding experiment in great tits. - Behav. Ecol. Sociobiol. 69: 777–785.

Goldstein, G., Flory, K. R., Browne, B. A., Majid, S., Ichida, J. M. and Burtt Jr., E. H. 2004. Bacterial Degradation of Black and White Feathers. - Auk 121: 656–659.

Golob, J. L., Margolis, E., Hoffman, N. G. and Fredricks, D. N. 2017. Evaluating the accuracy of amplicon-based microbiome computational pipelines on simulated human gut microbial communities. - BMC Bioinformatics 18: 283.

Gómez, D. 2006. AVICOL a program to analyse spectrometric data. Last update october 2011. Free executable available at http://sites.google.com/site/avicolprogram/ or from the author at dodogomez@yahoo.fr. in press.

González-Braojos, S., Vela, A. I., Ruiz-De-Castañeda, R., Briones, V., Cantarero, A. and Moreno, J. 2012. Is Nestling Growth Affected by Nest Reuse and Skin Bacteria in Pied Flycatchers *Ficedula hypoleuca?* - Acta Ornithol. 47: 119–127.

Gonzalez, J. C. T., Sheldon, B. C., Collar, N. J. and Tobias, J. A. 2013. A comprehensive molecular phylogeny for the hornbills (Aves: Bucerotidae). - Mol. Phylogenet. Evol. 67: 468–483.

Gosler, A. G., Higham, J. P. and Reynolds, S. J. 2005. Why are birds ' eggs speckled ? - Ecol. Lett. 8: 1105–1113.

Grether, G. F., Hudon, J. and Millie, D. F. 1999. Carotenoid limitation of sexual coloration along an environmental gradient in guppies. - Proc. R. Soc. London. Ser. B Biol. Sci. 266: 1317–1322.

Griggio, M., Valera, F., Casas, A. and Pilastro, A. 2005. Males prefer ornamented females: A field experiment of male choice in the rock sparrow. - Anim. Behav. 69: 1243–1250.

Grizard, S., Dini-Andreote, F., Tieleman, B. I. and Salles, J. F. 2014. Dynamics of bacterial and fungal communities associated with eggshells during incubation. - Ecol. Evol. 4: 1140–1157.

Grizard, S., Versteegh, M. A., Ndithia, H. K., Salles, J. F. and Tieleman, B. I. 2015. Shifts in Bacterial Communities of Eggshells and Antimicrobial Activities in Eggs during Incubation in a Ground-Nesting Passerine. - PLoS One: 10(4).

Gunderson, A. R. 2008. Feather-Degrading Bacteria: A New Frontier in Avian and Host–Parasite Research? - Auk 125: 972–979.

Gwinner, H. and Berger, S. 2005. European starlings: Nestling condition, parasites and green nest material during the breeding season. - J. Ornithol. 146: 365–371.

Haine, E. R. 2008. Symbiont-mediated protection. - Proc. R. Soc. B Biol. Sci. 275: 353–361.

Hamilton, W. D. and Zuk, M. 1982. Heritable true fitness and bright birds: a role for parasites? - Science (80-.). 218: 384–387.

Hanley, D. 2012. The function and evolution of egg colour in birds. - Electron. Theses Diss.: 382.

Hanley, D. and Doucet, S. M. 2009. Egg coloration in ring-billed gulls (Larus delawarensis): A test of the sexual signaling hypothesis. - Behav. Ecol. Sociobiol. 63: 719–729.

Hanley, D., Heiber, G. and Dearborn, D. C. 2008. Testing an Assumption of the Sexual-Signaling Hypothesis: Does Blue-Green Egg Color Reflect Maternal Antioxidant Capacity? - Condor 110: 767–771.

Hanley, D., Doucet, S. M. and Dearborn, D. C. 2010. A Blackmail Hypothesis for the Evolution of Conspicuous Egg Coloration in Birds. - Auk 127: 453–459.

Hargitai, R., Boross, N., Nyiri, Z. and Eke, Z. 2018a. Effects of food limitation on the intensity of blue-green and brown eggshell coloration: an experimental study with the canary. - J. Avian Biol.: e01486.

Hargitai, R., Herényi, M., Nagy, G. and Török, J. 2018b. Eggshell spotting pattern is related to hatching asynchrony, hematocrit value and growth of nestling great tits Parus major. - J. Avian Biol. 49: e01827.

Hart, N. S. 2001. The visual ecology of avian Photoreceptors. - Prog. Retin. Eye Res. 20, No. 5: 675 to 703.

Hart, N. S. 2002. Vision in the peafowl (Aves: Pavo cristatus). - J. Exp. Biol. 205: 3925–3935.

Harvey, P. H. and Pagel, M. D. 1991. The Comparative Method in Evolutionary Biology. - Oxford University Press.

He, Y., Caporaso, J. G., Jiang, X.-T., Sheng, H.-F., Huse, S. M., Rideout, J. R., Edgar, R. C., Kopylova, E., Walters, W. A., Knight, R. and Zhou, H.-W. 2015. Stability of operational taxonomic units: an important but neglected property for analyzing microbial diversity. - Microbiome 3: 20.

Heath-Heckman, E. A. C., Peyer, S. M., Whistler, C. A., Apicella, M. A., Goldman, W. E. and McFall-Ngai, M. J. 2013. Bacterial Bioluminescence Regulates Expression of a Host Cryptochrome Gene in the Squid-Vibrio Symbiosis (J Handelsman, Ed.). - MBio 4: e00167-13.

Heath, K. D. and Tiffin, P. 2007. Context dependence in the coevolution of plant and rhizobial mutualists. - Proc. R. Soc. B Biol. Sci. 274: 1905–1912.

Henry, L. P., Bruijning, M., Forsberg, S. K. G. and Ayroles, J. F. 2019. Can the microbiome

influence host evolutionary trajectories? - bioRxiv: 700237.

Hill, M. J. 1997. Intestinal flora and endogenous vitamin synthesis. - Eur. J. Cancer Prev. 6 Suppl 1: S43-45.

Hill, G. E. 2000. Energetic constraints on expression of carotenoid-based plumage coloration. - J. Avian Biol. 31: 559–566.

Hill, G. E. 2002. A red bird in a brown bag: the function and evolution of ornamental plumage coloration in the house finch. - Oxford University Press.

Hill, G. E. 2006. Environmental Regulation of Ornamental Coloration. - In: Hill, G. E. and Mcgraw, K. J. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 507–560.

Hill, G. E., Farmer, K. L. and Beck, M. L. 2004. The effect of mycoplasmosis on carotenoid plumage coloration in male house finches. - J. Exp. Biol. 207: 2095–2099.

Hird, S. M. 2017. Evolutionary Biology Needs Wild Microbiomes. - Front. Microbiol. 8: 725.

Hoffmann, J., Postma, E. and Schaub, M. 2015. Factors influencing double brooding in Eurasian Hoopoes Upupa epops. - Ibis (Lond. 1859). 157: 17–30.

Holveck, M.-J., Doutrelant, C., Guerreiro, R., Perret, P., Gomez, D. and Grégoire, A. 2010. Can eggs in a cavity be a female secondary sexual signal? Male nest visits and modelling of egg visual discrimination in blue tits. - Biol. Lett. 6: 453–457.

Holveck, M., Guerreiro, R., Perret, P., Doutrelant, C. and Grégoire, A. 2019. Eggshell coloration indicates female condition during egg-laying: a field experiment in blue tits. - Biol. J. Linn. Soc. XX: 1–20.

Honza, M., Požgayová, M., Procházka, P. and Cherry, M. I. 2011. Blue-green eggshell coloration is not a sexually selected signal of female quality in an open-nesting polygynous passerine. - Naturwissenschaften 98: 493–499.

Hornung, B. V. H., Zwittink, R. D. and Kuijper, E. J. 2019. Issues and current standards of controls in microbiome research. - FEMS Microbiol. Ecol. 95: fiz045.

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y. and Fukatsu, T. 2010. Wolbachia as a

bacteriocyte-associated nutritional mutualist. - Proc. Natl. Acad. Sci. 107: 769–774.

Hoyt, D. F. 1979. Practical methods of estimating volume and fresh weight of bird eggs. - Auk 96: 73–77.

Jacob, J. and Ziswiler, V. 1982. The uropygial gland. - In: Farner, D. S. et al. (eds), Avian biology, vol VI. Academic Press, pp. 359–362.

Jacob, S., Parthuisot, N., Vallat, A., Ramon-Portugal, F., Helfenstein, F. and Heeb, P. 2015. Microbiome affects egg carotenoid investment, nestling development and adult oxidative costs of reproduction in Great tits. - Funct. Ecol. 29: 1048–1058.

Janzen, D. H. 1985. The natural history of mutualism. - In: Boucher, D. H. (ed), The Biology of Mutualism: Ecology and Evolution. Oxford University Press, pp. 40–99.

Javůrková, V. G., Kreisinger, J., Procházka, P., Požgayová, M., Ševčíková, K., Brlík, V., Adamík, P., Heneberg, P. and Porkert, J. 2019. Unveiled feather microcosm: feather microbiota of passerine birds is closely associated with host species identity and bacteriocin-producing bacteria. - ISME J.: 2363–2376.

Jervis-Bardy, J., Leong, L. E. X., Marri, S., Smith, R. J., Choo, J. M., Smith-Vaughan, H. C., Nosworthy, E., Morris, P. S., O'Leary, S., Rogers, G. B. and Marsh, R. L. 2015. Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. - Microbiome 3: 19.

Jetz, W., Thomas, G. H., Joy, J. B., Hartmann, K. and Mooers, A. O. 2012. The global diversity of birds in space and time. - Nature 491: 444–448.

Johnsen, A., Vesterkjær, K. and Slagsvold, T. 2011. Do Male Pied Flycatchers (Ficedula hypoleuca) Adjust Their Feeding Effort According to Egg Colour? - Ethology 117: 309–317.

Johnstone, R. A. 1995. Sexual selection, honest advertisement and the handicap principle: reviewing the evidence. - Biol. Rev. 70: 1–65.

Kaltenpoth, M., Göttler, W., Herzner, G. and Strohm, E. 2005. Symbiotic Bacteria Protect Wasp Larvae from Fungal Infestation. - Curr. Biol. 15: 475–479.

Kaltz, O. and Koella, J. C. 2003. Host growth conditions regulate the plasticity of horizontal and vertical transmission in holospora undulata, a bacterial parasite of the protozoan paramecium caudatum. - Evolution (N. Y). 57: 1535–1542.

Karstens, L., Asquith, M., Davin, S., Fair, D., Gregory, W. T., Wolfe, A. J., Braun, J. and McWeeney, S. 2019. Controlling for Contaminants in Low-Biomass 16S rRNA Gene Sequencing Experiments (JA Gilbert, Ed.). - mSystems 4: e00290-19.

Kaur, H., Hughes, M. N., Green, C. J., Naughton, P., Foresti, R. and Motterlini, R. 2003. Interaction of bilirubin and biliverdin with reactive nitrogen species. - FEBS Lett. 543: 113–119.

Keebaugh, E. S., Yamada, R., Obadia, B., Ludington, W. B. and Ja, W. W. 2018. Microbial Quantity Impacts Drosophila Nutrition, Development, and Lifespan. - iScience 4: 247–259.

Kemp, A. C. 2001. Family Bucerotidae (Hornbills). - In: Del Hoyo, J. et al. (eds), Handbook of the birds of the world, Piciformes to Bucerotiformes, vol 6. Lynx Editi. pp. 436–523.

Kemp, D. J., Herberstein, M. E., Fleishman, L. J., Endler, J. a., Bennett, A. T. D., Dyer, A. G., Hart, N. S., Marshall, J. and Whiting, M. J. 2015. An Integrative Framework for the Appraisal of Coloration in Nature. - Am. Nat. 185: 000–000.

Kikuchi, Y., Hayatsu, M., Hosokawa, T., Nagayama, A., Tago, K. and Fukatsu, T. 2012. Symbiont-mediated insecticide resistance. - Proc. Natl. Acad. Sci. 109: 8618–8622.

Kilner, R. M. 2006. The evolution of egg colour and patterning in birds. - Biol. Rev. Camb. Philos. Soc. 81: 383–406.

Kinnaird, M. F. and O'Brien, T. G. 2007. The Ecology and Conservation of Asian Hornbills: Farmers of the Forest.

Knight, R., Vrbanac, A., Taylor, B. C., Aksenov, A., Callewaert, C., Debelius, J., Gonzalez, A., Kosciolek, T., McCall, L. I., McDonald, D., Melnik, A. V., Morton, J. T., Navas, J., Quinn, R. A., Sanders, J. G., Swafford, A. D., Thompson, L. R., Tripathi, A., Xu, Z. Z., Zaneveld, J. R., Zhu, Q., Caporaso, J. G. and Dorrestein, P. C. 2018. Best practices for analysing microbiomes. - Nat. Rev. Microbiol. 16: 410–422.

Kopylova, E., Navas-molina, J. A., Mercier, C., Xu, Z., Mahé, F., He, Y., Zhou, H.-W., Rognes, T., Caporaso, J. G. and Knight, R. 2016. Open-Source Sequence Clustering Methods Improve the State Of the Art. mSystems. 1: 1–16.

Kose, M. and Møller, A. P. 1999. Sexual selection, feather breakage and parasites: the importance of white spots in the tail of the barn swallow (Hirundo rustica). - Behav. Ecol. Sociobiol. 45: 430–436.

Kraaijeveld, K., Kraaijeveld-Smit, F. J. L. and Komdeur, J. 2007. The evolution of mutual ornamentation. - Anim. Behav. 74: 657–677.

Krist, M. and Grim, T. 2007. Are blue eggs a sexually selected signal of female collared flycatchers? A cross-fostering experiment. - Behav. Ecol. Sociobiol. 61: 863–876.

Kuczynski, J., Stombaugh, J., Walters, W. A., González, A., Caporaso, J. G. and Knight, R. 2012. Using QIIME to analyze 16s rRNA gene sequences from microbial communities. - Curr. Protoc. Microbiol. in press.

Labocha, M. K. and Hayes, J. P. 2012. Morphometric indices of body condition in birds: A review. - J. Ornithol. 153: 1–22.

Lahti, D. C. 2008. Population Differentiation and Rapid Evolution of Egg Color in Accordance With Solar Radiation. - Auk 125: 796–802.

Larsson, A. J. M., Stanley, G., Sinha, R., Weissman, I. L. and Sandberg, R. 2018. Computational correction of index switching in multiplexed sequencing libraries. - Nat. Methods 15: 305–307.

Law-Brown, J. and Meyers, P. R. 2003. *Enterococcus phoeniculicola sp . nov .*, a novel member of the enterococci isolated from the uropygial gland of the Red-billed Woodhoopoe , *Phoeniculus purpureus.* - Int. J. Syst. Evol. Microbiol. 53: 683–685.

Leclaire, S., Nielsen, J. F. and Drea, C. M. 2014. Bacterial communities in meerkat anal scent secretions vary with host sex, age, and group membership. - Behav. Ecol. 25: 996–1004.

Leclaire, S., Jacob, S., Greene, L. K., Dubay, G. R. and Drea, C. M. 2017. Social odours covary with bacterial community in the anal secretions of wild meerkats. - Sci. Rep. 7: 3240.

Leftwich, P. T., Hutchings, M. I. and Chapman, T. 2018. Diet, Gut Microbes and Host Mate Choice. - BioEssays 40: 1800053.

Leitner, S., Marshall, R. C., Leisler, B. and Catchpole, C. K. 2006. Male song quality, egg size and offspring sex in captive canaries (Serinus canaria). - Ethology 112: 554–563.

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D. and Gordon, J. I. 2005. Obesity alters gut microbial ecology. - Proc. Natl. Acad. Sci. 102: 11070–11075.

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R. and Gordon, J. I. 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. - Nat. Rev. Microbiol. 6: 776–788.

Li, D., Chen, B., Zhang, L., Gaur, U., Ma, T., Jie, H., Zhao, G., Wu, N., Xu, Z., Xu, H., Yao, Y., Lian, T., Fan, X., Yang, D., Yang, M., Zhu, Q. and Satkoski Trask, J. 2016. The musk chemical composition and microbiota of Chinese forest musk deer males. - Sci. Rep. 6: 18975.

Lindquist, N., Barber, P. H. and Weisz, J. B. 2005. Episymbiotic microbes as food and defence for marine isopods: unique symbioses in a hostile environment. - Proc. R. Soc. B Biol. Sci. 272: 1209–1216.

Linville, S. U., Breitwisch, R. and Schilling, A. J. 1998. Plumage brightness as an indicator of parental care in northern cardinals. - Anim. Behav. 55: 119–127.

Lopetuso, L. R., Scaldaferri, F., Petito, V. and Gasbarrini, A. 2013. Commensal Clostridia: leading players in the maintenance of gut homeostasis. - Gut Pathog. 5: 23.

López-Rull, I., Gil, M. and Gil, D. 2007. Spots in starling Sturnus unicolor eggs are good indicators of ectoparasite load by Carnus hemapterus (Diptera: Carnidae). - Ardeola 54: 131–134.

López-Rull, I., Pagán, I. and Macías Garcia, C. 2010. Cosmetic enhancement of signal coloration: Experimental evidence in the house finch. - Behav. Ecol. 21: 781–787.

Lozupone, C. and Knight, R. 2005. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. - Appl. Environ. Microbiol. 71: 8228–8235.

Lyu, N., Servedio, M. R., Lloyd, H. and Sun, Y. 2017. The evolution of postpairing male

mate choice. - Evolution (N. Y). 71: 1465–1477.

Macpherson, A. J. and Harris, N. L. 2004. Interactions between commensal intestinal bacteria and the immune system. - Nat. Rev. Immunol. 4: 478–485.

Maddison, W. . and Maddison, D. R. 2018. Mesquite: a modular system for evolutionary analysis. Version 3.6.: http://www.mesquiteproject.org.

Maier, R. M., Pepper, I. L. and Gerba, C. P. 2000. Environmental Microbiology. - Academic Press, San Diego, California.

Mainwaring, M. C., Hartley, I. R., Lambrechts, M. M. and Deeming, D. C. 2014. The design and function of birds' nests. - Ecol. Evol. 4: 3909–3928.

Mallows, C. L. 1973. Some Comments on Cp. - Technometrics 15: 661–675.

Maraci, Ö., Engel, K. and Caspers, B. A. 2018. Olfactory communication via microbiota: what is known in birds? - Genes (Basel). in press.

Martín-Platero, A. M., Valdivia, E., Ruíz-Rodríguez, M., Soler, J. J., Martín-Vivaldi, M., Maqueda, M. and Martínez-Bueno, M. 2006. Characterization of antimicrobial substances produced by *Enterococcus faecalis* MRR 10-3, isolated from the uropygial gland of the hoopoe (*Upupa epops*). - Appl. Environ. Microbiol. 72: 4245–4249.

Martín-Vivaldi, M., Palomino, J. J., Soler, M. and Soler, J. J. 1999. Determinants of reproductive success in the Hoopoe *Upupa epops*, a hole-nesting non-passerine bird with asynchronous hatching. - Bird Study 46: 205–216.

Martín-Vivaldi, M., Ruiz-Rodríguez, M., Soler, J. J., Peralta-Sánchez, J. M., Méndez, M., Valdivia, E., Martín-Platero, A. M. and Martínez-Bueno, M. 2009. Seasonal, sexual and developmental differences in hoopoe Upupa epops preen gland morphology and secretions: Evidence for a role of bacteria. - J. Avian Biol. 40: 191–205.

Martín-Vivaldi, M., Peña, A., Peralta-Sánchez, J. M., Sánchez, L., Ananou, S., Ruiz-Rodríguez, M. and Soler, J. J. 2010. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. - Proc. Biol. Sci. 277: 123–130.

Martín-Vivaldi, M., Soler, J. J., Peralta-Sánchez, J. M., Arco, L., Martín-Platero, A. M.,

Martínez-Bueno, M., Ruiz-Rodríguez, M. and Valdivia, E. 2014a. Special structures of hoopoe eggshells enhance the adhesion of symbiont-carrying uropygial secretion that increase hatching success. - J. Anim. Ecol. 83: 1289–1301.

Martín-Vivaldi, M., Doña, J., Romero Masegosa, J. and Soto Cárdenas, M. 2014b. Abubilla - Upupa epops Linnaeus, 1758. - En Encicl. Virtual los Vertebr. Españoles. Salvador. A., Morales, M. B. (Eds.). Mus. Nac. Ciencias Nat. Madrid. http//www.vertebradosibericos.org/

Martín-Vivaldi, M., Soler, J. J., Martínez-García, Á., Arco, L., Juárez-García-Pelayo, N., Ruiz-Rodríguez, M. and Martínez-Bueno, M. 2018. Acquisition of Uropygial Gland Microbiome by Hoopoe Nestlings. - Microb. Ecol. 76: 285–297.

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. - EMBnet.journal 17: 10–12.

Martin, T. E. and Badyaev, A. V 1996. Sexual dichromatism in birds: importance of nest predation and nest location for females versus males. - Evolution (N. Y). 50: 2454–2460.

Martínez-De La Puente, J., Merino, S., Moreno, J., Tomás, G., Morales, J., Lobato, E., García-Fraile, S. and Martínez, J. 2007. Are eggshell spottiness and colour indicators of health and condition in blue tits Cyanistes caeruleus? - J. Avian Biol. 38: 377–384.

Martínez-García, Á. 2015. Biodiversidad bacteriana de la secreción uropigial y la cáscara de los huevos de abubilla: estabilidad y adquisición.

Martínez-García, Á., Soler, J. J., Rodríguez-Ruano, S. M., Martínez-Bueno, M., Martín-Platero, A. M., Juárez-García, N. and Martín-Vivaldi, M. 2015. Preening as a Vehicle for Key Bacteria in Hoopoes. - Microb. Ecol. 70: 1024–1033.

Martínez-García, Á., Martín-Vivaldi, M., Rodríguez-Ruano, S. M., Peralta-Sánchez, J. M., Valdivia, E. and Soler, J. J. 2016a. Nest bacterial environment affects microbiome of hoopoe eggshells, but not that of the uropygial secretion. - PLoS One 11(7): e0158158.

Martínez-García, Á., Martín-Vivaldi, M., Ruiz-Rodríguez, M., Martínez-Bueno, M., Arco, L., Rodríguez-Ruano, S. M., Peralta-Sánchez, J. M. and Soler, J. J. 2016b. The Microbiome of the Uropygial Secretion in Hoopoes Is Shaped Along the Nesting Phase. - Microb.

Ecol. 72: 252–261.

Massaro, M., Davis, L. S. and Darby, J. T. 2003. Carotenoid-derived ornaments reflect parental quality in male and female yellow-eyed penguins (Megadyptes antipodes). - Behav. Ecol. Sociobiol. 55: 169–175.

Mazgajski, T. D. 2007. Effect of old nest material on nest site selection and breeding parameters in secondary hole nesters — a review. - Acta Ornithol. 42: 1–14.

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., Desantis, T. Z., Probst, A., Andersen, G. L., Knight, R. and Hugenholtz, P. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. - ISME J. 6: 610–618.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E., Rawls, J. F., Reid, A., Ruby, E. G., Rumpho, M., Sanders, J. G., Tautz, D. and Wernegreen, J. J. 2013. Animals in a bacterial world, a new imperative for the life sciences. - Proc. Natl. Acad. Sci. U. S. A. 110: 3229–3236.

Mcgraw, K. J. and Hill, G. E. 2004. Plumage color as a dynamic trait : carotenoid pigmentation of male house finches (Carpodacus mexicanus) fades during the breeding season. - Can. J. Zool 82: 734–738.

McGraw, K. J. and Ardia, D. R. 2003. Carotenoids, Immunocompetence, and the Information Content of Sexual Colors: An Experimental Test. - Am. Nat. 162: 704–712.

McKnight, D. T., Huerlimann, R., Bower, D. S., Schwarzkopf, L., Alford, R. A. and Zenger, K. R. 2019. microDecon: A highly accurate read-subtraction tool for the post-sequencing removal of contamination in metabarcoding studies. - Environ. DNA 1: 14–25.

Mikšík, I., Holáň, V. and Deyl, Z. 1996. Avian eggshell pigments and their variability. - Comp. Biochem. Physiol. 113: 607–612.

Miller, R. E., Trampel, D. W., Boever, W. J. and Kling, M. A. 1985. Carcinoma in the Casque of a Greater Indian Hornbill (Buceros bicornis). - J. Zoo Anim. Med. 16: 131–136.

Minich, J. J., Zhu, Q., Janssen, S., Hendrickson, R., Amir, A., Vetter, R., Hyde, J., Doty, M. M., Stillwell, K., Benardini, J., Kim, J. H., Allen, E. E., Venkateswaran, K. and Knight, R. 2018. KatharoSeq Enables High-Throughput Microbiome Analysis from Low-Biomass Samples (MJ McFall-Ngai, Ed.). - mSystems 3: e00218-17.

Møller, A. P. 1993. Sexual Selection in the Barn Swallow Hirundo rustica. III. Female Tail Ornaments. - Evolution (N. Y). 47: 417–431.

Møller, A. P. and Erritzoe, J. 1996. Parasite Virulence and Host Immune Defense : Host Immune Response is Related to Nest Reuse in Birds. - Evolution (N. Y). 50: 2066–2072.

Møller, A. P., Arriero, E., Lobato, E. and Merino, S. 2009. A meta-analysis of parasite virulence in nestling birds. - Biol. Rev. 84: 567–588.

Montgomerie, R. 2006. Cosmetic and adventitious colors. - In: Hill, E. and McGraw, K. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 399–427.

Morales, J., Sanz, J. J. and Moreno, J. 2006. Egg colour reflects the amount of yolk maternal antibodies and fledging success in a songbird. - Biol. Lett. 2: 334–336.

Morales, J., Moreno, J., Merino, S., Sanz, J. J., Tomás, G., Arriero, E., Lobato, E. and Martínez-De La Puente, J. 2007. Female ornaments in the Pied Flycatcher Ficedula hypoleuca: Associations with age, health and reproductive success. - Ibis (Lond. 1859). 149: 245–254.

Morales, J., Velando, A. and Moreno, J. 2008. Pigment allocation to eggs decreases plasma antioxidants in a songbird. - Behav. Ecol. Sociobiol. 63: 227–233.

Moran, N. A. 2006. Symbiosis. - Curr. Biol. 16: R866–R871.

Moran, N. A., McCutcheon, J. P. and Nakabachi, A. 2008. Genomics and Evolution of Heritable Bacterial Symbionts. - Annu. Rev. Genet. 42: 165–190.

Moreau, R. E. 1934. Breeding Habits of Hornbills. - Nature 134: 899.

Moreno, J. and Osorno, J. L. 2003. Avian egg colour and sexual selection: Does eggshell pigmentation reflect female condition and genetic quality? - Ecol. Lett. 6: 803–806.

Moreno, J., Briones, V., Merino, S., Ballesteros, C., Sanz, J. J. and Tomás, G. 2003. Beneficial Effects of Cloacal Bacteria on Growth and Fledging Size in Nestling Pied Flycatchers (Ficedula Hypoleuca) in Spain. - Auk 120: 784–790.

Moreno, J., Osorno, J. L., Morales, J., Merino, S. and Tomás, G. 2004. Egg Colouration and Male Parental Effort in the Pied Flycatcher Ficedula Hypoleuca. - J. Avian Biol. 35: 300–304.

Moreno, J., Morales, J., Lobato, E., Merino, S., Tomás, G. and Martínez-De La Puente, J. 2005. Evidence for the signaling function of egg color in the pied flycatcher Ficedula hypoleuca. - Behav. Ecol. 16: 931–937.

Moreno, J., Morales, J., Lobato, E., Merino, S., Tomás, G. and Martínez-De La Puente, J. 2006a. More colourful eggs induce a higher relative paternal investment in the pied flycatcher Ficedula hypoleuca: A cross-fostering experiment. - J. Avian Biol. 37: 555–560.

Moreno, J., Lobato, E., Morales, J., Merino, S., Tomás, G., Martínez-De La Puente, J., Sanz, J. J., Mateo, R. and Soler, J. J. 2006b. Experimental evidence that egg color indicates female condition at laying in a songbird. - Behav. Ecol. 17: 651–655.

Moreno, J., Lobato, E., Merino, S. and Martínez-De La Puente, J. 2008. Blue-green eggs in pied flycatchers: An experimental demonstration that a supernormal stimulus elicits improved nestling condition. - Ethology 114: 1078–1083.

Morton, J. T., Sanders, J., Quinn, R. A., McDonald, D., Gonzalez, A., Vázquez-Baeza, Y., Navas-Molina, J. A., Song, S. J., Metcalf, J. L., Hyde, E. R., Lladser, M., Dorrestein, P. C. and Knight, R. 2017. Balance Trees Reveal Microbial Niche Differentiation (JK Jansson, Ed.). - mSystems 2: e00162-16.

Mushegian, A. A., Walser, J.-C., Sullam, K. E. and Ebert, D. 2018. The microbiota of diapause: How host–microbe associations are formed after dormancy in an aquatic crustacean. - J. Anim. Ecol. 87: 400–413.

Naish, D. 2015. The Ecology and Conservation of Asian Hornbills: Farmers of the Forest. - Hist. Biol. 27: 954–956.

Navas-Molina, J. A., Peralta-Sánchez, J. M., González, A., McMurdie, P. J., Vázquez-Baeza, Y., Xu, Z., Ursell, L. K., Lauber, C., Zhou, H., Song, S. J., Huntley, J., Ackermann, G. L., Berg-Lyons, D., Holmes, S., Caporaso, J. G. and Knight, R. 2013. Advancing our understanding of the human microbiome using QIIME. - Elsevier Inc.

Negro, J. J., Margalida, A., Hiraldo, F. and Heredia, R. 1999. The function of the cosmetic coloration of bearded vultures : when art imitates life. - Anim. Behav. S8: 14–17.

Nicholls, E. M. and Rienits, K. G. 1971. Tryptophan derivatives and pigment in the hair of some Australian marsupials. - Int. J. Biochem. 2: 593–603.

Ödeen, A. and Håstad, O. 2003. Complex distribution of avian color vision systems revealed by sequencing the SWS1 opsin from total DNA. - Mol. Biol. Evol. 20: 855–861.

Oliver, K. M., Russell, J. A., Moran, N. A. and Hunter, M. S. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. - Proc. Natl. Acad. Sci. 100: 1803 LP – 1807.

Olson, V. A. and Owens, I. P. F. 1998. Costly sexual signals: are carotenoids rare, risky or required? - Trends Ecol. Evol. 13: 510–514.

Olsson, K. and Allander, K. 1995. Do Fleas, and/or Old Nest Material, Influence Nest-site preference in Hole-nesting Passerines? - Ethology 101: 160–170.

Olsson, P., Lind, O. and Kelber, A. 2015. Bird colour vision: behavioural thresholds reveal receptor noise. - J. Exp. Biol. 218: 184–193.

Orell, M., Rytkönen, S. and Ilomäki, K. 1993. Do pied flycatchers prefer nest boxes with old nest material? - Ann. Zool. Fennici 30: 313–316.

Pagel, M. 1997. Inferring evolutionary processes from phylogenies. - Zool. Scr. 26: 331–348.

Pagel, M. 1999. Inferring the historical patterns of biological evolution. - Nature 401: 877–884.

Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A. and Brown, P. O. 2007. Development of the Human Infant Intestinal Microbiota. - PLoS Biol. 5: e177.

Paradis, E., Claude, J. and Strimmer, K. 2004. APE : Analyses of Phylogenetics and

Evolution in R language. - Bioinformatics 20: 289–290.

Paz, L.-C., Schramm, A. and Lund, M. B. 2017. Biparental transmission of Verminephrobacter symbionts in the earthworm Aporrectodea tuberculata (Lumbricidae). - FEMS Microbiol. Ecol. 93: fix025.

Pearce, D. S., Hoover, B. A., Jennings, S., Nevitt, G. A. and Docherty, K. M. 2017. Morphological and genetic factors shape the microbiome of a seabird species (Oceanodroma leucorhoa) more than environmental and social factors. - Microbiome 5: 146.

Peig, J. and Green, A. J. 2010. The paradigm of body condition: A critical reappraisal of current methods based on mass and length. - Funct. Ecol. 24: 1323–1332.

Peralta-Sanchez, J. M., Møller, A. P., Martin-Platero, A. M. and Soler, J. J. 2010. Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: An experimental study. - Funct. Ecol. 24: 426–433.

Peralta-Sánchez, J. M., Martín-Vivaldi, M., Martín-Platero, A. M., Martínez-Bueno, M., Oñate, M., Ruiz-Rodríguez, M. and Soler, J. J. 2012. Avian life history traits influence eggshell bacterial loads: A comparative analysis. - Ibis (Lond. 1859). 154: 725–737.

Peralta-Sánchez, J. M., Soler, J. J., Martín-Platero, A. M., Knight, R., Martínez-Bueno, M. and Møller, A. P. 2014. Eggshell Bacterial Load Is Related to Antimicrobial Properties of Feathers Lining Barn Swallow Nests. - Microb. Ecol. 67: 480–487.

Pérez-Rodríguez, L., Mougeot, F. and Bortolotti, G. R. 2011. The effects of preen oils and soiling on the UV – visible reflectance of carotenoid-pigmented feathers. - Behav. Ecol. Sociobiol. 65: 1425–1435.

Piault, R., Gasparini, J., Bize, P., Paulet, M., McGraw, K. J. and Roulin, A. 2008. Experimental support for the makeup hypothesis in nestling tawny owls (Strix aluco). - Behav. Ecol. 19: 703–709.

Plard, F., Arlettaz, R. and Schaub, M. 2018. Hoopoe males experience intra-seasonal while females experience inter-seasonal reproductive costs. - Oecologia 186: 665–675.

Podofillini, S., Cecere, J. G., Griggio, M., Curcio, A., De Capua, E. L., Fulco, E., Pirrello, S.,

Saino, N., Serra, L., Visceglia, M. and Rubolini, D. 2018. Home, dirty home: effect of old nest material on nest-site selection and breeding performance in a cavity-nesting raptor. - Curr. Zool.: 1–10.

Poláček, M., Griggio, M., Mikšík, I., Bartíková, M., Eckenfellner, M. and Hoi, H. 2017. Eggshell coloration and its importance in postmating sexual selection. - Ecol. Evol. 7: 941–949.

Poonswad, P., Kemp, A. and Strange, M. 2013. Hornbills of the world: A photographic guide.

Price, M. N., Dehal, P. S. and Arkin, A. P. 2009. Fasttree: Computing large minimum evolution trees with profiles instead of a distance matrix. - Mol. Biol. Evol. 26: 1641–1650.

Prum, R. O. 2006. Anatomy, Physics, and Evolution of Structural Colors. - In: Hill, G. E. and Mcgraw, K. J. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 295–353.

Refsnider, J. M. and Janzen, F. J. 2010. Putting Eggs in One Basket: Ecological and Evolutionary Hypotheses for Variation in Oviposition-Site Choice. - Annu. Rev. Ecol. Evol. Syst. 41: 39–57.

Reichlin, T. S., Hobson, K. A., Van Wilgenburg, S. L., Schaub, M., Wassenaar, L. I., Martín-Vivaldi, M., Arlettaz, R. and Jenni, L. 2013. Conservation through connectivity: Can isotopic gradients in Africa reveal winter quarters of a migratory bird? - Oecologia 171: 591–600.

Renoult, J. P., Kelber, A. and Schaefer, H. M. 2017. Colour spaces in ecology and evolutionary biology. - Biol. Rev. 92: 292–315.

Reynolds, S. J., Martin, G. R. and Cassey, P. 2009. Is sexual selection blurring the functional significance of eggshell coloration hypotheses? - Anim. Behav. 78: 209–215.

Richard, F.-J. 2017. Symbiotic Bacteria Influence the Odor and Mating Preference of Their Hosts. - Front. Ecol. Evol. 5: 1–9.

Rideout, J. R., He, Y., Navas-Molina, J. A., Walters, W. A., Ursell, L. K., Gibbons, S. M., Chase, J., McDonald, D., Gonzalez, A., Robbins-Pianka, A., Clemente, J. C., Gilbert, J.

A., Huse, S. M., Zhou, H.-W., Knight, R. and Caporaso, J. G. 2014. Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. - PeerJ 2: e545.

Riehl, C. 2011. Paternal Investment and the "Sexually Selected Hypothesis" For the Evolution of Eggshell Coloration: Revisiting the Assumptions. - Auk 128: 175–179.

Riley, M. A. and Wertz, J. E. 2002. Bacteriocins: Evolution, Ecology, and Application. - Annu. Rev. Microbiol. 56: 117–137.

Rizzo, C. and Lo Giudice, A. 2018. Marine Invertebrates: Underexplored Sources of Bacteria Producing Biologically Active Molecules. - Diversity 10: 52.

Rodríguez-Ruano, S. M. 2015. Diversidad bacteriana en la glándula uropigial de la abubilla: dinámica estacional y beneficios asociados.

Rodríguez-Ruano, S. M., Martín-Vivaldi, M., Martín-Platero, A. M., López-López, J. P., Peralta-Sánchez, J. M., Ruiz-Rodríguez, M., Soler, J. J., Valdivia, E. and Martínez-Bueno, M. 2015. The hoopoe's uropygial gland hosts a bacterial community influenced by the living conditions of the bird. - PLoS One 10: 1–18.

Rodríguez-Ruano, S. M., Martín-Vivaldi, M., Peralta-Sánchez, J. M., García-Martín, A. B., Martínez-García, Á., Soler, J. J., Valdivia, E. and Martínez-Bueno, M. 2018. Seasonal and Sexual Differences in the Microbiota of the Hoopoe Uropygial Secretion. - Genes (Basel). 9: 407.

Rognes, T., Flouri, T., Nichols, B., Quince, C. and Mahé, F. 2016. VSEARCH: a versatile open source tool for metagenomics. - PeerJ 4: e2584.

Rolig, A. S., Parthasarathy, R., Burns, A. R., Bohannan, B. J. M. and Guillemin, K. 2015. Individual Members of the Microbiota Disproportionately Modulate Host Innate Immune Responses. - Cell Host Microbe 18: 613–620.

Rollins-Smith, L. A., Ramsey, J. P., Pask, J. D., Reinert, L. K. and Woodhams, D. C. 2011. Amphibian Immune Defenses against Chytridiomycosis: Impacts of Changing Environments. - Integr. Comp. Biol. 51: 552–562.

Rosvall, K. A. 2011. Intrasexual competition in females: evidence for sexual selection? -

Behav. Ecol. 22: 1131–1140.

Rubolini, D., Liker, A., Garamszegi, L. Z., Møller, A. P. and Saino, N. 2015. Using the birdtree.org website to obtain robust phylogenies for avian comparative studies: A primer. - Curr. Zool. 61: 959–965.

Ruiz-Castellano, C., Tomás, G., Ruiz-Rodríguez, M., Martín-Gálvez, D. and Soler, J. J. 2016. Nest material shapes eggs bacterial environment. - PLoS One 11: e0148894.

Ruiz-de-Castañeda, R., Burtt Jr, E. H., González-Braojos, S. and Moreno, J. 2012. Bacterial degradability of an intrafeather unmelanized ornament: a role for feather-degrading bacteria in sexual selection? - Biol. J. Linn. Soc. 105: 409–419.

Ruiz-De-Castañeda, R., Burtt, E. H., González-Braojos, S. and Moreno, J. 2015. Bacterial degradability of white patches on primary feathers is associated with breeding date and parental effort in a migratory bird. - Ibis (Lond. 1859). 157: 871–876.

Ruiz-Rodríguez, M., Azcárate-García, M., Ruiz-Castellano, C., Tomás, G., Díaz-Lora, S., Pérez-Contreras, T. and Soler, J. J. Antimicrobial capacity is related to body colouration and reproductive success in female spotless starlings. - Under Rev. in press.

Ruiz-Rodríguez, M., Valdivia, E., Soler, J. J., Martín-Vivaldi, M., Martín-Platero, A. M. and Martínez-Bueno, M. 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. - J. Exp. Biol. 212: 3621–6.

Ruiz-Rodríguez, M., Valdivia, E., Martín-Vivaldi, M., Martín-Platero, A. M., Martínez-Bueno, M., Méndez, M., Peralta-Sánchez, J. M. and Soler, J. J. 2012. Antimicrobial activity and genetic profile of *enteroccoci* isolated from hoopoes uropygial gland. - PLoS One 7: 1–6.

Ruiz-Rodríguez, M., Martínez-Bueno, M., Martín-Vivaldi, M., Valdivia, E. and Soler, J. J. 2013. Bacteriocins with a broader antimicrobial spectrum prevail in enterococcal symbionts isolated from the hoopoe's uropygial gland. - FEMS Microbiol. Ecol. 85: 495–502.

Ruiz-Rodríguez, M., Soler, J. J., Martín-Vivaldi, M., Martín-Platero, A. M., Méndez, M., Peralta-Sánchez, J. M., Ananou, S., Valdivia, E. and Martínez-Buenob, M. 2014.

Environmental factors shape the community of symbionts in the hoopoe uropygial gland more than genetic factors. - Appl. Environ. Microbiol. 80: 6714–6723.

Ruiz-Rodríguez, M., Tomás, G., Martín-Gálvez, D., Ruiz-Castellano, C. and Soler, J. J. 2015. Bacteria and the evolution of honest signals. The case of ornamental throat feathers in spotless starlings. - Funct. Ecol. 29: 701–709.

Ruiz-Rodríguez, M., Møller, A. P., Mousseau, T. A. and Soler, J. J. 2016. Defenses against keratinolytic bacteria in birds living in radioactively contaminated areas. - Sci. Nat. 103: 71.

Sachs, J. L., Skophammer, R. G. and Regus, J. U. 2011. Evolutionary transitions in bacterial symbiosis. - Proc. Natl. Acad. Sci. 108: 10800 LP – 10807.

Saino, N., Romano, M., Rubolini, D., Ambrosini, R., Caprioli, M., Milzani, A., Costanzo, A., Colombo, G., Canova, L. and Wakamatsu, K. 2013. Viability Is Associated with Melanin-Based Coloration in the Barn Swallow (Hirundo rustica). - PLoS One 8: e60426.

Sanz, J. J. and García-Navas, V. 2009. Eggshell pigmentation pattern in relation to breeding performance of blue tits Cyanistes caeruleus. - J. Anim. Ecol. 78: 31–41.

Scott, J. J., Oh, D.-C., Yuceer, M. C., Klepzig, K. D., Clardy, J. and Currie, C. R. 2008. Bacterial Protection of Beetle-Fungus Mutualism. - Science (80-.). 322: 63.

Searcy, W. A. and Nowicki, S. 2005. The evolution of animal communication. - Princeton Univ. Press, Princeton, NJ.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S. and Huttenhower, C. 2011. Metagenomic biomarker discovery and explanation. - Genome Biol. 12: R60.

Senar, J. C. and Pascual, J. 1997. Tarsus lenght predictor body size. - Ardea 85: 269–274.

Sender, R., Fuchs, S. and Milo, R. 2016. Revised Estimates for the Number of Human and Bacteria Cells in the Body. - PLOS Biol. 14: e1002533.

Sergio, F. and Newton, I. 2003. Occupancy as a measure of habitat quality. - J Anim Ecol. 72:

857–865.

Shapira, M. 2016. Gut Microbiotas and Host Evolution: Scaling Up Symbiosis. - Trends Ecol. Evol. 31: 539–549.

Sharon, G., Segal, D., M.Ringo, H., Hefetz, A., Zilber-Rosenberg, I. and Rosenberg, E. 2010. Commensal bacteria play a role in mating preference of Drosophila melanogaster. - PNAS 107: 20051–20056.

Shawkey, M. D., Pillai, S. R. and Hill, G. E. 2003. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. - J. Avian Biol. 34: 345–349.

Shawkey, M. D., Pillai, S. R. and Hill, G. E. 2009. Do feather-degrading bacteria affect sexually selected plumage color? - Naturwissenschaften 96: 123–128.

Sheldon, B. C. 2000. Differential allocation: Tests, mechanisms and implications. - Trends Ecol. Evol. 15: 397–402.

Shephard, K. L. 1994. Functions for fish mucus. - Rev. Fish Biol. Fish. 4: 401–429.

Sherwin, E., Bordenstein, S. R., Quinn, J. L., Dinan, T. G. and Cryan, J. F. 2019. Microbiota and the social brain. - Science (80-.). 366: eaar2016.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. and Ishikawa, H. 2000. Genome sequence of the endocellular bacterial symbiont of aphids Buchnera sp. APS. - Nature 407: 81–86.

Siefferman, L., Navara, K. J. and Hill, G. E. 2006. Egg coloration is correlated with female condition in eastern bluebirds (Sialia sialis). - Behav. Ecol. Sociobiol. 59: 651–656.

Singleton, D. R. and Harper, R. G. 1996. Bacteria in Old House Wren nests. - J F. Ornithol 69: 71–74.

Smith, H. G. and Montgomerie, R. 1991. Nestling American robins compete with siblings by begging. - Behav. Ecol. Sociobiol. 29: 307–312.

Soini, H. A., Whittaker, D. J., Wiesler, D., Ketterson, E. D. and Novotny, M. V. 2013. Chemosignaling diversity in songbirds: Chromatographic profiling of preen oil volatiles in different species. - J. Chromatogr. A 1317: 186–192.

Soler, J. J. and Møller, A. P. 1996. A comparative analysis of the evolution of variation in appearance of eggs of European passerines in relation to brood parasitism. - Behav. Ecol. 7: 89–94.

Soler, J. J., Moreno, J., Aviles, J. M. and Møller, A. P. 2005. Blue and green egg-color intensity is associated with parental effort and mating system in passerines: support for the sexual selection hypothesis. - Evolution (N. Y). 59: 636–644.

Soler, J. J., Martín-Vivaldi, M., Ruiz-Rodríguez, M., Valdivia, E., Martín-Platero, A. M., Martínez-Bueno, M., Peralta-Sánchez, J. M. and Méndez, M. 2008a. Symbiotic association between hoopoes and antibiotic-producing bacteria that live in their uropygial gland. - Funct. Ecol. 22: 864–871.

Soler, J. J., Navarro, C., Contreras, T. P., Avilés, J. M. and Cuervo, J. J. 2008b. Sexually Selected Egg Coloration in Spotless Starlings. - Am. Nat. 171: 183–194.

Soler, J. J., Martín-vivaldi, M., Peralta-sánchez, J. M. and Ruiz-rodríguez, M. 2010. Antibiotic-Producing Bacteria as a Possible Defence of Birds against Pathogenic Microorganisms. - Open Ornithol. J. 3: 93–100.

Soler, J. J., Martín-Vivaldi, M., Peralta-Sánchez, J. M., Arco, L. and Juárez-García-Pelayo, N. 2014. Hoopoes color their eggs with antimicrobial uropygial secretions. - Naturwissenschaften 101: 697–705.

Soler, J. J., Martínez-García, Á., Rodríguez-Ruano, S. M., Martínez-Bueno, M., Martín-Platero, A. M., Peralta-Sánchez, J. M. and Martín-Vivaldi, M. 2016. Nestedness of hoopoes' bacterial communities: symbionts from the uropygial gland to the eggshell. - Biol. J. Linn. Soc. 118: 763–773.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martín-Vivaldi, M., Martínez-de la Puente, J., Ruiz-Rodríguez, M. and Tomás, G. 2017. Telomere length and dynamics of spotless starling nestlings depend on nest-building materials used by parents. - Anim. Behav. 126: 89–100.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martínez-de la Puente, J., Ruiz-Rodríguez, M. and Tomás, G. 2018. Egg colouration predicts brood size, telomere length and body condition of spotless starling fledglings. - J. Avian Biol. 49: 1–12.

Soler, J. J., Morales, J., Cuervo, J. J. and Moreno, J. 2019. Conspicuousness of passerine females is associated with the nest-building behaviour of males. - Biol. J. Linn. Soc. 126: 824–835.

Solís, J. C. and de Lope, F. 1995. Nest and Egg Crypsis in the Ground-Nesting Stone Curlew Burhinus oedicnemus. - J. Avian Biol. 26: 135–138.

Spicer, M. E., Pruitt, J. N. and Keiser, C. N. 2019. Spiders, microbes and sex: Bacterial exposure on copulatory organs alters mating behaviour in funnel-web spiders. - Ethology 125: 677–685.

Spor, A., Koren, O. and Ley, R. 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. - Nat. Rev. Microbiol. 9: 279–290.

Stanback, M. T. and Rockwell, E. K. 2003. Nest-site fidelity in Eastern Bluebirds (Sialia sialis) depends on the quality of alternative cavities. - Auk 120: 1029–1032.

Statsoft, I. 2006. STATISTICA (data analysis software system), version 7. Available at www.statsoft.com.

Steinert, M., Hentschel, U. and Hacker, J. 2000. Symbiosis and pathogenesis: evolution of the microbe-host interaction. - Naturwissenschaften 87: 1—11.

Stoddard, M. C. and Prum, R. O. 2008. Evolution of avian plumage color in a tetrahedral colour space: a phylogenetic analysis of New World buntings. - Am. Nat. 171: 755–776.

Stoddard, M. C. and Osorio, D. 2019. Animal Coloration Patterns: Linking Spatial Vision to Quantitative Analysis. - Am. Nat. 193: 000–000.

Stoddard, M. C., Fayet, A. L., Kilner, R. M. and Hinde, C. A. 2012. Egg speckling patterns do not advertise offspring quality or influence male provisioning in great tits. - PLoS One 7: e40211.

Suedmeyer, W. K., McCaw, D. and Turnquist, S. 2001. Attempted Photodynamic Therapy of Squamous Cell Carcinoma in the Casque of a Great Hornbill (Buceros bicornis). - J. Avian Med. Surg. 15: 44–49.

Sumasgutner, P., Vasko, V., Varjonen, R. and Korpimäki, E. 2014. Public information

revealed by pellets in nest sites is more important than ecto-parasite avoidance in the settlement decisions of Eurasian kestrels. - Behav. Ecol. Sociobiol. 68: 2023–2034.

Surmacki, A. and Nowakowski, J. K. 2007. Soil and preen waxes influence the expression of carotenoid-based plumage coloration. - Naturwissenschaften 94: 829–835.

Suzuki, T. A. 2017. Links between Natural Variation in the Microbiome and Host Fitness in Wild Mammals. - Integr. Comp. Biol. 57: 756–769.

Swynnerton, C. F. M. 1916. On the coloration of the mouths and eggs of birds. II. On the coloration of eggs. - Ibis (Lond. 1859). 4: 529–606.

Swynnerton, C. F. M. 1918. Rejections by birds of eggs unlike their own: with remarks on some of the cuckoo problems. - Ibis (Lond. 1859).: 127–154.

Team, R. D. C. 2019. R: A Language and Environment for Statistical Computing (RDC Team, Ed.). - R Found. Stat. Comput. in press.

Theis, K. R., Venkataraman, A., Dycus, J. A., Koonter, K. D., Schmitt-Matzen, E. N., Wagner, A. P., Holekamp, K. E. and Schmidt, T. M. 2013. Symbiotic bacteria appear to mediate hyena social odors. - Proc. Natl. Acad. Sci. 110: 19832–19837.

Tomás, G., Merino, S., Moreno, J., Sanz, J. J., Morales, J. and García-Fraile, S. 2006. Nest Weight and Female Health in the Blue Tit (Cyanistes Caeruleus). - Auk 123: 1013–1021.

Tomás, G., Merino, S., Moreno, J. and Morales, J. 2007. Consequences of nest reuse for parasite burden and female health and condition in blue tits, Cyanistes caeruleus. - Anim. Behav. 73: 805–814.

Tomás, G., Martín-Gálvez, D., Ruiz-Castellano, C., Ruiz-Rodríguez, M., Peralta-Sánchez, J. M., Martín-Vivaldi, M. and Soler, J. J. 2018. Ectoparasite Activity During Incubation Increases Microbial Growth on Avian Eggs. - Microb. Ecol. 76: 555–564.

Trail, P. W. 2007. African hornbills: keystone species threatened by habitat loss, hunting and international trade. - Ostrich 78: 609–613.

Trivers, R. L. 1972. Parental investment and sexual selection. - In: Campbell, B. (ed), Sexual selection and the descent of man 1871-1971. pp. 136–179.

Tschumi, M., Schaub, M. and Arlettaz, R. 2014. Territory occupancy and parental quality as proxies for spatial prioritization of conservation areas. - PLoS One 9: e97679.

Uchida, Y. 1970. On the color change in Japanese crested ibis. A new type of cosmetic coloration in birds. - Misc Rep Yamashina Inst Ornithol 6: 54–72. In Japanese, with English summary.

Umesaki, Y., Setoyama, H., Matsumoto, S., Imaoka, A. and Itoh, K. 1999. Differential Roles of Segmented Filamentous Bacteria and Clostridia in Development of the Intestinal Immune System. - Infect. Immun. 67: 3504 LP – 3511.

Underwood, T. and Sealy, S. 2002. Adaptive significance of egg coloration. - In: Deeming DC (ed), Avian incubation: behaviour, environment, and evolution. Oxford Uni. pp. 280–298.

van Dongen, W. F., White, J., Brandl, H. B., Moodley, Y., Merkling, T., Leclaire, S., Blanchard, P., Danchin, É., Hatch, S. A. and Wagner, R. H. 2013. Age-related differences in the cloacal microbiota of a wild bird species. - BMC Ecol. 13: 11.

van Veelen, H. P. J., Falcao Salles, J. and Tieleman, B. I. 2017. Multi-level comparisons of cloacal, skin, feather and nest-associated microbiota suggest considerable influence of horizontal acquisition on the microbiota assembly of sympatric woodlarks and skylarks. - Microbiome 5: 156.

van Veelen, H. P. J., Salles, J. F. and Tieleman, B. I. 2018. Microbiome assembly of avian eggshells and their potential as transgenerational carriers of maternal microbiota. - ISME J. 12: 1375–1388.

van Wijk, R. E., Schaub, M. and Bauer, S. 2017. Dependencies in the timing of activities weaken over the annual cycle in a long-distance migratory bird. - Behav. Ecol. Sociobiol. 71: 73.

van Wijk, R. E., Schaub, M., Hahn, S., Juárez-García-Pelayo, N., Schäfer, B., Viktora, L., Martín-Vivaldi, M., Zischewski, M. and Bauer, S. 2018. Diverse migration strategies in hoopoes (Upupa epops) lead to weak spatial but strong temporal connectivity. - Sci. Nat. 105: 42.

Victoria, J. K. 1972. Clutch characteristics and egg discriminative ability of African village weaverbird Ploceus cucullatus. - Ibis (Lond. 1859). 114: 367–376.

Videler, H., Geertjes, G. J. and Videler, J. J. 1999. Biochemical characteristics and antibiotic properties of the mucous envelope of the queen parrotfish. - J. Fish Biol. 54: 1124–1127.

Viseshakul, N., Charoennitikul, W., Kitamura, S., Kemp, A., Thong-Aree, S., Surapunpitak, Y., Poonswad, P. and Ponglikitmongkol, M. 2011. A phylogeny of frugivorous hornbills linked to the evolution of Indian plants within Asian rainforests. - J. Evol. Biol. 24: 1533–1545.

Wallace AR 1889. Darwinism: an exposition of the theory of natural selection, with some of its applications.

Walters, L. A. and Getty, T. 2010. Are brighter eggs better? Egg color and parental investment by House Wrens. - J. F. Ornithol. 81: 155–166.

Wang, B., Wan, L., Wang, A. and Li, L. M. 2017. An adaptive decorrelation method removes Illumina DNA base-calling errors caused by crosstalk between adjacent clusters. - Sci. Rep. 7: 41348.

Warnecke, L., Turner, J. M., Bollinger, T. K., Lorch, J. M., Misra, V., Cryan, P. M., Wibbelt, G., Blehert, D. S. and Willis, C. K. R. 2012. Inoculation of bats with European Geomyces destructans supports the novel pathogen hypothesis for the origin of white-nose syndrome. - Proc. Natl. Acad. Sci. 109: 6999–7003.

Weiß, B. M., Kücklich, M., Thomsen, R., Henkel, S., Jänig, S., Kulik, L., Birkemeyer, C. and Widdig, A. 2018. Chemical composition of axillary odorants reflects social and individual attributes in rhesus macaques. - Behav. Ecol. Sociobiol. 72: 65.

Werren, J. H. 2012. Symbionts provide pesticide detoxification. - Proc. Natl. Acad. Sci. 109: 8364–8365.

Werren, J. H., Baldo, L. and Clark, M. E. 2008. Wolbachia: master manipulators of invertebrate biology. - Nat. Rev. Microbiol. 6: 741–751.

Wesolowski, T. and Maziarz, M. 2012. Dark tree cavities – a challenge for hole nesting birds? - J. Avian Biol. 43: 454–460.

Westcott, S. L. and Schloss, P. D. 2015. De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. - PeerJ 3: e1487.

Whittaker, D. J. and Theis, K. R. 2016. Bacterial Communities Associated with Junco Preen Glands : Preliminary Ramifications for Chemical Signaling. - In: Schulte, B. A. et al. (ed), Chemical Signals in Vertebrates 13. Springer International Publishing, pp. 105–117.

Whittaker, D. J., Soini, H. A., Atwell, J. W., Hollars, C., Novotny, M. V and Ketterson, E. D. 2010. Songbird chemosignals: volatile compounds in preen gland secretions vary among individuals, sexes, and populations. - Behav. Ecol. 21: 608–614.

Whittaker, D. J., Richmond, K. M., Miller, A. K., Kiley, R., Bergeon Burns, C., Atwell, J. W. and Ketterson, E. D. 2011. Intraspecific preen oil odor preferences in dark-eyed juncos (Junco hyemalis). - Behav. Ecol. 22: 1256–1263.

Whittaker, D. J., Gerlach, N. M., Slowinski, S. P., Corcoran, K. P., Winters, A. D., Soini, H. A., Novotny, M. V., Ketterson, E. D. and Theis, K. R. 2016. Social Environment Has a Primary Influence on the Microbial and Odor Profiles of a Chemically Signaling Songbird. - Front. Ecol. Evol. 4: 1–15.

Whittaker, D. J., Slowinski, S. P., Greenberg, J. M., Alian, O., Winters, A. D., Ahmad, M. M., Burrell, M. J. E., Soini, H. A., Novotny, M. V., Ketterson, E. D. and Theis, K. R. 2019. Experimental evidence that symbiotic bacteria produce chemical cues in a songbird. - J. Exp. Biol.: jeb.202978.

Wright, E. S. and Vetsigian, K. H. 2016. Quality filtering of Illumina index reads mitigates sample cross-talk. - BMC Genomics 17: 876.

Zahavi, A. 1975. Mate selection-A selection for a handicap. - J. Theor. Biol. 53: 205–214.

Zahavi, A. and Zahavi, A. 1997. The handicap principle; a missing piece of Darwin's puzzle.

Zamzow, J. P. and Losey, G. S. 2002. Ultraviolet radiation absorbance by coral reef fish mucus: Photo-protection and visual communication. - Environ. Biol. Fishes 63: 41–47.

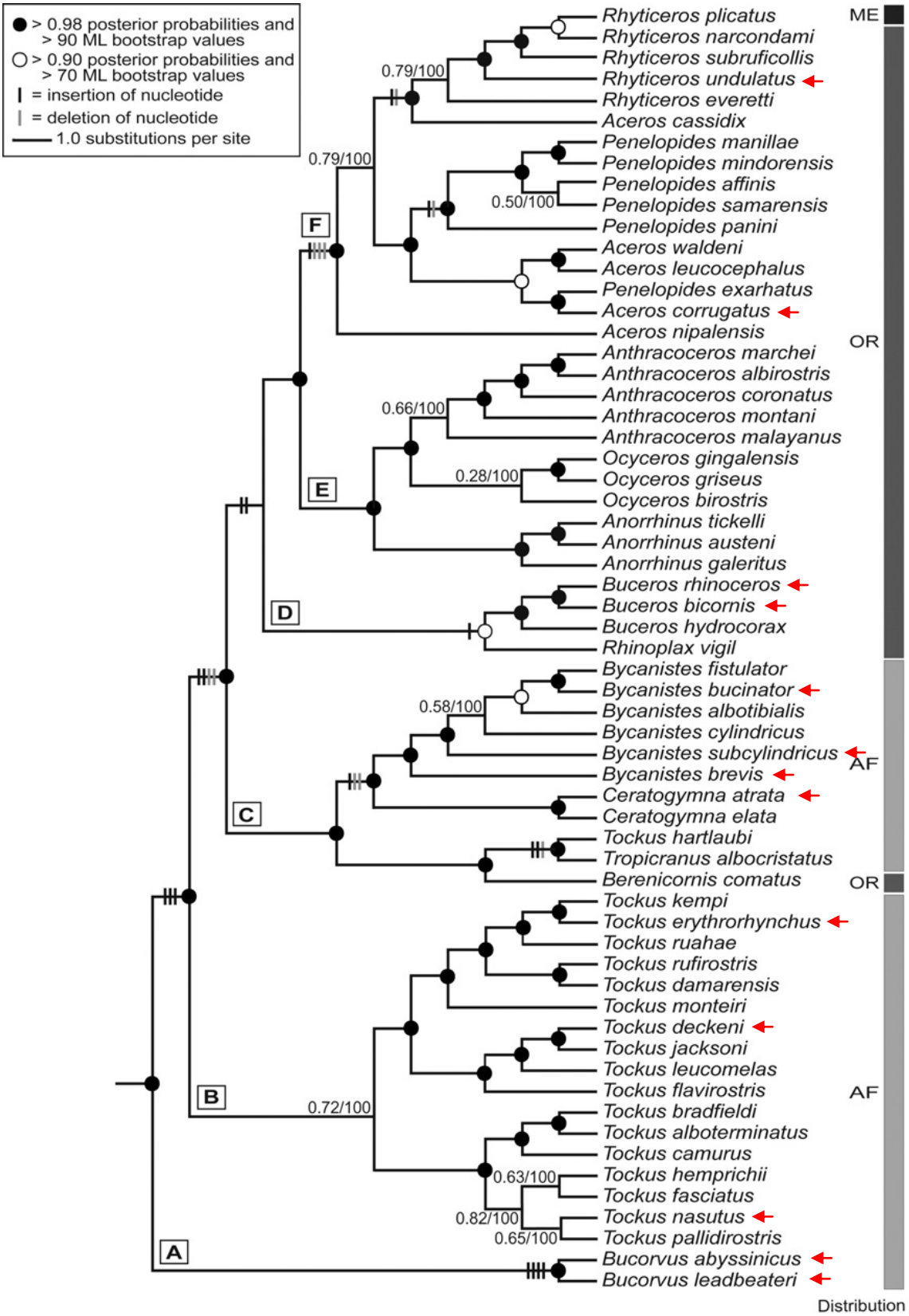
Zinter, M. S., Mayday, M. Y., Ryckman, K. K., Jelliffe-Pawlowski, L. L. and DeRisi, J. L.

2019. Towards precision quantification of contamination in metagenomic sequencing experiments. - Microbiome 7: 62.

SUPPLEMENTARY MATERIAL

Tabla S1. Database used for this study. CFU/ml averages (Log transformed) and prevalence (Prev.) per species, sample and medium are shown.

	log CFU ml^{-1} TSA Beak	Prev. KF Beak	N	log CFU ml^{-1} TSA Casque	N	log CFU ml^{-1} FMA Casque	N	White feathers	Casque length	Casque width	Casque length X width	Colour casque	Colour beak	Colour beak + casque	Uropygial colour	Total body colouration
Bucorvus leadbeateri	3.00	0.33	12	3.63	6	1.60	6	0.11	0.4	0.4	0.16	0	0	0	no	2
Bucorvus abyssinicus	3.40	0.00	4	3.29	4	3.02	4	0.11	0.4	0.9	0.36	0	0.13	0.13	no	2
Tockus nasutus	4.11	0.50	2	3.03	2	2.72	2	0.62	0.7	0.4	0.28	1	0.75	1.75	no	2
Tockus erythrorhynchus	2.99	0.00	2	-	0	-	0	0.58	0.6	0.3	0.18	1	1	2	no	3
Tockus deckeni	2.27	0.60	5	2.37	2	1.64	2	0.52	0.9	0.4	0.36	1	1	2	no	3
Aceros corrugatus	1.30	0.00	1	1.30	1	-1	1	0.4	0.5	0.6	0.3	1	1	2	yes	3
Aceros undulatus	-	-	0	3.20	1	3.11	1	0.58	0.7	0.5	0.35	0	0	0	no	2
Buceros rhinoceros	3.80	0.40	5	3.71	5	3.70	4	0.4	0.75	0.8	0.6	1	0.35	1.35	yes	4
Buceros bicornis	3.24	0.00	4	2.07	1	1.77	1	0.64	0.8	0.7	0.56	1	0.7	1.7	yes	4
Ceratogymna atrata	3.39	0.40	6	4.40	3	1.71	2	0.1	1	1	1	0	0	0	no	0
Bycanistes bucinator	3.13	0.66	3	2.27	3	1.25	3	0.25	1	0.9	0.9	0	0	0	no	1
Bycanistes brevis	4.00	0.83	6	4.72	5	2.14	5	0.24	1	1	1	0	0	0	no	0
Bycanistes subcylindricus	3.83	0.66	3	4.50	1	4.55	1	0.38	1	1	1	0	0	0	no	0


● > 0.98 posterior probabilities and > 90 ML bootstrap values
○ > 0.90 posterior probabilities and > 70 ML bootstrap values
| = insertion of nucleotide
| = deletion of nucleotide
—— 1.0 substitutions per site
Rhyticeros plicatus
Rhyticeros narcondami
Rhyticeros subruficollis
Rhyticeros undulatus
Rhyticeros everetti
Aceros cassidix
Penelopides manillae
Penelopides mindorensis
Penelopides affinis
Penelopides samarensis
Penelopides panini
Aceros waldeni
Aceros leucocephalus
Penelopides exarhatus
Aceros corrugatus
Aceros nipalensis
Anthracoceros marchei
Anthracoceros albirostris
Anthracoceros coronatus
Anthracoceros montani
Anthracoceros malayanus
Ocyceros gingalensis
Ocyceros griseus
Ocyceros birostris
Anorrhinus tickelli
Anorrhinus austeni
Anorrhinus galeritus
Buceros rhinoceros
Buceros bicornis
Buceros hydrocorax
Rhinoplax vigil
Bycanistes fistulator
Bycanistes bucinator
Bycanistes albotibialis
Bycanistes cylindricus
Bycanistes subcylindricus
Bycanistes brevis
Ceratogymna atrata
Ceratogymna elata
Tockus hartlaubi
Tropicranus albocristatus
Berenicornis comatus
Tockus kempi
Tockus erythrorhynchus
Tockus ruahae
Tockus rufirostris
Tockus damarensis
Tockus monteiri
Tockus deckeni
Tockus jacksoni
Tockus leucomelas
Tockus flavirostris
Tockus bradfieldi
Tockus alboterminatus
Tockus camurus
Tockus hemprichii
Tockus fasciatus
Tockus nasutus
Tockus pallidirostris
Bucorvus abyssinicus
Bucorvus leadbeateri
0.79/100
0.79/100
0.50/100
0.66/100
0.28/100
0.58/100
0.72/100
0.63/100
0.82/100
0.65/100
A
B
C
D
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ME
OR
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OR
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Distribution

Figure S1. Phylogeny for the hornbills (Figure 3 of Gonzalez et al. 2013). Red arrows indicate the species used in this study. Letters from A to F indicate major clades: A (*Bucorvus* clade); B (*Tockus* clade); C (*Berenicornis* clade); D (*Rhinoplax* clade); E (*Anorrhinus* clade) and F (*Aceros* clade).

DISCUSIÓN GENERAL

En esta tesis se ha querido comprobar si las glándulas de abubillas y de calaos albergan comunidades bacterianas simbiontes diferentes entre individuos, y si se han seleccionado señales para transmitir la presencia de esas bacterias como indicativo de calidad. De esta manera, las predicciones de esta tesis eran las siguientes: (I) Las abubillas son capaces de incorporar a su glándula bacterias beneficiosas del material del nido cuando estos han sido previamente usados por otra abubilla. (II) Las propiedades de la secreción de las hembras de abubilla son usadas por los machos para inferir la calidad de las hembras. Así, incrementarán su aporte de cebas cuando estén emparejados con hembras cuyas secreciones sean de mayor calidad. (III) Las secreciones uropigiales pigmentadas de calaos contendrán más bacterias que las no pigmentadas. Todos los resultados obtenidos en la tesis apoyan la hipótesis general de que en abubillas y calaos, las coloraciones cosméticas de la secreción uropigial pueden estar siendo seleccionadas como señales de calidad asociada a la posesión de comunidades bacterianas simbiontes. En el caso de las abubillas, los machos respondieron a los cambios de la coloración de los huevos ajustando su aporte de cebas. En el caso de los calaos se confirma que algunas especies también mantienen simbiosis con bacterias en sus secreciones, algo que no se conocía hasta el momento. Sin embargo, los resultados no apoyan nuestra predicción de que la asociación predomine en las especies con secreciones coloreadas, ya que las bacterias estuvieron también presentes e incluso fueron más abundantes en las especies con secreciones no coloreadas. No obstante, algunos simbiontes encontrados en las especies que poseían secreciones coloreadas podrían ser responsables de las propiedades especiales de la secreción, por sus capacidades biosintéticas particulares. Además, el estudio comparativo de los ornamentos de estas especies muestra asociaciones entre estos y grupos de bacterias, tanto patógenas como productoras de sustancias defensivas, e interacciones entre estos grupos bacterianos entre sí. A continuación se discute la importancia de estos resultados, las posibles limitaciones de estudio, así como futuras líneas de investigación interesantes que permitan comprender mejor la simbiosis entre bacterias y aves.

Uno de los factores clave para el establecimiento y la evolución de la simbiosis, es el modo de transmisión de los simbiontes (Bright y Bulgheresi 2010). Varios mecanismos

han sido propuestos para explicar la compleja comunidad bacteriana de la secreción uropigial de la abubilla (Rodríguez-Ruano et al. 2018). Estudios anteriores han señalado que las abubillas suelen reutilizar las cavidades para su reproducción (Cramp 1998, Martín-Vivaldi et al. 1999, van Wijk et al. 2017). Por tanto, una posibilidad es que esas bacterias simbiontes se transmitan a través del material del nido. Concretamente, si la reutilización del material viejo de los nidos que habían sido previamente usados por otra abubilla podría actuar como una fuente de bacterias potencialmente beneficiosas. Si este fuese el caso, se esperaría una selección de esos nidos por parte de la abubilla. Sin embargo, los resultados obtenidos indican que las abubillas muestran una preferencia por las cajas nido con material de nido experimental independientemente de si el material agregado proviene de nidos de abubilla previamente utilizados o no. Aunque las características del material del nido no afectaron a su éxito reproductivo, sí afectaron a las cargas bacterianas de la cáscara del huevo y a la composición de la comunidad bacteriana de la glándula uropigial. Además, varios OTUs de la secreción uropigial de las hembras se asociaron positivamente con el éxito de eclosión. Estos resultados obtenidos apoyan lo que ya sugerían otros estudios previos, que una función principal de la secreción uropigial de las hembras es proteger a los huevos de una infección microbiana (Martín-Vivaldi et al. 2014a y Soler et al. 2014), además de actuar como una señal de calidad para el macho.

A pesar de no seleccionar los nidos con material previamente usado por otra abubilla (material viejo), se encontró una preferencia por las cajas nido que contenían material en su interior, en lugar de las que estaban vacías. Este resultado coincide con lo esperado, posiblemente debido a los numerosos beneficios que proporciona el material del nido. Algunos de ellos son que puede ayudar a mejorar la absorción de agua, el aislamiento térmico y, por lo tanto, mejorar la eficiencia en la incubación (Mazgajski 2007, Mainwaring et al.2014, Podofillini et al.2018), además de minimizar el riesgo de rotura de los huevos (Podofillini et al. 2018).

El hecho de que no se haya encontrado un efecto del material del nido experimental podría ser debido a que la elección de la caja nido también podría depender de la disponibilidad de nidos apropiados (Stanback y Rockwell 2003) y de las características del territorio (Tschumi et al. 2014). Sin embargo, es improbable que esta posibilidad explique nuestros resultados ya que las cajas nido experimentales eran nuevas (nunca antes usadas) e instaladas en ubicaciones con un diseño experimental por pares. Adicionalmente, la edad y la experiencia previa en la reproducción podrían afectar a la

elección de nidos en las abubillas. No tenemos un criterio fiable para estimar la edad en las hembras de esa temporada de reproducción, por lo que no podemos controlarlo en los análisis. Sin embargo, hay muy pocas recapturas debido a la alta mortalidad y a la alta dispersión de esta especie (van Wijk et al.2018). Por lo tanto, la mayoría de las hembras eran individuos nuevos que llegaron de otros lugares a nuestra área de estudio. Además, las nuevas cajas nido experimentales eran nuevas para todas las abubillas que llegaron a la zona, independientemente de su edad (recapturas o no recapturas) y ambos, individuos de mayor y menor edad, usaron cajas nido nuevas (experimentales) y viejas. Otra posibilidad podría ser que la elección de la caja nido se viera afectada por la comunidad bacteriana de las abubillas individuales. Aunque esta última explicación alternativa es improbable, no se puede descartar.

A pesar de no mostrar preferencia por nidos con material viejo de nido de abubilla, encontramos apoyo al efecto esperado del material de nido experimental en la composición de la secreción uropigial de las hembras y en la carga bacteriana de las cáscaras de los huevos. Este resultado muestra, por primera vez en las abubillas, que el material del nido podría ser una fuente de cepas para su incorporación a la glándula uropigial que podría interactuar con otros componentes de la comunidad.

Dentro de la glándula uropigial se encontraron dos OTUs que se vieron muy afectados por la presencia del material de nido viejo de abubilla, pero en direcciones opuestas. OTU29 solo estaba presente en nidos con material de nido viejo de abubilla, mientras que OTU12 era mucho más frecuente en nidos sin dicho material viejo (sólo serrín). Esto sugiere que OTU29 solo se incorpora a la comunidad bacteriana de la secreción cuando las abubillas usan cavidades donde sus congéneres se han reproducido previamente. No se sabe en qué medida la incorporación de estas bacterias en la secreción es beneficiosa o no para el hospedador, o incluso si alguna de ellas es potencialmente patógena, ya que no se han encontrado efectos del experimento sobre el éxito reproductivo o sobre la actividad antimicrobiana de las secreciones. Sin embargo, dado que ambos están correlacionados con la abundancia de otros componentes de la comunidad, podrían tener un papel en la dinámica de la microbiota de la glándula uropigial.

Los resultados muestran que el material viejo del nido podría afectar a la comunidad microbiana establecida en las glándulas de las hembras. De esta forma, las hembras podrían adquirir bacterias del material viejo del nido. Esto también podría explicar las diferencias encontradas en la comunidad bacteriana de la secreción uropigial

entre hembras y pollos, ya que estos últimos tenían seis OTUs no presentes en las hembras. Otra posibilidad no exclusiva es que los pollos podrían adquirir esos OTUs del nido después de que la hembra saliese, pero no de fuentes presentes en el material viejo del nido. Las hembras permanecen dentro del nido hasta que los primeros pollos tienen ocho días de edad y los pollos dejan el nido a los 24-30 días (Martín-Vivaldi et al. 2014b). De esta manera, los OTUs presentes sólo en las glándulas de los pollos podrían obtenerse de los restos de comida, heces, pollos muertos, etc. Otra posibilidad es que las glándulas de los pollos podrían ser menos selectivas en la adquisición de cepas del medio ambiente que las hembras. La asociación de la edad con la madurez del sistema inmune podría determinar o controlar la microbiota de la glándula uropigial. Esta posibilidad también es consistente con la idea de que los animales jóvenes albergan simbiontes más raros y transitorios que los adultos (por ejemplo, Palmer et al. 2007). Cualquiera sea la razón, otros estudios también encontraron un mayor número total de OTUs bacterianos en pollos que en adultos (van Dongen et al. 2013, Whittaker et al. 2016). De esta manera, nuestro resultado podría ajustarse a esta tendencia general de las aves. Sin embargo, la microbiota de los materiales del nido no ha sido analizada. Por lo tanto, es posible que los resultados encontrados se deban a efectos indirectos del material del nido o de las condiciones ambientales ya que varios estudios han encontrado que las condiciones ambientales tienen un papel importante en la carga bacteriana que podría influir en el microbioma final (Palmer et al. 2007, Peralta-Sánchez et al. 2012, Brandl et al. 2014, D'Alba y Shawkey 2015, Ruiz-Castellano et al.2016, Whittaker et al.2016, Martín-Vivaldi et al.2018).

Los resultados obtenidos en la carga bacteriana de la cáscara del huevo, coinciden con varios estudios experimentales que también detectaron en varias especies de aves una influencia del material del nido en la microbiota de la cáscara del huevo (Brandl et al.2014, Grizard et al.2014, Martínez-García et al.2016a, Ruiz-Castellano et al. 2016, van Veelen et al.2018). Estas bacterias provienen principalmente de las heces, de las plumas, del material del nido, del tracto digestivo y de la piel de la hembra (Peralta-Sanchez et al. 2010, van Veelen et al. 2017, 2018). Además, la secreción uropigial podría alcanzar las cáscaras de huevo y, por lo tanto, debido a sus propiedades antimicrobianas, determinar al menos parcialmente su microbiota (Peralta-Sánchez et al. 2012). En el caso de las abubillas, la secreción uropigial también contiene bacterias simbiontes productoras de bacteriocinas. Además, las hembras untan activamente las cáscaras de huevo con su secreción uropigial (Soler et al. 2014), llenando estructuras especiales (criptas) de la

cáscara, que mejoran la adhesión de la secreción (Martín-Vivaldi et al., 2014b). Por tanto, dado que se ha encontrado un efecto del material experimental del nido en la comunidad bacteriana de la secreción uropigial de las hembras, los efectos detectados en la carga bacteriana de la cáscara de los huevos no solo pueden estar directamente determinados por la microbiota del material del nido, sino también podrían estar indirectamente determinados por su efecto en la comunidad bacteriana de la secreción de la hembra.

La ausencia de efectos del tipo de material del nido en el éxito reproductivo sugiere que las cepas bacterianas adquiridas por el nido en las abubillas no son especialmente importantes o que estas bacterias son funcionalmente redundantes. Los componentes centrales del microbioma habitual pueden adquirirse verticalmente, probablemente mantenidos de una a otra temporada de reproducción en reservorios del cuerpo, o pueden obtenerse horizontalmente a través de fuentes ambientales como el material del nido (Ruiz-Rodríguez et al. 2014, Martínez-García et al.2016b, Martín-Vivaldi et al.2018, Rodríguez-Ruano et al.2018). Este resultado podría explicar por qué las abubillas no han desarrollado una preferencia por los nidos con material de nido viejo de abubilla.

Los efectos de la reutilización de nidos en la eficacia biológica de los pollos y en su éxito reproductivo no están del todo claros. Algunos estudios encontraron efectos negativos (Tomás et al. 2007; González-Braojos et al., 2012). Sin embargo, la mayoría de los estudios coinciden con nuestros resultados, no encontrando que la reutilización de nidos afecte al éxito de eclosión o a la reproducción (revisión en Mazgajski, 2007; Martínez-García et al., 2016a; Podofillini et al., 2018). Sin embargo, a pesar de que el experimento no afectó al éxito reproductivo, varios OTUs de las secreciones uropigiales de las hembras se asociaron positivamente con el éxito de eclosión. Estos OTUs pertenecen al filo *Firmicutes*, como los *enterococos*, que son productores de bacteriocinas. Las bacteriocinas protegen a las plumas de las bacterias degradadadoras y a los embriones (Martín-Platero et al., 2006; Soler et al., 2008; Martín-Vivaldi et al., 2014b). El experimento no afectó a estas bacterias y, por lo tanto, es poco probable que provengan del material del nido, por lo que es necesario continuar explorando su origen. Otra posible explicación de la ausencia de un efecto significativo en el éxito reproductivo podría ser el pequeño tamaño de muestra obtenido, insuficiente para detectar efectos estadísticamente significativos.

La información proporcionada por la descripción de toda la comunidad microbiana que habita estas glándulas (Rodríguez-Ruano et al.2018 y el Capítulo I) conducirá al siguiente paso interesante de estudiar el conjunto completo de interacciones entre ellos y sus efectos sobre la salud de las aves y su éxito reproductivo. Además, sería importante analizar la microbiota del propio material del nido para comprender cómo interactúa con la composición bacteriana de la secreción uropigial y poder reforzar así los resultados y conclusiones derivados de este experimento.

El hecho de que haya una variación en la coloración de la secreción en nuestra población de estudio podría indicar una diferencia entre individuos en la concentración de las bacterias simbiontes, en el tipo de comunidad bacteriana que han adquirido y en su capacidad antimicrobiana. Un estudio previo demostró que, después de la eliminación de las bacterias simbiontes de la glándula uropigial de la abubilla mediante el uso de antibióticos, las secreciones se volvieron menos marrones y de un color rojo más intenso (Martín-Vivaldi et al., 2010). Además, la variación natural en la saturación de los colores de secreción se relacionó negativamente con el poder antimicrobiano de tales secreciones (Soler et al., 2014). Dado que casi todas las bacterias que crecen a partir de las secreciones en TSA son *enterococos* productores de bacteriocinas (Soler et al 2008), todas estas evidencias apuntan a que la saturación de la secreción es una señal válida de su potencial antimicrobiano mediado por los simbiontes de la glándula. Los machos no pueden evaluar directamente la coloración de la secreción uropigial de sus hembras, pero pueden hacerlo mediante la evaluación de la coloración de la cáscara de huevo. Por tanto, el color del huevo podría reflejar no solo la abundancia, sino también la composición de la comunidad bacteriana alojada en la glándula de la hembra (Martínez-García et al., 2016; Rodríguez-Ruano et al., 2018) que serían transferidos a los huevos después de la puesta (Soler et al., 2016). De esta manera, podrían haber evolucionado distintos comportamientos y adaptaciones para señalizar a los machos esa capacidad antimicrobiana y que estos sean capaces de detectar esas señales. Una posible adaptación podría ser el comportamiento de la hembra de manchar activamente los huevos con su secreción uropigial (Soler et al. 2014). Así, el macho podría utilizar esa señal de calidad para invertir acordemente, influyendo en su cuidado parental. Los resultados obtenidos, tanto correlacional como experimentalmente, apoyan esta predicción. Los machos de abubilla invirtieron menos en nidos de hembras que contenían huevos con una coloración más saturada, que indica una menor abundancia de bacterias simbióticas en su secreción. Esta tesis muestra la primera

demostración experimental de los beneficios asociados con la señal cosmética de las hembras, lo que sugiere que la coloración de las cáscaras de huevo de la abubilla es un indicador de la calidad sexual de las hembras seleccionado después del apareamiento en términos de capacidades antimicrobianas. Previos estudios han encontrado una relación entre la coloración de la cáscara de huevo y la inversión parental del macho. Sin embargo, la mayoría de ellos se han centrado en la coloración intrínseca causada por pigmentos como la biliverdina y/o protoporfirina, depositados dentro del oviducto durante la formación de la cáscara del huevo (English & Montgomerie, 2011; Hanley et al., 2008; Moreno, Morales, et al., 2006; Moreno et al., 2004; Soler, Martín-Vivaldi, et al., 2008). Por tanto, nuestro estudio es pionero en mostrar el efecto de la coloración cosmética de los huevos causada por la secreción uropigial en la inversión del macho.

Los resultados obtenidos son consistentes con el segundo supuesto de la hipótesis SSEC que relaciona la inversión del macho con la coloración de los huevos (Moreno y Osorno 2003). En cuanto al primer supuesto, no se ha testado si la señal de color cosmético de la cáscara de huevo es un indicador costoso y fiable de la calidad de la hembra, pero se sabe que las secreciones uropigiales pueden ser un recurso limitado de las hembras y colorear los huevos es una actividad que consume mucho tiempo. Por tanto, la capacidad antimicrobiana de la secreción uropigial podría ser un indicador de la calidad fenotípica de la hembra. Además, es una señal difícil de engañar ya que su propiedad antimicrobiana no solo depende de la hembra, sino que es el resultado de las características de la comunidad microbiana de la secreción, algo difícil de suplantar para obtener un color determinado. Esto, sumado al hecho de que la secreción uropigial protege a los embriones de infecciones patógenas y aumenta el éxito de eclosión (Martín-Vivaldi et al. 2014a), dificultaría el hecho de que se produjese un cambio de color sin que variase la comunidad microbiana. Todo esto indica que el color de la secreción uropigial en abubillas podría ser una señal honesta. Teniendo en cuenta esta suposición, junto con nuestros hallazgos sobre cómo la coloración de la cáscara de huevo influye en el aporte de cebas del macho, nuestros resultados respaldarían la hipótesis SSEC. Además, muestran que la función de señalización del color de los huevos puede ser producida por cosméticos, revelando así un mecanismo novedoso por el cual la selección sexual puede producir señales post-apareamiento en los huevos (como se sugiere en Soler et al.2014).

Se han propuesto varias funciones adaptativas para explicar la variación intra e interespecífica en la coloración de la cáscara de huevo en escenarios de, por ejemplo,

interacciones depredador-presa, parasitismo de cría, termorregulación, resistencia a la rotura o selección sexual (Cherry & Gosler, 2010; Gosler, Higham, & Reynolds, 2005; Kilner, 2006; Moreno y Osorno, 2003; Reynolds, Martin y Cassey, 2009; Soler y Møller, 1996; Soler et al., 2005, 2018). Algunas de las explicaciones alternativas acerca del uso de la secreción uropigial para colorear los huevos son que inicialmente podría evolucionar para camuflarse en el fondo, ya que los huevos de color marrón verdoso son menos visibles que los grises azulados iniciales, o para evitar a los depredadores debido a sus volátiles malolientes (Martín- Vivaldi et al., 2010). Sin embargo, las hembras podrían tener otra estrategia antidepredatoria, ya que es común que defequen en sus huevos cuando son capturadas (Martín-Vivaldi, Doña, et al., 2014). Una posibilidad no adaptativa es que las hembras esparzan sus huevos con su secreción uropigial para proteger sus plumas ventrales, que están en contacto cercano con los huevos y con el material del nido, donde la prevalencia de parásitos y microbios es alta. Si este fuera el caso, las cáscaras de huevo podrían mancharse al estar en contacto con las plumas ventrales manchadas. Sin embargo, las grabaciones de video han mostrado cómo las hembras manchan activamente las cáscaras de huevo con su secreción uropigial (Martín-Vivaldi, Soler, et al., 2014). Por lo tanto, incluso con otras posibles funciones de la coloración cosmética, el macho se beneficiaría de poder interpretar esas pistas, que terminarían evolucionando en señales.

El efecto general detectado del color de la cáscara de huevo en el aporte del macho depende exclusivamente de la fase de incubación, lo que sugiere que el efecto se detiene cuando la coloración de la cáscara del huevo no es visible. En esta especie, las hembras dependen exclusivamente del macho para obtener alimento durante el periodo de incubación y hasta que los pollos tienen aproximadamente ocho días de edad (Martín-Vivaldi, Doña, et al., 2014). Por lo tanto, una mayor inversión de los machos en esta etapa podría ser importante para las hembras, permitiendo una incubación más eficiente de los huevos. Otros estudios también han detectado que la asociación entre la coloración de los huevos y la inversión del macho ocurre sólo durante las primeras etapas del período de anidación, desapareciendo posteriormente (English & Montgomerie, 2011; Soler, Navarro, et al., 2008). Durante la fase de pollos algunas otras variables tales como la tasa de cebas de la hembra, el número de pollos, la fecha de puesta y la condición física de la hembra podrían tener una mayor influencia en el aporte de cebas del macho. Sin embargo, es posible que nuestro índice de cebas del macho durante la fase de pollos haya sido subestimado. Esto se debe a que los machos pueden dar las presas a la hembra cuando esta

se encuentra fuera del nido en lugar de alimentarlos directamente (Martín-Vivaldi, Doña, et al., 2014). No obstante, las hembras generalmente permanecen dentro del nido durante los primeros ocho días después de la eclosión (Martín-Vivaldi, Doña, et al., 2014) y no se detectó ningún efecto de la coloración de la cáscara de huevo en el aporte de los machos cuando el primer pollo tenía tres días. Es posible que las señales de comportamiento como la intensidad de pedida de alimento de los pollos se conviertan en un predictor más importante de la calidad de la descendencia y de las necesidades de la tasa de aporte del macho a medida que crecen (Moreno, Morales, et al., 2006; Smith y Montgomerie, 1991). El hecho de que los machos de abubillas alimenten a las hembras durante el período de incubación (Martín-Vivaldi, Doña, et al., 2014) nos permite comprobar directamente si el aporte de cebas del macho a la hembra son causadas por el color de los huevos en lugar de por las características de los pollos. La condición física de la hembra durante la fase de incubación fue un predictor del aporte del macho, pero no afectó al efecto del color de los huevos durante la incubación, y fue especialmente importante durante las fases de pollos, especialmente cuando el primer pollo tenía 11 días. Esto sugiere que la inversión diferencial de los machos detectada en nuestros análisis, no fue causada únicamente por la evaluación de la condición física de la hembra.

Se ha demostrado experimentalmente mediante el uso de antibióticos, que cuando las bacterias son eliminadas de la glándula uropigial, no solo las secreciones se vuelven más claras si no que también la cantidad de volátiles químicos asociados a la secreción se reduce drásticamente (Martín-Vivaldi et al. 2009, 2010). Por tanto, la secreción depositada en la cáscara de los huevos por las hembras modifica no solo el color, sino también el olor de la puesta. De esta manera, los machos podrían usar una o ambas características para ajustar su aporte. El experimento realizado del intercambio de puestas no permite descartar la posibilidad de que los machos usen el olor de la cáscara de los huevos impregnadas con la secreción, en vez de su color. Sin embargo, sus propias hembras producen continua y diariamente una gran cantidad de secreción por lo que es probable que el intenso olor de tales secreciones frescas (Obs. pers.) sustituya el posible olor de las secreciones secas provenientes de los huevos adoptivos, es decir, de la otra hembra. Por tanto, aunque no podemos descartar por completo que otras propiedades de la secreción que cubren los huevos sean las señales detectadas por los machos, la explicación más probable de nuestro resultado es que detectaron el cambio en el color del huevo.

Además de la coloración del huevo, el experimento de intercambio de huevos mostró cómo su tamaño podría tener también una influencia en el aporte del macho. Concretamente, este tendía a ser menor cuando aumentaba el tamaño de los huevos de las nidadas experimentales. Esta tendencia podría ser debida a que las hembras pongan huevos más grandes cuando se combinan con machos de alta calidad (Cunningham y Russell 2000, Leitner et al. 2006), una posibilidad que necesita más investigación.

Algunos autores han utilizado la luz tenue de los nidos dentro de las cavidades (Wesolowski y Maziarz 2012) como un argumento en contra de cómo la coloración de huevos podría funcionar como señales en esos entornos (Reynolds et al. 2009, pero ver Avilés et al. 2011). Sin embargo, las aves poseen grandes habilidades de discriminación del color, pudiendo discriminar en condiciones de poca luz (Olsson et al. 2015). Algunos estudios han demostrado cómo las aves que anidan en cavidades pueden discriminar entre los parámetros de color dentro de la cavidad del nido (Avilés y Soler 2009, Avilés et al. 2010, 2011, Holveck et al. 2010). Además, tanto el modelo visual aviar bajo condiciones de poca luz y el experimento de discriminación en ambientes oscuros respaldan la posibilidad de que la señal sexual en general, y las de la coloración de huevos en particular, funcionan dentro de la cavidad del nido (Holveck et al. 2010, Avilés et al. 2011). Todo ello apoya la posibilidad de la evolución de estas señales de color de los huevos de las aves. Tanto en el Capítulo II como en el Capítulo III se han analizado los parámetros visuales teniendo en cuenta los estímulos de los conos de las aves dentro de la cavidad de un nido, y por lo tanto, todas las evidencias apoyan a ese papel también en la abubilla.

La posibilidad de que las coloraciones de huevos reflejen la capacidad antimicrobiana mediada por las secreciones uropigiales cosméticas también se puede aplicar a otras especies de aves (Soler et al. 2014). Esto se debe a que las secreciones uropigiales tienen generalmente sustancias antimicrobianas (Jacob y Ziswiler 1982) que pueden alcanzar las cáscaras de huevo durante la incubación, debido a su contacto con las plumas del vientre, previamente manchadas con ella (Cook et al. 2005). Además, es conocido cómo la secreción uropigial cambia la coloración de diferentes superficies corporales de las aves, como las plumas y los picos (Kemp 2001, Montgomerie 2006, Delhey et al. 2007, Piault et al. 2008, Amat et al. al.2011, Pérez-Rodríguez et al.2011). Por lo tanto, es probable que también pudieran cambiar el color de las cáscaras de los huevos. De esta manera, sería interesante estudiar la posibilidad de que la coloración del

huevo debido al uso cosmético de la secreción uropigial tenga componentes sexuales en otras especies además de en las abubillas.

El uso de cosméticos sucede en una amplia variedad de animales, incluidos peces, mamíferos y aves (revisado en Delhey et al. 2007). De manera similar a las abubillas, se ha demostrado cómo los peces de los arrecifes tropicales secretan compuestos bioquímicos con antibióticos en la mucosa epitelial (Shephard 1994, Videler et al. 1999). Estos compuestos son negros a la longitud de onda UV (Zamzow y Losey 2002) por lo que su color podría indicar no solo las características de la mucosa, sino también la calidad individual en términos de capacidad de obtención de fuentes de alimentación ricas en bloqueadores UV (Zamzow y Losey 2002). En cuanto a los mamíferos, se ha visto como el canguro rojo (*Megaleia rufa*) y la zarigüeya gris (*Trichosurus vulpecula*) poseen parches pigmentados de color en el pelaje debido a la secreción de las glándulas integumentarias (Nicholls y Rienits 1971) siendo un carácter sexualmente dimórfico debido a la coloración cosmética. Dos estudios en aves han mostrado cómo el cambio de color del plumaje debido a la tinción deliberada con cosméticos (tierra de color rojo hierro o secreción uropigial) puede ayudar a los individuos a comunicar su calidad (Negro et al. 1999, Amat et al. 2011) en escenarios de comunicación social, incluida la elección de pareja (Amat et al. 2011). A diferencia de estos y algunos otros ejemplos de coloraciones cosméticas, la coloración de las cáscaras de huevo de las abubillas muestra no solo las capacidades fisiológicas de los individuos, sino también las propiedades de sus simbiontes microbianos. Hasta donde sabemos, las abubillas que pintan sus huevos con su propia secreción uropigial es el primer ejemplo de animales que usan una coloración cosmética para mostrar las características de sus bacterias simbiontes productoras de sustancias antimicrobianas. Es probable que este comportamiento haya sido mantenido y seleccionado por la inversión diferencial del aporte de cebas de los machos en un proceso típicamente seleccionado sexualmente después del apareamiento (Burley 1988, Sheldon 2000), como lo demostraron nuestros resultados experimentales.

Las propiedades simbiontes de la comunidad microbiana de la secreción uropigial de las abubillas podría ser un fenómeno más extendido, pudiendo existir también en otras especies de aves (Soler et al. 2010). En los últimos años, se han aislado e identificado en la glándula uropigial, nuevas especies bacterianas en diferentes aves, lo que sugiere que es un ambiente especial que causa una especialización de microorganismos. Sin embargo, en ningún caso, las secreciones son visiblemente alteradas por la presencia de bacterias, tal y

como sucede en las abubillas europeas y en las abubillas arbóreas (Law-brown y Meyers 2003,Soler et al. 2008a, Martín-Vivaldi et al. 2010,). No obstante, el orden *Bucerotiformes*, clado hermano de las abubillas formado por 61 especies de calaos (Gonzalez et al. 2013), posee varias similitudes en cuanto a su secreción uropigial. Algunas de sus especies, tanto machos como hembras, presentan secreciones uropigiales coloreadas, que usan para teñir distintas partes de su cuerpo, posiblemente con una función ornamental (Kemp 2001, Delhey et al. 2007, Poonswad et al. 2013), pudiendo transferir mensajes adicionales con la señal de la coloración.

Los resultados obtenidos en los calaos muestran pruebas claras de la presencia de simbiontes bacterianos asociados a sus glándulas uropigiales. Para varias de sus especies del linaje africano, el estudio microscópico de la secreción extendida mostró que las bacterias pueden ser muy abundantes y ocupar toda la secreción, lo que sugiere que pueden vivir dentro de la glándula uropigial. Para otras especies, el cultivo y las imágenes microscópicas sugieren que este también puede ser el caso, aunque la evidencia no es tan clara. El análisis de la composición del microbioma asociados al penacho de la glándula uropigial y a sus alrededores indica que algunos taxones son típicos de este entorno en especies o grupos particulares de calaos. Por lo tanto, podrían ser simbiontes especializados que viven alrededor o incluso dentro de la glándula uropigial. Todos estos resultados concuerdan con la hipótesis de que el orden *Bucerotiformes*, puede mostrar asociaciones similares a su grupo hermano del orden *Upupiformes*, en el que se ha demostrado ampliamente una estrecha asociación entre las glándulas uropigiales y las bacterias mutualistas que viven dentro de ella.

Varias especies de calaos tienen bacterias abundantes en las secreciones extraídas del interior de la glándula. Este resultado se basa tanto en el cultivo como en la visualización de las secreciones en un microscopio y, por lo tanto, tiene un soporte similar al de las especies de aves en las que este tipo de relación se ha estudiado más a fondo: la abubilla (Soler et al., 2008; Rodríguez-Ruano et al., 2018). Otros estudios recientes han obtenido un crecimiento bacteriano a partir de muestras de secreción uropigial de diferentes grupos de aves, pero no han examinado las secreciones en un microscopio y, por lo tanto, no se ha comprobado el grado en que dichas bacterias ocupan las secreciones (Braun et al., 2016; Braun et al., 2018b; Braun et al., 2018c; Braun et al., 2019a; Braun et al., 2019b). Hasta donde sabemos, las glándulas uropigiales de los calaos no tienen ampollas agrandadas para almacenar secreciones (Jacob y Ziswiler, 1982), como es el caso

de la abubilla, donde las secreciones se acumulan y las bacterias simbióticas proliferan (Fig. 1, Soler et al., 2008). No se sabe dónde se ubican dentro de las glándulas. Para averiguarlo, se necesita un enfoque anatómico utilizando glándulas fijas de especímenes fallecidos para teñir las bacterias en su posición natural.

Las dos especies con mayor evidencia de bacterias abundantes que viven dentro de las secreciones son: *Bucorvus leadbeateri* y *Bycanistes brevis*. Ambas con más del 50% de secreciones con crecimiento bacteriano y dos de cada tres preparaciones microscópicas con células bacterianas abundantes teñidas en todas las secreciones. De hecho, el crecimiento prevaleció en todas las especies del linaje africano, excepto en *Ceratogymna atrata*, que también presentaba bajas densidades de bacterias en imágenes microscópicas. A pesar de que el tamaño de las muestras de varias de estas especies es bajo y sus imágenes microscópicas no están disponibles, el patrón general para las especies africanas es que presentan de forma más evidente y frecuente bacterias en sus secreciones en comparación con las especies asiáticas. Sin embargo, este resultado debe tomarse con precaución ya que los tamaños de muestra son más pequeños para las especies asiáticas. No obstante, para tres de cada cuatro especies de este linaje no hubo crecimiento al cultivar sus secreciones, un hecho que nunca ocurrió en las especies africanas. Este resultado es contrario al esperado, debido a nuestra hipótesis acerca de que las secreciones coloreadas de los calaos asiáticos eran debido a las bacterias alojadas en sus glándulas. Sin embargo, muchas bacterias no son cultivables en las condiciones utilizadas (Baumann y Moran, 1997) y se necesitan otros enfoques, especialmente la secuenciación del ADN bacteriano presente en las secreciones, para tener un conocimiento completo de la composición del microbioma total presente en estos entornos. Por ejemplo, en la abubilla solo se detectaron *enterococos* en sus secreción mediante los métodos de cultivo (Soler et al., 2008; Ruiz-Rodríguez et al., 2012). Sin embargo, con el método de ARISA y de secuenciación de alto rendimiento (Pyrosequencing e Illumina) se ha demostrado que sus secreciones poseen una comunidad compleja de muchos taxones diferentes (Rodríguez-Ruano et al., 2015; Martínez-García et al., 2016; Martín-Vivaldi et al., 2017; Rodríguez-Ruano et al., 2018).

Curiosamente, cuando aplicamos métodos de secuenciación a las muestras tomadas de diferentes partes del cuerpo en calaos, se descubrió que en las dos especies/géneros con mayor evidencia de la presencia de bacterias dentro de la glándula uropigial, el análisis GNEISS identificó taxones particulares asociados con muestras de la glándula. En

Bucorvus leadbeateri (así como en todo el género *Bucorvus*), cuatro OTUs de Actinobacteria se asociaron significativamente con la base del pico, el casco y la glándula uropigial en comparación con las partes restantes del cuerpo. Entre ellas, una *Propionibacterium* (*Propionibacteraceae*) fue especialmente abundante en comparación con las otras tres *Corynebacteraceae* no identificadas en la glándula uropigial. Una *Cardiobacteriaceae* no identificada (P. *Proteobacteria*) también fue típica de la glándula uropigial y del casco. La mayoría de estos OTUs asociados a la glándula uropigial y al casco son *Corynebacteriaceae*. Por lo tanto, de la misma familia (no sabemos si del mismo género) que los simbiontes que se encuentran en las glándulas uropigiales de pavos y gansos egipcios (Braun et al., 2016; Braun et al., 2018b). Estas bacterias son *Actynomycetales*, un grupo bien conocido por su capacidad de producir antibióticos (Goodfellow y Cross, 1984). Además, suelen estar generalmente involucrados en asociaciones protectoras con animales, como las hormigas cultivadoras de hongos (Barke et al., 2010; Cafaro et al., 2011) y otros grupos (Piel, 2004; Florez et al., 2015). Las especies de *Corynebacterium* se aislaron previamente de las cloacas y otras partes del cuerpo en varias especies de aves (revisado en Braun et al., 2016). También se han detectado varios *Actinomycetales* no identificados en la comunidad presente dentro de las glándulas uropigiales de abubillas (Rodríguez-Ruano et al., 2015; Rodríguez-Ruano et al., 2018). Las *cardiobacteraceae* son un grupo de *Gammaproteobacterias* anaeróbicas facultativas con varias especies involucradas en enfermedades del pericardio y pulmonares en el hombre y en otros animales, incluidas las aves, y aparentemente poco frecuentes en muestras no clínicas (Moore et al., 2014). Por lo tanto, no podemos evaluar la explicación de su asociación con las glándulas en esta especie.

En el caso del género *Bycanistes*, los taxones bacterianos asociados a su glándula uropigial fueron *Enterococcus casseliflavus*. Los *enterococos* son los mutualistas más estudiados presentes en las glándulas uropigiales de *Upupiformes* (Law-Brown y Meyers, 2003; Martin-Platero et al., 2006; Soler et al., 2008; Ruiz-Rodríguez et al., 2012; Rodríguez-Ruano et al., 2018). Además, es conocida su capacidad de producción de antimicrobianos (Martin-Platero et al., 2006; Ruiz-Rodríguez et al., 2012; Ruiz-Rodríguez et al., 2013) y han demostrado tener efectos beneficiosos en los huéspedes (Soler et al., 2008; Ruiz-Rodríguez et al., 2009; Martín-Vivaldi et al., 2014). Por lo tanto, es también un candidato principal para mantener una relación similar en las glándulas de calaos. No

podemos estar seguros de su papel con la evidencia disponible, pero sin duda es un caso que merece más investigación.

Finalmente, las glándulas de *Buceros rhinoceros* estaban claramente asociadas con un grupo de *Rhodobacteraceae* y otros taxones en comparación con otras especies y partes del cuerpo. Este es un resultado interesante por dos razones. Primero, *Buceros rhinoceros* es una especie asiática con coloración cosmética causada por secreciones uropigiales de color amarillo anaranjado, y también es la especie asiática de calao en la que hemos encontrado una mayor prevalencia (tres de tres) de crecimiento bacteriano a partir de secreciones inoculadas. Toda esta evidencia sugiere que hay bacterias especiales vinculadas a las secreciones uropigiales coloreadas en esta especie, tal y como se predijo para los calaos asiáticos. *Rhodobacteraceae* es una de las familias más diversas del dominio Bacteria, con una variedad de ecologías y capacidades fisiológicas. También posee la particularidad de su pigmentación frecuente amarilla-naranja-rosa, que ocurre no solo en miembros fotosintéticos (que presentan Bchl a y carotenoides de clase esferoideno), sino también en miembros no fototróficos (Pujalte et al., 2014). De los tres géneros encontrados en calaos, tanto *Paracoccus* (nueve sOTUs detectados) como *Rubellimicrobium* (un sOTU) son conocidos productores de carotenoides (Pujalte et al., 2014). Aunque la mayoría de las *Rhodobacteraceae* habitan en ambientes marinos, existen casos conocidos de simbiosis con invertebrados marinos y con la rizosfera de plantas, por lo que podría haber evolucionado una relación también con los calaos. Esta relación es muy interesante, ya que estas bacterias amarillo-rojas se encontraron en las secreciones y manchas de color amarillo-naranja del cuerpo de calaos. Solo se puede especular la posibilidad de que tales bacterias estén involucradas en la producción del color de la secreción. No se tiene evidencia de que las aves en este parque zoológico estén provistas de tierra coloreada o fuentes especiales de alimentos enriquecidos con carotenoides u otras formas artificiales por las cuales las bacterias de color podrían haber sido incorporadas por las aves, pero la ubicación particular sugiere que está estrechamente relacionada con la secreción uropigial.

En cuanto a la relación evolutiva en los calaos entre sus ornamentos, encontramos que el grado de exageración de dos adornos, concretamente, las áreas de plumaje blanco y la superficie coloreada del pico, se correlacionan positivamente a través de la filogenia de los calaos. Además la variación en la ornamentación entre especies está relacionada con diferencias en las cargas bacterianas en diferentes partes del cuerpo. Por otra parte, la

prevalencia en la glándula uropigial de un grupo particular de bacterias conocidas por su producción de bacteriocinas (*enterococos*) se relacionó con la abundancia de bacterias patógenas potenciales (queratinolíticas) en el adorno distintivo de los calaos: el casco.

Una crítica principal que se puede hacer a nuestro estudio es que las muestras provienen de individuos mantenidos en cautividad, en diferentes zoológicos, y que las muestras se toman en diferentes estaciones. A esto hay que añadir el bajo tamaño de muestra obtenido para algunas especies y partes del cuerpo. Esto probablemente haya impedido detectar asociaciones con taxones particulares en las especies de calaos con muestras de menor tamaño. No hemos muestreado individuos silvestres, por lo que no podemos estar seguros de que las cargas microbianas encontradas en los individuos mantenidos en cautividad reflejen la de los silvestres. De hecho, algunos estudios han demostrado cómo los microbiomas difieren entre individuos mantenidos en cautividad y silvestres (Hird 2017). Un ejemplo de ello ocurre en las abubillas, cuyo microbioma de la glándula uropigial es ligeramente mayor en individuos silvestres, en comparación con los mantenidos en cautividad. Sin embargo, esto sólo ocurre en una pequeña fracción de las cepas bacterianas del microbioma, con microbiomas centrales muy similares (Martínez-García 2015, Rodríguez-Ruano 2015). Una razón principal para el uso de individuos mantenidos en cautividad es la gran dificultad para obtener muestras de la naturaleza, especialmente en las grandes especies de calaos asiáticos. Aunque no podemos descartar por completo el efecto del cautiverio en nuestros resultados, todos los individuos muestreados fueron mantenidos en cautividad, las especies con secreciones coloridas conocidas también las tenían en cautiverio, y por nuestra experiencia con abubillas sabemos que los componentes típicos de los microbiomas uropigiales, y las propiedades particulares de las secreciones uropigiales se mantienen también en condiciones de cautividad. De esta manera, se espera que esto mismo se pueda aplicar a los calaos. Incluso si el cautiverio hubiese afectado a nuestros resultados, aún se podrían sacar las conclusiones de que los patrones en especímenes mantenidos en cautividad y silvestres deben mostrar relaciones similares para la coloración y el tamaño del microbioma. Esto sugiere que la mayor parte de la variación explicada por los zoológicos se debe a la diferente agrupación de especies ubicadas en diferentes zoológicos.

Existe una variación geográfica en la presencia de cepas bacterianas particulares (Dequiedt et al. 2009). De este modo, es posible que las diferentes gestiones en los zoológicos, incluidas las fuentes de alimentos, afectan a las comunidades bacterianas

presentes en el entorno donde viven estos calaos. Sin embargo, se espera que en su entorno particular, los animales sean colonizados por un conjunto particular de bacterias típicas o capaces de vivir en cada parte del cuerpo en particular, por lo que deben ser ecológicamente equivalentes. En primer lugar, algunos componentes de los microbiomas pueden transmitirse verticalmente y, por lo tanto, mantenerse a lo largo de generaciones (Darby y Douglas 2003, Sachs et al. 2011, Martín-Vivaldi et al. 2018). En segundo lugar, parte de (Martín-Vivaldi et al. (2018)) o la totalidad de los ensamblajes se pueden obtener del ambiente que causa que las especies o las cepas ocupen un nicho particular dentro del microbioma, causando el microbioma en un ambiente específico (tanto en condiciones de cautividad como silvestres). Esto refleja los efectos locales causados por patrones naturales de colonización microbiana. Incluso si estos efectos pueden variar entre los zoológicos de manera diferente a los causados por la dispersión en la naturaleza, dado que para la mayoría de las especies tenemos muestras de diferentes zoológicos, el zoológico de origen probablemente haya diluido en lugar de haber causado efectos. Dado que se han realizado los análisis con enfoques ponderados y no ponderados, los resultados también están controlados por los posibles efectos de especies con menos individuos derivados de un menor número de zoológicos. Además, los resultados indicaron que la variación debido a los zoológicos es relativamente baja cuando se analizaron las cargas bacterianas de *Bucorvus leadbeateri* (la especie con más individuos muestreados en diferentes zoológicos).

Con respecto a la variación en la temporada de muestreo de diferentes individuos, se sabe que, al menos, la composición del microbioma de la secreción uropigial y su función señalizadora pueden variar según la temporada y el sexo en varias especies (Tuttle et al. 2014, Rodríguez-Ruano et al.2018). Las muestras de calaos utilizadas en el presente estudio se recolectaron en diferentes estaciones (ver Métodos) y, por lo tanto, no se puede descartar la posibilidad de que las diferencias en la carga bacteriana entre especies puedan verse influenciadas por la temporada en la que se tomaron las muestras. No obstante, ninguna de las muestras se obtuvo durante la temporada de reproducción por lo que nuestros resultados pueden ser válidos solo fuera de la temporada de reproducción. En cualquier caso, las diferentes especies pueden diferir en su relación con las bacterias entre las estaciones, como es el caso de las abubillas (secreciones oscuras y microbiomas complejos solo durante la temporada de reproducción) (Martín-Vivaldi et al. 2009, Martínez-García et al. 2016) y abubillas arbóreas (secreciones oscuras y bacterias

especializadas que viven en las glándulas durante todo el año (Law-Brown y Meyers 2003, Burger et al. 2004). Por tanto, si la temporada tiene alguna influencia en las variables medidas, no debería causar efectos sino diluirlos. No podemos estar seguros de si algunos aspectos pueden desaparecer mientras que otros se vuelven evidentes durante la temporada de reproducción. Finalmente, en cuanto al sexo, no se encontraron diferencias en la carga bacteriana entre ellos y, por lo tanto, esto no debería haber sesgado nuestros resultados.

Los resultados obtenidos muestran que las especies con secreciones coloreadas tienen una mayor superficie coloreada de rojo-naranja-amarillo en el casco y en el pico, lo que sugiere que la coloración cosmética es la forma principal de aumentar estos colores por especies con secreciones coloridas. El hecho de que el casco explicara esta relación más que el pico, sugiere que esta estructura única en los calaos podría haber evolucionado parcialmente para mostrar el color de sus secreciones uropigiales. Con respecto a la coloración y a la carga bacteriana, las especies con mayores superficies coloreadas en el pico tenían cargas bacterianas más bajas en sus tegumentos. Este resultado puede interpretarse de diferentes maneras. Primero, puede ser consecuencia de que las secreciones pigmentadas que cubren el pico impidan la supervivencia de las bacterias en la superficie a causa de sus propiedades antimicrobianas. Sin embargo, la inclusión de la variable coloración cosmética en el modelo no cambió los resultados, lo que sugiere que no fue debido a los efectos antimicrobianos directos de las secreciones coloreadas. Alternativamente, esta relación encontrada puede indicar que las especies que invierten en este tipo de adornos son más capaces de hacer frente y combatir las bacterias a través de defensas evolucionadas, o al contrario, las especies menos expuestas a las bacterias han podido desarrollar señales dependientes de carotenoides más exageradas. El primer escenario es compatible con estudios en otras especies de aves, como en el estornino negro, en el que se ha encontrado que la coloración del pico refleja diferencias interindividuales en las defensas antimicrobianas (Ruiz-Rodríguez et al.2020, en revisión). No se pueden probar directamente estas alternativas, ya que no se ha estimado la inmunocompetencia de las diferentes especies de calaos. En cualquier caso, la última interpretación parece más plausible, ya que la estimación de la carga bacteriana del pico es probablemente la muestra que más refleja la exposición global a las bacterias del entorno, tanto del ambiente como del alimento. El hecho de que se haya encontrado esta relación solo con un medio general (TSA) también respalda esta explicación.

Además, las especies con una mayor superficie coloreada en el pico, tenían más superficie de plumaje blanco en su cuerpo. Esto puede deberse a la existencia de señales múltiples. En primer lugar, la coloración del pico podría indicar el acceso a los carotenoides y, por lo tanto, su calidad individual. En segundo lugar, la proporción de plumas blancas en el cuerpo podría indicar la calidad individual a través de su estado de degradación (Ruiz-De-Castañeda et al. 2015) ya que son más fácilmente degradables que las plumas melanizadas (Goldstein et al. 2004; Azcárate-García et al. 2020). Varios estudios en otras especies han demostrado cómo los parches de plumaje blanco son un predictor de la calidad fenotípica, ya que dependen de la condición e influyen en el éxito reproductivo (Doucet et al. 2004; revisado en Prum 2006). Además, las áreas blancas pueden teñirse con cosméticos, teniendo también un papel importante en la señalización sexual.

El casco es una estructura exagerada y sexualmente dimorfa única en las especies de calaos, por lo que se predijo que habría evolucionado para señalar la calidad individual. El tamaño de esta estructura es muy variable entre las especies y en algunos (por ejemplo, el género *Tockus*) es pequeño y difícil de distinguir del pico (Kemp 2001, Poonswad et al. 2013). Por lo tanto, no se pueden descartar diferentes funciones del casco dependiendo de la especie (Gamble 2007). Sin embargo, no se encontró una relación entre la coloración o el tamaño del casco y la carga bacteriana. Esto podría deberse al pequeño número de especies incluidas en nuestro estudio. Sin embargo, cuando consideramos el número de estructuras coloreadas de amarillo, naranja o rojo del cuerpo, se relacionada negativamente con la carga de bacterias mesófílas del casco. Este resultado podría interpretarse de la misma manera que la relación negativa entre la superficie coloreada del pico y las bacterias mesófílas, ya que la superficie coloreada del pico se relacionó con la del casco. Un resultado notable del casco es la relación positiva entre la prevalencia de *enterococos* en la glándula uropigial y la carga de bacterias queratinolíticas del casco. Este resultado parece reflejar la asociación entre la evolución de las secreciones uropigiales capaces de albergar *enterococos*, con la selección causada por bacterias queratinolíticas. Estas bacterias pueden deteriorar la apariencia de una estructura exagerada de queratina como lo es el casco. Las secreciones uropigiales de las aves pueden tener propiedades antimicóticas (Bandyopadhyay y Bhattacharyya 1996) y antibacterianas (Shawkey et al. 2003), pero en abubillas y abubillas arbóreas esta capacidad está mediada por *enterococos* simbiontes (Martín-Platero et al. 2006, Ruiz-Rodríguez et al.2013). Dado que también se

han encontrado bacterias en las secreciones de calaos (Capítulo IV), es posible que algunas de estas bacterias sean *enterococos* u otros productores de antimicrobianos, y que esta relación simbiótica haya evolucionado como un medio para proteger el casco. Esto podría explicar por qué muchas especies de calaos manchan el casco con secreciones uropigiales (Kemp 2001, Poonswad et al.2013, Naish 2015). Por lo tanto, este comportamiento podría haber evolucionado para proteger el casco de bacterias queratinolíticas y de enfermedades como el carcinoma, muy común en el casco de *Buceros bicornis* (Miller et al. 1985, Suedmeyer et al. 2001). El hecho de que se haya encontrado una relación positiva entre la superficie coloreada del casco y la coloración cosmética también respalda esta opinión.

La aplicación del conocimiento sobre las estructuras de señalización del color de los calaos se podría utilizar para la conservación y la cría en cautividad de estas especies en peligro de extinción. Comprender las posibles relaciones con la carga bacteriana en *Bucerotiformes* abriría nuevos conocimientos sobre el estudio de la coevolución y la selección sexual. Por ejemplo, sería interesante saber si las bacterias presentes en las secreciones facilitan a las aves combatir los patógenos (es decir, el uso de antimicrobianos y/o carotenoides del metabolismo bacteriano). Más investigación es necesaria para determinar si la coloración ornamental es un rasgo sexualmente seleccionado en calaos. Sería necesario un análisis sólido de una lista más larga de especies que cubran un rango filogenético más amplio, así como más individuos por especie en poblaciones naturales para llegar a conclusiones firmes. La manipulación experimental de los colores basados en carotenoides sería particularmente reveladora.

En resumen, los resultados de esta tesis en conjunto ponen de manifiesto la importancia que los microorganismos tienen en la evolución de las señales en abubillas y calaos. En las abubillas se ha descubierto que parte de estos microorganismos pueden obtenerse vía horizontal, a través del material del nido previamente usado por otra abubilla. Sin embargo, no se ha encontrado un efecto de ese material en el éxito reproductivo. En cuanto a las señales causadas por la presencia de bacterias, se ha encontrado cómo la coloración cosmética de sus huevos es una señal sexual post-apareamiento de la calidad antimicrobiana de la hembra que influye en el aporte de cebas del macho. En cuanto a los calaos, se han encontrado bacterias en la secreción de su glándula uropigial en varias especies. Algunos de sus taxones podrían proporcionarles ventajas en su lucha frente a los parásitos patógenos, o incluso proporcionar sustancias

coloreadas utilizadas para pintar partes de su cuerpo. Por otra parte, parece existir una relación evolutiva entre su coloración y su carga bacteriana de los calaos. En conjunto, estos hallazgos sugieren que el grupo de los calaos es un nuevo modelo de estudio de gran interés para comprender la evolución de simbiosis mutualistas con bacterias en aves, así como de señales asociadas a esa relación.

CONCLUSIONES

1. Las abubillas prefieren utilizar las cajas nido con material durante la época de cría en vez de las cajas nido vacías. Entre las cajas con material, no tienen preferencia por aquellas que contienen material de un nido previamente usado por otra abubilla.

2. El tipo de material del nido afecta a la carga bacteriana de las cáscaras de los huevos y a la composición de la comunidad bacteriana de la secreción uropigial de la hembra.

3. El tipo de material del nido no tiene efectos en el éxito reproductor de la abubilla. No obstante, varios OTUs de la secreción de la hembra se han relacionado con el éxito de eclosión.

4. La saturación del color cosmético de la cáscara de los huevos de abubilla se relaciona negativamente con la carga bacteriana de la secreción uropigial de la hembra.

5. El aporte de cebas del macho disminuye a medida que aumenta la saturación del color cosmético de los huevos. Por tanto, la coloración cosmética de la cáscara del huevo debido a la secreción uropigial, podría actuar como una señal sexual post apareamiento en las hembras de abubilla.

6. En las glándulas uropigiales de calaos también se ha detectado la presencia de bacterias simbiontes, al igual que en las abubillas. Las especies de calaos Africanas generalmente presentan una mayor carga microbiana que las especies Asiáticas.

7. En la secreción uropigial coloreada de una especie Asiática de calao (*Buceros rhinoceros*) se ha puesto de manifiesto la presencia de la Familia *Rhodobacteraceae* (Proteobacterias), conocida por sus propiedades pigmentantes y

por formar interacciones mutualistas en otras especies. Este grupo taxonómico se ha podido detectar tanto en su secreción uropigial como en las partes coloreadas de su cuerpo.

8. El grado de exageración de dos adornos en calaos, concretamente, las áreas de plumaje blanco y la superficie coloreada del pico, se correlacionan positivamente a través de la filogenia de 13 especies de calaos.

9. La variación en la ornamentación entre especies de calaos está relacionada con diferencias en la carga bacteriana de las diferentes partes del cuerpo.

10. La prevalencia en la glándula uropigial de *enterococos* se relaciona con la abundancia de bacterias queratinolíticas en el adorno distintivo de los calaos: el casco. En conjunto, estos hallazgos sugieren que el grupo de los calaos es un nuevo modelo de estudio de gran interés para comprender la evolución de simbiosis mutualistas con bacterias en aves, así como de señales asociadas a esa relación.

CONCLUSIONS

1. Hoopoes prefer to breed in nest boxes with material versus empty ones. Among the boxes with material, they have no preference for those that contained material from a nest previously used by another hoopoe.

2. The type of nest material has an effect on the bacterial load of the eggshells, and the composition of the bacterial community of uropygial secretion of the female.

3. The type of nest material has no effect on reproductive success of hoopoes. However, several OTUs of the uropygial secretion of the female is related to hatching success.

4. The saturation of the cosmetic colour of the eggshells of hoopoes is negatively related to the abundance of symbiotic bacteria in the female's uropygial secretion.

5. Male´s investment is lower in nest containing eggs with a more saturated colouration. Therefore, the cosmetic colour of the eggshells due to uropygial secretion could act as a post-mating sexual signal in the female of hoopoes.

6. Hornbills maintain an association with bacteria in their uropygial glands. African species generally contain higher bacterial loads in their secretions than Asiatic ones.

7. In the coloured uropygial secretion of an Asian species of hornbill (*Buceros rhinoceros*), the Family *Rhodobacteraceae* (Proteobacteria), known for its pigmenting properties and for forming mutualistic interactions in other species, has been revealed. This taxonomic group has been detected both in its uropygial secretion and in the coloured parts of its body.

8. The degree of exaggeration of two ornaments in hornbills, specifically, the white plumage areas and the coloured surface of the beak, are positively correlated through the phylogeny of 13 hornbills species.

9. The variation in ornamentation between hornbills species is related to differences in the bacterial loads of different parts of the body.

10. The prevalence in the uropygial gland of a particular group of *enterococci* was related to the abundance of keratinolytic bacteria in the distinctive ornament of hornbills: the casque. Together, these findings suggest that the group of hornbills is a new study model of great interest to understand the evolution of mutualistic symbiosis with bacteria in birds, as well as of signals associated with that relationship.

BIBLIOGRAFÍA

Alberdi, A., Aizpurua, O., Bohmann, K., Zepeda-Mendoza, M. L. and Gilbert, M. T. P. 2016. Do Vertebrate Gut Metagenomes Confer Rapid Ecological Adaptation? - Trends Ecol. Evol. 31: 689–699.

Alexander, G. D., Houston, D. C. and Campbell, M. 1994. A possible acoustic function for the casque structure in hornbills (Aves: *Bucerotidae*). - J. Zool. 233: 57–67.

Amat, J. A., Rendón, M. A., Garrido-Fernández, J., Garrido, A., Rendón-martos, M. and Pérez-gálvez, A. 2011. Greater flamingos *Phoenicopterus roseus* use uropygial secretions as make-up. - Behav. Ecol. 65: 665–673.

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Zech Xu, Z., Kightley, E. P., Thompson, L. R., Hyde, E. R., Gonzalez, A. and Knight, R. 2017. Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. - mSystems 2: e00191-16.

Amundsen, T. 2000. Why are female birds ornamented? - Trends Ecol. Evol. 15: 149–155.

Amundsen, T. 2018. Sex roles and sexual selection: Lessons from a dynamic model system. - Curr. Zool. 64: 363–392.

Andersson, M. 1994. Sexual selection (Princeton Univ. Press, Ed.).

Andersson, M. and Simmons, L. W. 2006. Sexual selection and mate choice. - Trends Ecol. Evol. 21: 296–302.

Archie, E. A. and Theis, K. R. 2011. Animal behaviour meets microbial ecology. - Anim. Behav. 82: 425–436.

Arlettaz, R., Schaub, M., Fournier, J., Reichlin, T. S., Sierro, A., Watson, J. E. M. and Braunisch, V. 2010. From Publications to Public Actions: When Conservation Biologists Bridge the Gap between Research and Implementation. - Bioscience 60: 835–842.

Aronesty, E. 2011. ea-utils: Command-line tools for processing biological sequencing data.: https://github.com/ExpressionAnalysis/ea-utils.

Avilés, J. M. and Soler, J. J. 2009. Nestling colouration is adjusted to parent visual performance in altricial birds. - J. Evol. Biol. 22: 376–386.

Avilés, J. M., Soler, J. J. and Pérez-Contreras, T. 2006. Dark nests and egg colour in birds: a possible functional role of ultraviolet reflectance in egg detectability. - Proc. Biol.

Sci. 273: 2821–2829.

Avilés, J. M., Pérez-Contreras, T., Navarro, C. and Soler, J. J. 2009. Male spotless starlings adjust feeding effort based on egg spots revealing ectoparasite load. - Anim. Behav. 78: 993–999.

Avilés, J. M., Vikan, J. R., Fossøy, F., Antonov, A., Moksnes, A., Røskaft, E. and Stokke, B. G. 2010. Avian colour perception predicts behavioural responses to experimental brood parasitism in chaffinches. - J Evol Biol 23: 293–301.

Avilés, J. M., Soler, J. J. and Hart, N. S. 2011. Sexual selection based on egg colour: Physiological models and egg discrimination experiments in a cavity-nesting bird. - Behav. Ecol. Sociobiol. 65: 1721–1730.

Azcárate-García, M., González-Braojos, S., Díaz-Lora, S., Ruiz-Rodríguez, M., Martin-Vivaldi, M., Martínez-Bueno, M., Moreno, J. and Soler, J. J. 2020. Interspecific variation in deterioration and degradability of avian feathers: The evolutionary role of the microbiome. - J. Avian Biol.: in press.

Baker, L. J., Freed, L. L., Easson, C. G., Lopez, J. V, Fenolio, D., Sutton, T. T., Nyholm, S. V and Hendry, T. A. 2019. Diverse deep-sea anglerfishes share a genetically reduced luminous symbiont that is acquired from the environment. - Elife 8: e47606.

Bandyopadhyay, A. and Bhattacharyya, S. P. 1996. Influence of fowl uropygial gland and its secretory lipid components on growth of skin surface bacteria of fowl. - Indian J. Exp. Biol. 34: 48–52.

Banning, J. L., Weddle, A. L., Wahl III, G. W., Simon, M. A., Lauer, A., Walters, R. L. and Harris, R. N. 2008. Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, *Hemidactylium scutatum.* - Oecologia 156: 423–429.

Barbieri, E., K. Barry, A. Child, and N. Wainwright. 1997. Antimicrobial activity in the microbial community of the accessory nidamental gland and egg cases of Loligo pealei (Cephalopoda: *Loliginidae*). Biological Bulletin 193(2):275-276.

Barke, J., R. Seipke, S. Gruschow, D. Heavens, N. Drou, M. Bibb, R. Goss, D. Yu, and M. Hutchings. 2010. A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus.* Bmc Biology 8(1):109.

Barr, J. J., Auro, R., Furlan, M., Whiteson, K. L., Erb, M. L., Pogliano, J., Stotland, A., Wolkowicz, R., Cutting, A. S., Doran, K. S., Salamon, P., Youle, M. and Rohwer, F.

2013. Bacteriophage adhering to mucus provide a non–host-derived immunity. - Proc. Natl. Acad. Sci. 110: 10771–10776.

Baumann, P., and N. A. Moran. 1997. Non-cultivable microorganisms from symbiotic associations of insects and other hosts. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 72(1):39-48.

Beltran-Bech, S. and Richard, F.-J. 2014. Impact of infection on mate choice. - Anim. Behav. 90: 159–170.

Bennett, G. M. and Moran, N. A. 2015. Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. - Proc. Natl. Acad. Sci. 112: 10169–10176.

Bentley, D. R., Balasubramanian, S., Swerdlow, H. P., Smith, G. P., Milton, J., Brown, C. G., Hall, K. P., Evers, D. J., Barnes, C. L., Bignell, H. R., Boutell, J. M., Bryant, J., Carter, R. J., Keira Cheetham, R., Cox, A. J., Ellis, D. J., Flatbush, M. R., Gormley, N. A., Humphray, S. J., Irving, L. J., Karbelashvili, M. S., Kirk, S. M., Li, H., Liu, X., Maisinger, K. S., Murray, L. J., Obradovic, B., Ost, T., Parkinson, M. L., Pratt, M. R., Rasolonjatovo, I. M. J., Reed, M. T., Rigatti, R., Rodighiero, C., Ross, M. T., Sabot, A., Sankar, S. V., Scally, A., Schroth, G. P., Smith, M. E., Smith, V. P., Spiridou, A., Torrance, P. E., Tzonev, S. S., Vermaas, E. H., Walter, K., Wu, X., Zhang, L., Alam, M. D., Anastasi, C., Aniebo, I. C., Bailey, D. M. D., Bancarz, I. R., Banerjee, S., Barbour, S. G., Baybayan, P. A., Benoit, V. A., Benson, K. F., Bevis, C., Black, P. J., Boodhun, A., Brennan, J. S., Bridgham, J. A., Brown, R. C., Brown, A. A., Buermann, D. H., Bundu, A. A., Burrows, J. C., Carter, N. P., Castillo, N., Catenazzi, M. C. E., Chang, S., Neil Cooley, R., Crake, N. R., Dada, O. O., Diakoumakos, K. D., Dominguez-Fernandez, B., Earnshaw, D. J., Egbujor, U. C., Elmore, D. W., Etchin, S. S., Ewan, M. R., Fedurco, M., Fraser, L. J., Fuentes Fajardo, K. V., Scott Furey, W., George, D., Gietzen, K. J., Goddard, C. P., Golda, G. S., Granieri, P. A., Green, D. E., Gustafson, D. L., Hansen, N. F., Harnish, K., Haudenschild, C. D., Heyer, N. I., Hims, M. M., et al. 2008. Accurate whole human genome sequencing using reversible terminator chemistry. - Nature 456: 53–59.

Boisbunon, A., Canu, S., Fourdrinier, D., Strawderman, W. and Wells, M. T. 2013. AIC , Cp and estimators of loss for elliptically symmetric distributions. - hal-00851206

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., Mills, D. A. and Caporaso, J. G. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. - Nat. Methods 10: 57–59.

Bolyen, E., J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet, G. A. Al-Ghalith, H. Alexander, E. J. Alm, M. Arumugam, F. Asnicar, Y. Bai, J. E. Bisanz, K. Bittinger, A. Brejnrod, C. J. Brislawn, C. T. Brown, B. J. Callahan, A. M. Caraballo-Rodríguez, J. Chase, E. K. Cope, R. Da Silva, C. Diener, P. C. Dorrestein, G. M. Douglas, D. M. Durall, C. Duvallet, C. F. Edwardson, M. Ernst, M. Estaki, J. Fouquier, J. M. Gauglitz, S. M. Gibbons, D. L. Gibson, A. Gonzalez, K. Gorlick, J. Guo, B. Hillmann, S. Holmes, H. Holste, C. Huttenhower, G. A. Huttley, S. Janssen, A. K. Jarmusch, L. Jiang, B. D. Kaehler, K. B. Kang, C. R. Keefe, P. Keim, S. T. Kelley, D. Knights, I. Koester, T. Kosciolek, J. Kreps, M. G. I. Langille, J. Lee, R. Ley, Y.-X. Liu, E. Loftfield, C. Lozupone, M. Maher, C. Marotz, B. D. Martin, D. McDonald, L. J. McIver, A. V. Melnik, J. L. Metcalf, S. C. Morgan, J. T. Morton, A. T. Naimey, J. A. Navas-Molina, L. F. Nothias, S. B. Orchanian, T. Pearson, S. L. Peoples, D. Petras, M. L. Preuss, E. Pruesse, L. B. Rasmussen, A. Rivers, M. S. Robeson, P. Rosenthal, N. Segata, M. Shaffer, A. Shiffer, R. Sinha, S. J. Song, J. R. Spear, A. D. Swafford, L. R. Thompson, P. J. Torres, P. Trinh, A. Tripathi, P. J. Turnbaugh, S. Ul-Hasan, J. J. J. van der Hooft, F. Vargas, Y. Vázquez-Baeza, E. Vogtmann, M. von Hippel, W. Walters, Y. Wan, M. Wang, J. Warren, K. C. Weber, C. H. D. Williamson, A. D. Willis, Z. Z. Xu, J. R. Zaneveld, Y. Zhang, Q. Zhu, R. Knight, and J. G. Caporaso. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 37(8):852-857. doi: 10.1038/s41587-019-0209-9

Brandl, H. B., van Dongen, W. F. D., Darolová, A., Kristofík, J., Majtan, J. and Hoi, H. 2014. Composition of Bacterial Assemblages in Different Components of Reed Warbler Nests and a Possible Role of Egg Incubation in Pathogen Regulation. - PLoS One 9(12): 1–19.

Braun, M. S., Zimmermann, S., Danner, M., Rashid, H. and Wink, M. 2016. Corynebacterium uropygiale sp. nov., isolated from the preen gland of Turkeys *(Meleagris gallopavo)*. - Syst. Appl. Microbiol. 39: 88–92.

Braun, M. S., Wang, E., Zimmermann, S. and Wink, M. 2018a. *Corynebacterium heidelbergense sp. nov.*, isolated from the preen glands of Egyptian geese (*Alopochen aegyptiacus*). - Syst. Appl. Microbiol. 41: 564–569.

Braun, M. S., Wang, E., Zimmermann, S., Boutin, S. and Wink, M. 2018b. *Kocuria uropygioeca sp. nov*. and *Kocuria uropygialis sp. nov*., isolated from the preen glands

of Great Spotted Woodpeckers (*Dendrocopos major*). - Syst. Appl. Microbiol. 41: 38–43.

Braun, M. S., Sporer, F., Zimmermann, S. and Wink, M. 2018c. Birds, feather-degrading bacteria and preen glands: The antimicrobial activity of preen gland secretions from turkeys (*Meleagris gallopavo*) is amplified by keratinase. - FEMS Microbiol. Ecol. 94: fiy117.

Braun, M. S., Wang, E., Zimmermann, S., Boutin, S., Wagner, H. and Wink, M. 2019a. *Kocuria tytonicola*, new bacteria from the preen glands of American barn owls (*Tyto furcata*). - Syst. Appl. Microbiol. 42: 198–204.

Braun, M. S., Wang, E., Zimmermann, S., Wagner, H. and Wink, M. 2019b. *Kocuria tytonis sp. nov.*, isolated from the uropygial gland of an American barn owl (*Tyto furcata*). - Int. J. Syst. Evol. Microbiol. 69: 447–451.

Bright, M. and Bulgheresi, S. 2010. A complex journey: transmission of microbial symbionts. - Nat. Rev. Microbiol. 8: 218–230.

Brulez, K., Pike, T. W. and Reynolds, S. J. 2015. Egg signalling: The use of visual, auditory, and chemical stimuli. - In: Deeming, D. C. and Reynolds, S. J. (eds), Nest, Egg, and Incubation. New ideas about avian reproduction. Oxford University Press, pp. 127–141.

Brunetti, A. E., Lyra, M. L., Melo, W. G. P., Andrade, L. E., Palacios-Rodríguez, P., Prado, B. M., Haddad, C. F. B., Pupo, M. T. and Lopes, N. P. 2019. Symbiotic skin bacteria as a source for sex-specific scents in frogs. - Proc. Natl. Acad. Sci. 116: 2124–2129.

Bulla, M., Šálek, M. and Gosler, A. G. 2012. Eggshell spotting does not predict male incubation but marks thinner areas of a shorebird's shells. - Auk 129: 26–35.

Burger, B. V, Reiter, B., Borzyk, O. and du Plessis, M. A. 2004. Avian Exocrine Secretions. I. Chemical Characterization of the Volatile Fraction of the Uropygial Secretion of the Green Woodhoopoe, *Phoeniculus purpureus.* - J. Chem. Ecol. 30: 1603–1611.

Burley, N. 1986. Sexual Selection for Aesthetic Traits in Species with Biparental Care. - Am. Nat. 127: 415–445.

Burley, N. 1988. The Differential Allocation hypothesis. An experimental test. - Am. Nat. 132: 611–628.

Cafaro, M. J., M. Poulsen, A. E. F. Little, S. L. Price, N. M. Gerardo, B. Wong, A. E. Stuart, B. Larget, P. Abbot, and C. R. Currie. 2011. Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. Proceedings of the Royal Society B-Biological Sciences 278(1713):1814-1822.

Callahan, B. J., McMurdie, P. J. and Holmes, S. P. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. - ISME J. 11: 2639–2643.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., González Peña, A., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., Mcdonald, D., Muegge, B., Pirrung, M., Reeder, J., Sevinsky, J., Turnbaugh, P., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J. and Knight, R. 2010. QIIME allows analysis of high-throughput community sequencing data. - Nat Methods. 7: 335–336.

Caruso, V., Song, X., Asquith, M. and Karstens, L. 2019. Performance of Microbiome Sequence Inference Methods in Environments with Varying Biomass (SM Gibbons, Ed.). - mSystems 4: e00163-18.

Chapman, T., Arnqvist, G., Bangham, J. and Rowe, L. 2003. Sexual conflict. - Trends Ecol. Evol. 18: 41–47.

Chau, R., J. A. Kalaitzis, and B. A. Neilan. 2011. On the origins and biosynthesis of tetrodotoxin. Aquatic Toxicology 104(1):61-72.

Cherry, M. I. and Gosler, A. G. 2010. Avian eggshell coloration: New perspectives on adaptive explanations. - Biol. J. Linn. Soc. 100: 753–762.

Chiarello, M., Villéger, S., Bouvier, C., Auguet, J. C. and Bouvier, T. 2017. Captive bottlenose dolphins and killer whales harbor a species-specific skin microbiota that varies among individuals. - Sci. Rep. 7: 15269.

Clayton, D. H. 1991. The influence of parasites on host sexual selection. - Parasitol. Today 7: 329–334.

Clutton-Brock, T. H. 1991. The evolution of parental care (Princeton University Press, Ed.).

Clutton-Brock, T. 2007. Sexual Selection in Males and Females. - Science (80). 318: 1882–1885.

Clutton-Brock, T. 2009. Sexual selection in females. - Anim. Behav. 77: 3–11.

Cook, M. I., Beissinger, S. R., Toranzos, G. A. and Arendt, W. J. 2005. Incubation

reduces microbial growth on eggshells and the opportunity for trans-shell infection. - Ecol. Lett. 8: 532–537.

Corti, M., Romano, A., Costanzo, A., Bentz, A. B., Navara, K. J., Parolini, M., Saino, N. and Rubolini, D. 2018. Protoporphyrin-based eggshell pigmentation predicts hatching success and offspring sex ratio in the barn swallow. - J. Avian Biol. 49: 1–17.

Cramp, S. 1998. The complete birds of the western Palearctic on CD-ROM. - Oxford University Press.

Cunningham, E. J. A. and Russell, A. F. 2000. Egg investment is influenced by male attractiveness in the mallard. - Nature 404: 74–77.

Currie, C. R., M. Poulsen, J. Mendenhall, J. J. Boomsma, and J. Billen. 2006. Coevolved Crypts and Exocrine Glands Support Mutualistic Bacteria in Fungus-Growing Ants. Science 311:81-83.

Currie, C. R., Scott, J. A., Summerbell, R. C. and Malloch, D. 1999. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. - Nature 398: 701–704.

D'Alba, L. and Shawkey, M. D. 2015. Mechanisms of antimicrobial defense in avian eggs. - J. Ornithol. 156: S399–S408.

Darby, A. C. and Douglas, A. E. 2003. Elucidation of the Transmission Patterns of an Insect-Borne Bacterium. - Appl. Environ. Microbiol. 69: 4403 LP – 4407.

Darwin, C. 1871. The descent of man and selection in relation to sex (J Murray, Ed.).

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A. and Callahan, B. J. 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. - Microbiome 6: 226.

de Goffau, M. C., Lager, S., Salter, S. J., Wagner, J., Kronbichler, A., Charnock-Jones, D. S., Peacock, S. J., Smith, G. C. S. and Parkhill, J. 2018. Recognizing the reagent microbiome. - Nat. Microbiol. 3: 851–853.

Delhey, K., Peters, A. and Kempenaers, B. 2007. Cosmetic Coloration in Birds: Occurrence, Function, and Evolution. - Am. Nat. 169: S145–S158.

Dequiedt, S., Thioulouse, J., Jolivet, C., Saby, N. P. A., Lelievre, M., Maron, P.-A., Martin, M. P., Prévost-Bouré, N. C., Toutain, B., Arrouays, D., Lemanceau, P. and Ranjard, L. 2009. Biogeographical patterns of soil bacterial communities. - Environ. Microbiol. Rep. 1: 251–255.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P. and Andersen, G. L. 2006. Greengenes, a chimera-checked 16S

rRNA gene database and workbench compatible with ARB. - Appl. Environ. Microbiol. 72: 5069–5072.

Devaynes, A., Antunes, A., Bedford, A. and Ashton, P. 2018. Progression in the bacterial load during the breeding season in nest boxes occupied by the Blue Tit and its potential impact on hatching or fledging success. - J. Ornithol. 159: 1009–1017.

Díaz-Lora, S., Pérez-Contreras, T., Azcárate-García, M., Peralta-Sánchez, J. M., Martínez-Bueno, M., Soler, J. J. and Martín-Vivaldi, M. Cosmetic coloration of cross-fostered eggs affects male parental investment in the Hoopoe (*Upupa epops*). - Under Rev. in press.

Díaz-Lora, S., Pérez-Contreras, T., Azcárate-García, M., Martínez-Bueno, M., Soler, J. J. and Martín-Vivaldi, M. Hoopoe (*Upupa epops*) male feeding effort is related to female cosmetic egg colouration. - Journal of Avian Biology: Under Rev.

Díaz-Lora, S., Martín-Vivaldi, M., Juárez-García-Pelayo, N., Azcárate-García, M., Rodríguez-Ruano, S., M., Martínez-Bueno, M. and Soler, J. J. 2019. Data from: experimental old nest material predicts hoopoe *Upupa epops* eggshell and uropygial gland microbiota. − Dryad Digital Repository.

Dillon, R. J., Vennard, C. T., Buckling, A. and Charnley, A. K. 2005. Diversity of locust gut bacteria protects against pathogen invasion. - Ecol. Lett. 8: 1291–1298.

Douglas, A. E. 1998. Nutritional Interactions in Insect-Microbial Symbioses: Aphids and Their Symbiotic Bacteria *Buchnera*. - Annu. Rev. Entomol. 43: 17–37.

Dumbrell, A. J., Ferguson, R. M. W. and Clark, D. R. 2017. Microbial Community Analysis by Single-Amplicon High-Throughput Next Generation Sequencing: Data Analysis – From Raw Output to Ecology. - In: McGenity, T. J. et al. (eds), Springer. Hydrocarbon and Lipid Microbiology Protocols: Microbial Quantitation, Community Profiling and Array Approaches, pp. 155–206.

Dunlap, P. V., J. C. Ast, S. Kimura, A. Fukui, T. Yoshino, and H. Endo. 2007. Phylogenetic analysis of host-symbiont specificity and codivergence in bioluminescent symbioses. Cladistics 23(5):507-532.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. - Bioinformatics 26: 2460–2461.

Edgar, R. C. 2017. UNCROSS: Filtering of high-frequency cross-talk in 16S amplicon reads. - PeerJ 5: e3889.

Edward, D. A. and Chapman, T. 2011. The evolution and significance of male mate

choice. - Trends Ecol. Evol. 26: 647–654.

Eisenhofer, R., Minich, J. J., Marotz, C., Cooper, A., Knight, R. and Weyrich, L. S. 2019. Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. - Trends Microbiol. 27: 105–117.

Ellegaard, K. M. and Engel, P. 2016. Beyond 16S rRNA Community Profiling: Intra-Species Diversity in the Gut Microbiota . - Front. Microbiol. 7: 1475.

Endler, J. A. 1990. On the measurement and classification of color in studies of animal color patterns. - Biol. J. Linn. Soc. 41: 315–352.

Endler, J. A. and Mielke, P. W. 2005. Comparing color patterns as birds see them. - Biol. J. Linn. Soc. 86: 405–431.

English, P. A. and Montgomerie, R. 2011. Robin's egg blue: Does egg color influence male parental care? - Behav. Ecol. Sociobiol. 65: 1029–1036.

Erckmann, W., Beletsky, L., Orians, G., Johnsen, T., Sharbaugh, S. and D´Antonio, C. 1990. Old nests as cues for nest-site selection: an experimental test with Red-winged Blackbirds. - Condor 92: 113–117.

Ezenwa, V. O. and Williams, A. E. 2014. Microbes and animal olfactory communication: Where do we go from here? - BioEssays 36: 847–854.

Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M. and Xavier, J. B. 2012. Animal Behavior and the Microbiome. - Science (80). 338: 198–199.

Fitzpatrick, C. L. and Servedio, M. R. 2018. The evolution of male mate choice and female ornamentation: A review of mathematical models. - Curr. Zool. 64: 323–333.

Flórez, L. V. and Kaltenpoth, M. 2017. Symbiont dynamics and strain diversity in the defensive mutualism between *Lagria* beetles and *Burkholderia*. - Environ. Microbiol. 19: 3674–3688.

Flórez, L. V, Biedermann, P. H. W., Engl, T. and Kaltenpoth, M. 2015. Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. - Nat. Prod. Rep. 32: 904–936.

Folstad, I. and Karter, A. J. 1992. Parasites, bright males, and the immunocompetence handicap. - Am. Nat. 139: 603–622.

Fons, M., Gomez, A. and Karjalainen, T. 2000. Mechanisms of Colonisation and Colonisation Resistance of the Digestive Tract Part 2: Bacteria/Bacteria Interactions. - Microb. Ecol. Health Dis. 12: 240–246.

Fontaine, J. J. and Martin, T. E. 2006. Parent birds assess nest predation risk and adjust

their reproductive strategies. - Ecol. Lett. 9: 428–434.

Fox, H. M. and Vevers, G. 1960. The nature of animal colors.

Friedman, J. and Alm, E. J. 2012. Inferring Correlation Networks from Genomic Survey Data. - PLoS Comput. Biol. 8: e1002687.

Fronstin, R. B., Doucet, S. M. and Christians, J. K. 2016. Haematocrit, eggshell colouration and sexual signaling in the European starling (*Sturnus vulgaris*). - BMC Ecol. in press.

Gamble, K. C. 2007. Internal Anatomy of the Hornbill Casque Described by Radiography , Contrast Radiography , and Computed Tomography. - J. Avian Med. Surg. 21: 38–49.

Gamble, K. C. 2012. Chapter 37. Squamous Cell Carcinoma in Buceros Hornbills. - In: Miller, R. E. and Fowler, M. (eds), Fowler's Zoo and Wild Animal Medicine. pp. 281–285.

Garamszegi, Z. and Møller, A. P. 2010. Effects of sample size and intraspecific variation in phylogenetic comparative studies : a meta-analytic review. - Biol. Rev. 85: 797–805.

Garamszegi, L. Z. and Møller, A. P. 2011. Nonrandom Variation in Within-Species Sample Size and Missing Data in Phylogenetic Comparative Studies. - Syst. Biol. 60: 876–880.

Gaston, A. J., De Forest, L. N. and G.Noble, D. 1993. Egg Recognition and Egg Stealing in Murres (*Uria spp.*). - Anim. Behav. 45: 301–306.

Gawryszewski, F. M. 2018. Color vision models: Some simulations, a general n-dimensional model, and the colourvision R package. - Ecol. Evol.: 8159–8170.

Giard, J.-C., Laplace, J.-M., Rincé, A., Pichereau, V., Benachour, A., Leboeuf, C., Flahaut, S., Auffray, Y. and Hartke, A. 2001. The stress proteome of *Enterococcus faecalis*. - Electrophoresis 22: 2947–2954.

Gil-Turnes, M. S. and Fenical, W. 1992. Embryos of *Homarus americanus* are Protected by Epibiotic Bacteria. - Biol. Bull. 182: 105–108.

Giordano, M., Costantini, D., Pick, J. L. and Tschirren, B. 2015. Female oxidative status, egg antioxidant protection and eggshell pigmentation: a supplemental feeding experiment in great tits. - Behav. Ecol. Sociobiol. 69: 777–785.

Goldstein, G., Flory, K. R., Browne, B. A., Majid, S., Ichida, J. M. and Burtt Jr., E. H. 2004. Bacterial Degradation of Black and White Feathers. - Auk 121: 656–659.

Golob, J. L., Margolis, E., Hoffman, N. G. and Fredricks, D. N. 2017. Evaluating the accuracy of amplicon-based microbiome computational pipelines on simulated human gut microbial communities. - BMC Bioinformatics 18: 283.

Gómez, D. 2006. AVICOL a program to analyse spectrometric data. Last update october 2011. Free executable available at http://sites.google.com/site/avicolprogram/ or from the author at dodogomez@yahoo.fr.

González-Braojos, S., Vela, A. I., Ruiz-De-Castañeda, R., Briones, V., Cantarero, A. and Moreno, J. 2012. Is Nestling Growth Affected by Nest Reuse and Skin Bacteria in Pied Flycatchers *Ficedula hypoleuca?* - Acta Ornithol. 47: 119–127.

Gonzalez, J. C. T., Sheldon, B. C., Collar, N. J. and Tobias, J. A. 2013. A comprehensive molecular phylogeny for the hornbills (Aves: *Bucerotidae*). - Mol. Phylogenet. Evol. 67: 468–483.

Goodfellow, M., and T. Cross. 1984. The Biology of Actinomycetes. Academic Press, London.

Gosler, A. G., Higham, J. P. and Reynolds, S. J. 2005. Why are birds ' eggs speckled ? - Ecol. Lett. 8: 1105–1113.

Grether, G. F., Hudon, J. and Millie, D. F. 1999. Carotenoid limitation of sexual coloration along an environmental gradient in guppies. - Proc. R. Soc. London. Ser. B Biol. Sci. 266: 1317–1322.

Griggio, M., Valera, F., Casas, A. and Pilastro, A. 2005. Males prefer ornamented females: A field experiment of male choice in the rock sparrow. - Anim. Behav. 69: 1243–1250.

Grizard, S., Dini-Andreote, F., Tieleman, B. I. and Salles, J. F. 2014. Dynamics of bacterial and fungal communities associated with eggshells during incubation. - Ecol. Evol. 4: 1140–1157.

Grizard, S., Versteegh, M. A., Ndithia, H. K., Salles, J. F. and Tieleman, B. I. 2015. Shifts in Bacterial Communities of Eggshells and Antimicrobial Activities in Eggs during Incubation in a Ground-Nesting Passerine. - PLoS One: 10(4).

Gunderson, A. R. 2008. Feather-Degrading Bacteria: A New Frontier in Avian and Host–Parasite Research? - Auk 125: 972–979.

Gwinner, H. and Berger, S. 2005. European starlings: Nestling condition, parasites and green nest material during the breeding season. - J. Ornithol. 146: 365–371.

Hackett, S. J., R. T. Kimball, S. Reddy, R. C. K. Bowie, E. L. Braun, M. J. Braun, J. L. Chojnowski, W. A. Cox, K. L. Han, J. Harshman, C. J. Huddleston, B. D. Marks, K. J. Miglia, W. S. Moore, F. H. Sheldon, D. W. Steadman, C. C. Witt, and T. Yuri. 2008. A Phylogenomic Study of Birds Reveals Their Evolutionary History. Science 320(5884):1763-1768.

Haine, E. R. 2008. Symbiont-mediated protection. - Proc. R. Soc. B Biol. Sci. 275: 353–361.

Hamilton, W. D. and Zuk, M. 1982. Heritable true fitness and bright birds: a role for parasites? - Science (80-.). 218: 384–387.

Hanley, D. 2012. The function and evolution of egg colour in birds. - Electron. Theses Diss.: 382.

Hanley, D. and Doucet, S. M. 2009. Egg coloration in ring-billed gulls (*Larus delawarensis*): A test of the sexual signaling hypothesis. - Behav. Ecol. Sociobiol. 63: 719–729.

Hanley, D., Heiber, G. and Dearborn, D. C. 2008. Testing an Assumption of the Sexual-Signaling Hypothesis: Does Blue-Green Egg Color Reflect Maternal Antioxidant Capacity? - Condor 110: 767–771.

Hanley, D., Doucet, S. M. and Dearborn, D. C. 2010. A Blackmail Hypothesis for the Evolution of Conspicuous Egg Coloration in Birds. - Auk 127: 453–459.

Hargitai, R., Boross, N., Nyiri, Z. and Eke, Z. 2018a. Effects of food limitation on the intensity of blue-green and brown eggshell coloration: an experimental study with the canary. - J. Avian Biol.: e01486.

Hargitai, R., Herényi, M., Nagy, G. and Török, J. 2018b. Eggshell spotting pattern is related to hatching asynchrony, hematocrit value and growth of nestling great tits Parus major. - J. Avian Biol. 49: e01827.

Hart, N. S. 2001. The visual ecology of avian Photoreceptors. - Prog. Retin. Eye Res. 20, No. 5: 675 to 703.

Hart, N. S. 2002. Vision in the peafowl (Aves: *Pavo cristatus*). - J. Exp. Biol. 205: 3925–3935.

Harvey, P. H. and Pagel, M. D. 1991. The Comparative Method in Evolutionary Biology. - Oxford University Press.

He, Y., Caporaso, J. G., Jiang, X.-T., Sheng, H.-F., Huse, S. M., Rideout, J. R., Edgar, R. C., Kopylova, E., Walters, W. A., Knight, R. and Zhou, H.-W. 2015. Stability of

operational taxonomic units: an important but neglected property for analyzing microbial diversity. - Microbiome 3: 20.

Heath-Heckman, E. A. C., Peyer, S. M., Whistler, C. A., Apicella, M. A., Goldman, W. E. and McFall-Ngai, M. J. 2013. Bacterial Bioluminescence Regulates Expression of a Host Cryptochrome Gene in the Squid-Vibrio Symbiosis (J Handelsman, Ed.). - MBio 4: e00167-13.

Heath, K. D. and Tiffin, P. 2007. Context dependence in the coevolution of plant and rhizobial mutualists. - Proc. R. Soc. B Biol. Sci. 274: 1905–1912.

Henry, L. P., Bruijning, M., Forsberg, S. K. G. and Ayroles, J. F. 2019. Can the microbiome influence host evolutionary trajectories? - bioRxiv: 700237.

Herring, P. J. 2007. Sex with the lights on? A review of bioluminescent sexual dimorphism in the sea. Journal of the Marine Biological Association of the United Kingdom 87(4):829-842.

Hildebrandt, B., and M. Schaub (2017). The effects of hatching asynchrony on growth and mortality patterns in Eurasian Hoopoe *Upupa epops* nestlings. Ibis:0–3.

Hill, M. J. 1997. Intestinal flora and endogenous vitamin synthesis. - Eur. J. Cancer Prev. 6 Suppl 1: S43-45.

Hill, G. E. 2000. Energetic constraints on expression of carotenoid-based plumage coloration. - J. Avian Biol. 31: 559–566.

Hill, G. E. 2002. A red bird in a brown bag: the function and evolution of ornamental plumage coloration in the house finch. - Oxford University Press.

Hill, G. E. 2006. Environmental Regulation of Ornamental Coloration. - In: Hill, G. E. and Mcgraw, K. J. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 507–560.

Hill, G. E., Farmer, K. L. and Beck, M. L. 2004. The effect of mycoplasmosis on carotenoid plumage coloration in male house finches. - J. Exp. Biol. 207: 2095–2099.

Hird, S. M. 2017. Evolutionary Biology Needs Wild Microbiomes. - Front. Microbiol. 8: 725.

Hoffmann, J., Postma, E. and Schaub, M. 2015. Factors influencing double brooding in Eurasian Hoopoes *Upupa epops*. - Ibis (Lond. 1859). 157: 17–30.

Holveck, M.-J., Doutrelant, C., Guerreiro, R., Perret, P., Gomez, D. and Grégoire, A. 2010. Can eggs in a cavity be a female secondary sexual signal? Male nest visits and modelling of egg visual discrimination in blue tits. - Biol. Lett. 6: 453–457.

Holveck, M., Guerreiro, R., Perret, P., Doutrelant, C. and Grégoire, A. 2019. Eggshell coloration indicates female condition during egg-laying: a field experiment in blue tits. - Biol. J. Linn. Soc. XX: 1–20.

Honza, M., Požgayová, M., Procházka, P. and Cherry, M. I. 2011. Blue-green eggshell coloration is not a sexually selected signal of female quality in an open-nesting polygynous passerine. - Naturwissenschaften 98: 493–499.

Hornung, B. V. H., Zwittink, R. D. and Kuijper, E. J. 2019. Issues and current standards of controls in microbiome research. - FEMS Microbiol. Ecol. 95: fiz045.

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y. and Fukatsu, T. 2010. Wolbachia as a bacteriocyte-associated nutritional mutualist. - Proc. Natl. Acad. Sci. 107: 769–774.

Hoyt, D. F. 1979. Practical methods of estimating volume and fresh weight of bird eggs. - Auk 96: 73–77.

Hwang, D. F., O. Arakawa, T. Saito, T. Noguchi, U. Simidu, K. Tsukamoto, Y. Shida, and K. Hashimoto. 1989. Tetrodotoxin-producing bacteria from the blue-ringed octopus *Octopus maculosus*. Marine Biology 100(3):327-332. doi: 10.1007/BF00391147

Jacob, J. and Ziswiler, V. 1982. The uropygial gland. - In: Farner, D. S. et al. (eds), Avian biology, vol VI. Academic Press, pp. 359–362.

Jacob, S., Parthuisot, N., Vallat, A., Ramon-Portugal, F., Helfenstein, F. and Heeb, P. 2015. Microbiome affects egg carotenoid investment, nestling development and adult oxidative costs of reproduction in Great tits. - Funct. Ecol. 29: 1048–1058.

Janssen, S., D. McDonald, A. Gonzalez, J. A. Navas-Molina, L. Jiang, Z. Z. Xu, K. Winker, D. M. Kado, E. Orwoll, M. Manary, S. Mirarab, and R. Knight. 2018. Phylogenetic Placement of Exact Amplicon Sequences Improves Associations with Clinical Information. mSystems 3(3):e00021-00018. doi: 10.1128/mSystems.00021-18

Janzen, D. H. 1985. The natural history of mutualism. - In: Boucher, D. H. (ed), The Biology of Mutualism: Ecology and Evolution. Oxford University Press, pp. 40–99.

Javůrková, V. G., Kreisinger, J., Procházka, P., Požgayová, M., Ševčíková, K., Brlík, V., Adamík, P., Heneberg, P. and Porkert, J. 2019. Unveiled feather microcosm: feather microbiota of passerine birds is closely associated with host species identity and bacteriocin-producing bacteria. - ISME J.: 2363–2376.

Jervis-Bardy, J., Leong, L. E. X., Marri, S., Smith, R. J., Choo, J. M., Smith-Vaughan, H.

C., Nosworthy, E., Morris, P. S., O'Leary, S., Rogers, G. B. and Marsh, R. L. 2015. Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. - Microbiome 3: 19.

Jetz, W., Thomas, G. H., Joy, J. B., Hartmann, K. and Mooers, A. O. 2012. The global diversity of birds in space and time. - Nature 491: 444–448.

Johnsen, A., Vesterkjær, K. and Slagsvold, T. 2011. Do Male Pied Flycatchers (*Ficedula hypoleuca*) Adjust Their Feeding Effort According to Egg Colour? - Ethology 117: 309–317.

Johnstone, R. A. 1995. Sexual selection, honest advertisement and the handicap principle: reviewing the evidence. - Biol. Rev. 70: 1–65.

Kaltenpoth, M., Göttler, W., Herzner, G. and Strohm, E. 2005. Symbiotic Bacteria Protect Wasp Larvae from Fungal Infestation. - Curr. Biol. 15: 475–479.

Kaltz, O. and Koella, J. C. 2003. Host growth conditions regulate the plasticity of horizontal and vertical transmission in holospora undulata, a bacterial parasite of the protozoan paramecium caudatum. - Evolution (N. Y). 57: 1535–1542.

Karstens, L., Asquith, M., Davin, S., Fair, D., Gregory, W. T., Wolfe, A. J., Braun, J. and McWeeney, S. 2019. Controlling for Contaminants in Low-Biomass 16S rRNA Gene Sequencing Experiments (JA Gilbert, Ed.). - mSystems 4: e00290-19.

Kaur, H., Hughes, M. N., Green, C. J., Naughton, P., Foresti, R. and Motterlini, R. 2003. Interaction of bilirubin and biliverdin with reactive nitrogen species. - FEBS Lett. 543: 113–119.

Keebaugh, E. S., Yamada, R., Obadia, B., Ludington, W. B. and Ja, W. W. 2018. Microbial Quantity Impacts Drosophila Nutrition, Development, and Lifespan. - iScience 4: 247–259.

Kemp, A. C. 2001. Family *Bucerotidae* (Hornbills). - In: Del Hoyo, J. et al. (eds), Handbook of the birds of the world, Piciformes to Bucerotiformes, vol 6. Lynx Editi. pp. 436–523.

Kemp, D. J., Herberstein, M. E., Fleishman, L. J., Endler, J. a., Bennett, A. T. D., Dyer, A. G., Hart, N. S., Marshall, J. and Whiting, M. J. 2015. An Integrative Framework for the Appraisal of Coloration in Nature. - Am. Nat. 185: 000–000.

Kikuchi, Y., Hayatsu, M., Hosokawa, T., Nagayama, A., Tago, K. and Fukatsu, T. 2012. Symbiont-mediated insecticide resistance. - Proc. Natl. Acad. Sci. 109: 8618–8622.

Kilner, R. M. 2006. The evolution of egg colour and patterning in birds. - Biol. Rev.

Camb. Philos. Soc. 81: 383–406.

Kimbell, J. R., and M. J. McFall-Ngai. 2003. The squid-vibrio symbioses: From demes to genes. Integrative and Comparative Biology 43(2):254-260.

Kinnaird, M. F. and O'Brien, T. G. 2007. The Ecology and Conservation of Asian Hornbills: Farmers of the Forest.

Knight, R., Vrbanac, A., Taylor, B. C., Aksenov, A., Callewaert, C., Debelius, J., Gonzalez, A., Kosciolek, T., McCall, L. I., McDonald, D., Melnik, A. V., Morton, J. T., Navas, J., Quinn, R. A., Sanders, J. G., Swafford, A. D., Thompson, L. R., Tripathi, A., Xu, Z. Z., Zaneveld, J. R., Zhu, Q., Caporaso, J. G. and Dorrestein, P. C. 2018. Best practices for analysing microbiomes. - Nat. Rev. Microbiol. 16: 410–422.

Kopylova, E., Navas-molina, J. A., Mercier, C., Xu, Z., Mahé, F., He, Y., Zhou, H.-W., Rognes, T., Caporaso, J. G. and Knight, R. 2016. Open-Source Sequence Clustering Methods Improve the State Of the Art. mSystems. 1: 1–16.

Kose, M. and Møller, A. P. 1999. Sexual selection, feather breakage and parasites: the importance of white spots in the tail of the barn swallow (*Hirundo rustica*). - Behav. Ecol. Sociobiol. 45: 430–436.

Kraaijeveld, K., Kraaijeveld-Smit, F. J. L. and Komdeur, J. 2007. The evolution of mutual ornamentation. - Anim. Behav. 74: 657–677.

Krist, M. and Grim, T. 2007. Are blue eggs a sexually selected signal of female *collared flycatchers*? A cross-fostering experiment. - Behav. Ecol. Sociobiol. 61: 863–876.

Kuczynski, J., Stombaugh, J., Walters, W. A., González, A., Caporaso, J. G. and Knight, R. 2012. Using QIIME to analyze 16s rRNA gene sequences from microbial communities. - Curr. Protoc. Microbiol.

Lago, J., L. P. Rodríguez, L. Blanco, J. M. Vieites, and A. G. Cabado. 2015. Tetrodotoxin, an Extremely Potent Marine Neurotoxin: Distribution, Toxicity, Origin and Therapeutical Uses. Mar Drugs 13(10):6384-6406. doi: 10.3390/md13106384

Labocha, M. K. and Hayes, J. P. 2012. Morphometric indices of body condition in birds: A review. - J. Ornithol. 153: 1–22.

Lahti, D. C. 2008. Population Differentiation and Rapid Evolution of Egg Color in Accordance With Solar Radiation. - Auk 125: 796–802.

Larsson, A. J. M., Stanley, G., Sinha, R., Weissman, I. L. and Sandberg, R. 2018. Computational correction of index switching in multiplexed sequencing libraries. - Nat. Methods 15: 305–307.

Law-Brown, J. 2001. Chemical defence in the red-billed woodhoopoe, *Phoeniculus purpureus*, University of Cape Town.

Law-Brown, J. and Meyers, P. R. 2003. *Enterococcus phoeniculicola sp . nov* ., a novel member of the enterococci isolated from the uropygial gland of the Red-billed Woodhoopoe , *Phoeniculus purpureus*. - Int. J. Syst. Evol. Microbiol. 53: 683–685.

Leclaire, S., Nielsen, J. F. and Drea, C. M. 2014. Bacterial communities in meerkat anal scent secretions vary with host sex, age, and group membership. - Behav. Ecol. 25: 996–1004.

Leclaire, S., Jacob, S., Greene, L. K., Dubay, G. R. and Drea, C. M. 2017. Social odours covary with bacterial community in the anal secretions of wild meerkats. - Sci. Rep. 7: 3240.

Leftwich, P. T., Hutchings, M. I. and Chapman, T. 2018. Diet, Gut Microbes and Host Mate Choice. - BioEssays 40: 1800053.

Leitner, S., Marshall, R. C., Leisler, B. and Catchpole, C. K. 2006. Male song quality, egg size and offspring sex in captive canaries (*Serinus canaria*). - Ethology 112: 554–563.

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D. and Gordon, J. I. 2005. Obesity alters gut microbial ecology. - Proc. Natl. Acad. Sci. 102: 11070–11075.

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R. and Gordon, J. I. 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. - Nat. Rev. Microbiol. 6: 776–788.

Li, D., Chen, B., Zhang, L., Gaur, U., Ma, T., Jie, H., Zhao, G., Wu, N., Xu, Z., Xu, H., Yao, Y., Lian, T., Fan, X., Yang, D., Yang, M., Zhu, Q. and Satkoski Trask, J. 2016. The musk chemical composition and microbiota of Chinese forest musk deer males. - Sci. Rep. 6: 18975.

Lindquist, N., Barber, P. H. and Weisz, J. B. 2005. Episymbiotic microbes as food and defence for marine isopods: unique symbioses in a hostile environment. - Proc. R. Soc. B Biol. Sci. 272: 1209–1216.

Linville, S. U., Breitwisch, R. and Schilling, A. J. 1998. Plumage brightness as an indicator of parental care in northern cardinals. - Anim. Behav. 55: 119–127.

Little, A. E. F., T. Murakami, U. G. Mueller, and C. R. Currie. 2006. Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. Biology Letters 2(1):12-16.

Lopetuso, L. R., Scaldaferri, F., Petito, V. and Gasbarrini, A. 2013. Commensal Clostridia: leading players in the maintenance of gut homeostasis. - Gut Pathog. 5: 23.

López-Rull, I., Gil, M. and Gil, D. 2007. Spots in starling *Sturnus unicolor* eggs are good indicators of ectoparasite load by *Carnus hemapterus* (Diptera: *Carnidae*). - Ardeola 54: 131–134.

López-Rull, I., Pagán, I. and Macías Garcia, C. 2010. Cosmetic enhancement of signal coloration: Experimental evidence in the house finch. - Behav. Ecol. 21: 781–787.

Lozupone, C. and Knight, R. 2005. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. - Appl. Environ. Microbiol. 71: 8228–8235.

Lyu, N., Servedio, M. R., Lloyd, H. and Sun, Y. 2017. The evolution of postpairing male mate choice. - Evolution (N. Y). 71: 1465–1477.

Macpherson, A. J. and Harris, N. L. 2004. Interactions between commensal intestinal bacteria and the immune system. - Nat. Rev. Immunol. 4: 478–485.

Maddison, W. . and Maddison, D. R. 2018. Mesquite: a modular system for evolutionary analysis. Version 3.6.: http://www.mesquiteproject.org.

Maier, R. M., Pepper, I. L. and Gerba, C. P. 2000. Environmental Microbiology. - Academic Press, San Diego, California.

Mainwaring, M. C., Hartley, I. R., Lambrechts, M. M. and Deeming, D. C. 2014. The design and function of birds' nests. - Ecol. Evol. 4: 3909–3928.

Mallows, C. L. 1973. Some Comments on Cp. - Technometrics 15: 661–675.

Maraci, Ö., Engel, K. and Caspers, B. A. 2018. Olfactory communication via microbiota: what is known in birds? - Genes (Basel). in press.

Martín-Platero, A. M., Valdivia, E., Ruíz-Rodríguez, M., Soler, J. J., Martín-Vivaldi, M., Maqueda, M. and Martínez-Bueno, M. 2006. Characterization of antimicrobial substances produced by *Enterococcus faecalis* MRR 10-3, isolated from the uropygial gland of the hoopoe (*Upupa epops*). - Appl. Environ. Microbiol. 72: 4245–4249.

Martín-Vivaldi, M., Palomino, J. J., Soler, M. and Soler, J. J. 1999. Determinants of reproductive success in the Hoopoe Upupa epops, a hole-nesting non-passerine bird

with asynchronous hatching. - Bird Study 46: 205–216.

Martín-Vivaldi, M., Ruiz-Rodríguez, M., Soler, J. J., Peralta-Sánchez, J. M., Méndez, M., Valdivia, E., Martín-Platero, A. M. and Martínez-Bueno, M. 2009. Seasonal, sexual and developmental differences in hoopoe *Upupa epops* preen gland morphology and secretions: Evidence for a role of bacteria. - J. Avian Biol. 40: 191–205.

Martín-Vivaldi, M., Peña, A., Peralta-Sánchez, J. M., Sánchez, L., Ananou, S., Ruiz-Rodríguez, M. and Soler, J. J. 2010. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. - Proc. Biol. Sci. 277: 123–130.

Martín-Vivaldi, M., Soler, J. J., Peralta-Sánchez, J. M., Arco, L., Martín-Platero, A. M., Martínez-Bueno, M., Ruiz-Rodríguez, M. and Valdivia, E. 2014a. Special structures of hoopoe eggshells enhance the adhesion of symbiont-carrying uropygial secretion that increase hatching success. - J. Anim. Ecol. 83: 1289–1301.

Martín-Vivaldi, M., Doña, J., Romero Masegosa, J. and Soto Cárdenas, M. 2014b. Abubilla - Upupa epops Linnaeus, 1758. - En Encicl. Virtual los Vertebr. Españoles. Salvador. A., Morales, M. B. (Eds.). Mus. Nac. Ciencias Nat. Madrid. http//www.vertebradosibericos.org/

Martín-Vivaldi, M., Soler, J. J., Martínez-García, Á., Arco, L., Juárez-García-Pelayo, N., Ruiz-Rodríguez, M. and Martínez-Bueno, M. 2018. Acquisition of Uropygial Gland Microbiome by Hoopoe Nestlings. - Microb. Ecol. 76: 285–297.

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. - EMBnet.journal 17: 10–12.

Martin, T. E. and Badyaev, A. V 1996. Sexual dichromatism in birds: importance of nest predation and nest location for females versus males. - Evolution (N. Y). 50: 2454–2460.

Martínez-De La Puente, J., Merino, S., Moreno, J., Tomás, G., Morales, J., Lobato, E., García-Fraile, S. and Martínez, J. 2007. Are eggshell spottiness and colour indicators of health and condition in blue tits *Cyanistes caeruleus*? - J. Avian Biol. 38: 377–384.

Martínez-García, Á. 2015. Biodiversidad bacteriana de la secreción uropigial y la cáscara de los huevos de abubilla: estabilidad y adquisición.

Martínez-García, Á., Soler, J. J., Rodríguez-Ruano, S. M., Martínez-Bueno, M., Martín-Platero, A. M., Juárez-García, N. and Martín-Vivaldi, M. 2015. Preening as a Vehicle for Key Bacteria in Hoopoes. - Microb. Ecol. 70: 1024–1033.

Martínez-García, Á., Martín-Vivaldi, M., Rodríguez-Ruano, S. M., Peralta-Sánchez, J. M.,

Valdivia, E. and Soler, J. J. 2016a. Nest bacterial environment affects microbiome of hoopoe eggshells, but not that of the uropygial secretion. - PLoS One 11(7): e0158158.

Martínez-García, Á., Martín-Vivaldi, M., Ruiz-Rodríguez, M., Martínez-Bueno, M., Arco, L., Rodríguez-Ruano, S. M., Peralta-Sánchez, J. M. and Soler, J. J. 2016b. The Microbiome of the Uropygial Secretion in Hoopoes Is Shaped Along the Nesting Phase. - Microb. Ecol. 72: 252–261.

Massaro, M., Davis, L. S. and Darby, J. T. 2003. Carotenoid-derived ornaments reflect parental quality in male and female yellow-eyed penguins (*Megadyptes antipodes*). - Behav. Ecol. Sociobiol. 55: 169–175.

Mazgajski, T. D. 2007. Effect of old nest material on nest site selection and breeding parameters in secondary hole nesters — a review. - Acta Ornithol. 42: 1–14.

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., Desantis, T. Z., Probst, A., Andersen, G. L., Knight, R. and Hugenholtz, P. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. - ISME J. 6: 610–618.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E., Rawls, J. F., Reid, A., Ruby, E. G., Rumpho, M., Sanders, J. G., Tautz, D. and Wernegreen, J. J. 2013. Animals in a bacterial world, a new imperative for the life sciences. - Proc. Natl. Acad. Sci. U. S. A. 110: 3229–3236.

Mcgraw, K. J. and Hill, G. E. 2004. Plumage color as a dynamic trait : carotenoid pigmentation of male house finches (*Carpodacus mexicanus*) fades during the breeding season. - Can. J. Zool 82: 734–738.

McGraw, K. J. and Ardia, D. R. 2003. Carotenoids, Immunocompetence, and the Information Content of Sexual Colors: An Experimental Test. - Am. Nat. 162: 704–712.

McKnight, D. T., Huerlimann, R., Bower, D. S., Schwarzkopf, L., Alford, R. A. and Zenger, K. R. 2019. microDecon: A highly accurate read-subtraction tool for the post-sequencing removal of contamination in metabarcoding studies. - Environ. DNA 1: 14–25.

Mikšík, I., Holáň, V. and Deyl, Z. 1996. Avian eggshell pigments and their variability. -

Comp. Biochem. Physiol. 113: 607–612.

Miller, R. E., Trampel, D. W., Boever, W. J. and Kling, M. A. 1985. Carcinoma in the Casque of a Greater Indian Hornbill (*Buceros bicornis*). - J. Zoo Anim. Med. 16: 131–136.

Minich, J. J., Zhu, Q., Janssen, S., Hendrickson, R., Amir, A., Vetter, R., Hyde, J., Doty, M. M., Stillwell, K., Benardini, J., Kim, J. H., Allen, E. E., Venkateswaran, K. and Knight, R. 2018. KatharoSeq Enables High-Throughput Microbiome Analysis from Low-Biomass Samples (MJ McFall-Ngai, Ed.). - mSystems 3: e00218-17.

Møller, A. P. 1993. Sexual Selection in the Barn Swallow *Hirundo rustica*. III. Female Tail Ornaments. - Evolution (N. Y). 47: 417–431.

Møller, A. P. and Erritzoe, J. 1996. Parasite Virulence and Host Immune Defense : Host Immune Response is Related to Nest Reuse in Birds. - Evolution (N. Y). 50: 2066–2072.

Møller, A. P., Arriero, E., Lobato, E. and Merino, S. 2009. A meta-analysis of parasite virulence in nestling birds. - Biol. Rev. 84: 567–588.

Montgomerie, R. 2006. Cosmetic and adventitious colors. - In: Hill, E. and McGraw, K. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 399–427.

Moore, E. R. B., S. A. Mihaylova, and M. Gomila. 2014. The Family *Cardiobacteriaceae*. In: E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt and F. Thompson, editors, The Prokaryotes: *Gammaproteobacteria*. Springer Berlin Heidelberg, Berlin, Heidelberg. p. 135-146.

Morales, J., Sanz, J. J. and Moreno, J. 2006. Egg colour reflects the amount of yolk maternal antibodies and fledging success in a songbird. - Biol. Lett. 2: 334–336.

Morales, J., Moreno, J., Merino, S., Sanz, J. J., Tomás, G., Arriero, E., Lobato, E. and Martínez-De La Puente, J. 2007. Female ornaments in the *Pied Flycatcher* Ficedula hypoleuca: Associations with age, health and reproductive success. - Ibis (Lond. 1859). 149: 245–254.

Morales, J., Velando, A. and Moreno, J. 2008. Pigment allocation to eggs decreases plasma antioxidants in a songbird. - Behav. Ecol. Sociobiol. 63: 227–233.

Moran, N. A. 2006. Symbiosis. - Curr. Biol. 16: R866–R871.

Moran, N. A., McCutcheon, J. P. and Nakabachi, A. 2008. Genomics and Evolution of Heritable Bacterial Symbionts. - Annu. Rev. Genet. 42: 165–190.

Moreau, R. E. 1934. Breeding Habits of Hornbills. - Nature 134: 899.

Moreno, J. and Osorno, J. L. 2003. Avian egg colour and sexual selection: Does eggshell pigmentation reflect female condition and genetic quality? - Ecol. Lett. 6: 803–806.

Moreno, J., Briones, V., Merino, S., Ballesteros, C., Sanz, J. J. and Tomás, G. 2003. Beneficial Effects of Cloacal Bacteria on Growth and Fledging Size in Nestling Pied Flycatchers (*Ficedula Hypoleuca*) in Spain. - Auk 120: 784–790.

Moreno, J., Osorno, J. L., Morales, J., Merino, S. and Tomás, G. 2004. Egg Colouration and Male Parental Effort in the Pied Flycatcher *Ficedula Hypoleuca*. - J. Avian Biol. 35: 300–304.

Moreno, J., Morales, J., Lobato, E., Merino, S., Tomás, G. and Martínez-De La Puente, J. 2005. Evidence for the signaling function of egg color in the pied flycatcher *Ficedula hypoleuca*. - Behav. Ecol. 16: 931–937.

Moreno, J., Morales, J., Lobato, E., Merino, S., Tomás, G. and Martínez-De La Puente, J. 2006a. More colourful eggs induce a higher relative paternal investment in the pied flycatcher *Ficedula hypoleuca*: A cross-fostering experiment. - J. Avian Biol. 37: 555–560.

Moreno, J., Lobato, E., Morales, J., Merino, S., Tomás, G., Martínez-De La Puente, J., Sanz, J. J., Mateo, R. and Soler, J. J. 2006b. Experimental evidence that egg color indicates female condition at laying in a songbird. - Behav. Ecol. 17: 651–655.

Moreno, J., Lobato, E., Merino, S. and Martínez-De La Puente, J. 2008. Blue-green eggs in pied flycatchers: An experimental demonstration that a supernormal stimulus elicits improved nestling condition. - Ethology 114: 1078–1083.

Morton, J. T., Sanders, J., Quinn, R. A., McDonald, D., Gonzalez, A., Vázquez-Baeza, Y., Navas-Molina, J. A., Song, S. J., Metcalf, J. L., Hyde, E. R., Lladser, M., Dorrestein, P. C. and Knight, R. 2017. Balance Trees Reveal Microbial Niche Differentiation (JK Jansson, Ed.). - mSystems 2: e00162-16.

Mushegian, A. A., Walser, J.-C., Sullam, K. E. and Ebert, D. 2018. The microbiota of diapause: How host–microbe associations are formed after dormancy in an aquatic crustacean. - J. Anim. Ecol. 87: 400–413.

Naish, D. 2015. The Ecology and Conservation of Asian Hornbills: Farmers of the Forest. - Hist. Biol. 27: 954–956.

Navas-Molina, J. A., Peralta-Sánchez, J. M., González, A., McMurdie, P. J., Vázquez-Baeza, Y., Xu, Z., Ursell, L. K., Lauber, C., Zhou, H., Song, S. J., Huntley, J.,

Ackermann, G. L., Berg-Lyons, D., Holmes, S., Caporaso, J. G. and Knight, R. 2013. Advancing our understanding of the human microbiome using QIIME. - Elsevier Inc.

Negro, J. J., Margalida, A., Hiraldo, F. and Heredia, R. 1999. The function of the cosmetic coloration of bearded vultures : when art imitates life. - Anim. Behav. S8: 14–17.

Nicholls, E. M. and Rienits, K. G. 1971. Tryptophan derivatives and pigment in the hair of some Australian marsupials. - Int. J. Biochem. 2: 593–603.

Nyholm, S. V., and M. J. McFall-Ngai. 1998. Sampling the light-organ microenvironment of Euprymna scolopes: Description of a population of host cells in association with the bacterial symbiont *Vibrio fischeri*. Biological Bulletin 195(2):89-97.

Ödeen, A. and Håstad, O. 2003. Complex distribution of avian color vision systems revealed by sequencing the SWS1 opsin from total DNA. - Mol. Biol. Evol. 20: 855–861.

Oliver, K. M., Russell, J. A., Moran, N. A. and Hunter, M. S. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. - Proc. Natl. Acad. Sci. 100: 1803 LP – 1807.

Olson, V. A. and Owens, I. P. F. 1998. Costly sexual signals: are carotenoids rare, risky or required? - Trends Ecol. Evol. 13: 510–514.

Olsson, K. and Allander, K. 1995. Do Fleas, and/or Old Nest Material, Influence Nest-site preference in Hole-nesting Passerines? - Ethology 101: 160–170.

Olsson, P., Lind, O. and Kelber, A. 2015. Bird colour vision: behavioural thresholds reveal receptor noise. - J. Exp. Biol. 218: 184–193.

Orell, M., Rytkönen, S. and Ilomäki, K. 1993. Do pied flycatchers prefer nest boxes with old nest material? - Ann. Zool. Fennici 30: 313–316.

Pagel, M. 1997. Inferring evolutionary processes from phylogenies. - Zool. Scr. 26: 331–348.

Pagel, M. 1999. Inferring the historical patterns of biological evolution. - Nature 401: 877–884.

Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A. and Brown, P. O. 2007. Development of the Human Infant Intestinal Microbiota. - PLoS Biol. 5: e177.

Paradis, E., Claude, J. and Strimmer, K. 2004. APE : Analyses of Phylogenetics and Evolution in R language. - Bioinformatics 20: 289–290.

Paz, L.-C., Schramm, A. and Lund, M. B. 2017. Biparental transmission of *Verminephrobacter* symbionts in the earthworm *Aporrectodea tuberculata*

(Lumbricidae). - FEMS Microbiol. Ecol. 93: fix025.

Pearce, D. S., Hoover, B. A., Jennings, S., Nevitt, G. A. and Docherty, K. M. 2017. Morphological and genetic factors shape the microbiome of a seabird species (*Oceanodroma leucorhoa*) more than environmental and social factors. - Microbiome 5: 146.

Peig, J. and Green, A. J. 2010. The paradigm of body condition: A critical reappraisal of current methods based on mass and length. - Funct. Ecol. 24: 1323–1332.

Peralta-Sanchez, J. M., Møller, A. P., Martin-Platero, A. M. and Soler, J. J. 2010. Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: An experimental study. - Funct. Ecol. 24: 426–433.

Peralta-Sánchez, J. M., Martín-Vivaldi, M., Martín-Platero, A. M., Martínez-Bueno, M., Oñate, M., Ruiz-Rodríguez, M. and Soler, J. J. 2012. Avian life history traits influence eggshell bacterial loads: A comparative analysis. - Ibis (Lond. 1859). 154: 725–737.

Peralta-Sánchez, J. M., Soler, J. J., Martín-Platero, A. M., Knight, R., Martínez-Bueno, M. and Møller, A. P. 2014. Eggshell Bacterial Load Is Related to Antimicrobial Properties of Feathers Lining Barn Swallow Nests. - Microb. Ecol. 67: 480–487.

Pérez-Rodríguez, L., Mougeot, F. and Bortolotti, G. R. 2011. The effects of preen oils and soiling on the UV – visible reflectance of carotenoid-pigmented feathers. - Behav. Ecol. Sociobiol. 65: 1425–1435.

Piault, R., Gasparini, J., Bize, P., Paulet, M., McGraw, K. J. and Roulin, A. 2008. Experimental support for the makeup hypothesis in nestling tawny owls (*Strix aluco*). - Behav. Ecol. 19: 703–709.

Piel, J. 2004. Metabolites from symbiotic bacteria. Natural Product Reports 21(4):519-538.

Plard, F., Arlettaz, R. and Schaub, M. 2018. Hoopoe males experience intra-seasonal while females experience inter-seasonal reproductive costs. - Oecologia 186: 665–675.

Podofillini, S., Cecere, J. G., Griggio, M., Curcio, A., De Capua, E. L., Fulco, E., Pirrello, S., Saino, N., Serra, L., Visceglia, M. and Rubolini, D. 2018. Home, dirty home: effect of old nest material on nest-site selection and breeding performance in a cavity-nesting raptor. - Curr. Zool.: 1–10.

Poláček, M., Griggio, M., Mikšík, I., Bartíková, M., Eckenfellner, M. and Hoi, H. 2017.

Eggshell coloration and its importance in postmating sexual selection. - Ecol. Evol. 7: 941–949.

Poonswad, P., Kemp, A. and Strange, M. 2013. Hornbills of the world: A photographic guide.

Price, M. N., Dehal, P. S. and Arkin, A. P. 2009. Fasttree: Computing large minimum evolution trees with profiles instead of a distance matrix. - Mol. Biol. Evol. 26: 1641–1650.

Prum, R. O. 2006. Anatomy, Physics, and Evolution of Structural Colors. - In: Hill, G. E. and Mcgraw, K. J. (eds), Bird colouration I. Mechanisms and measurements. Harvard University Press, pp. 295–353.

Pujalte, M. J., T. Lucena, M. A. Ruvira, D. R. Arahal, and M. C. Macián. 2014. The Family Rhodobacteraceae. In: E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt and F. Thompson, editors, The Prokaryotes: *Alphaproteobacteria* and *Betaproteobacteria*. Springer Berlin Heidelberg, Berlin, Heidelberg. p. 439-512.

Refsnider, J. M. and Janzen, F. J. 2010. Putting Eggs in One Basket: Ecological and Evolutionary Hypotheses for Variation in Oviposition-Site Choice. - Annu. Rev. Ecol. Evol. Syst. 41: 39–57.

Reichlin, T. S., Hobson, K. A., Van Wilgenburg, S. L., Schaub, M., Wassenaar, L. I., Martín-Vivaldi, M., Arlettaz, R. and Jenni, L. 2013. Conservation through connectivity: Can isotopic gradients in Africa reveal winter quarters of a migratory bird? - Oecologia 171: 591–600.

Renoult, J. P., Kelber, A. and Schaefer, H. M. 2017. Colour spaces in ecology and evolutionary biology. - Biol. Rev. 92: 292–315.

Reynolds, S. J., Martin, G. R. and Cassey, P. 2009. Is sexual selection blurring the functional significance of eggshell coloration hypotheses? - Anim. Behav. 78: 209–215.

Richard, F.-J. 2017. Symbiotic Bacteria Influence the Odor and Mating Preference of Their Hosts. - Front. Ecol. Evol. 5: 1–9.

Rideout, J. R., He, Y., Navas-Molina, J. A., Walters, W. A., Ursell, L. K., Gibbons, S. M., Chase, J., McDonald, D., Gonzalez, A., Robbins-Pianka, A., Clemente, J. C., Gilbert, J. A., Huse, S. M., Zhou, H.-W., Knight, R. and Caporaso, J. G. 2014. Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. - PeerJ 2: e545.

Riehl, C. 2011. Paternal Investment and the "Sexually Selected Hypothesis" For the Evolution of Eggshell Coloration: Revisiting the Assumptions. - Auk 128: 175–179.

Riley, M. A. and Wertz, J. E. 2002. Bacteriocins: Evolution, Ecology, and Application. - Annu. Rev. Microbiol. 56: 117–137.

Rizzo, C. and Lo Giudice, A. 2018. Marine Invertebrates: Underexplored Sources of Bacteria Producing Biologically Active Molecules. - Diversity 10: 52.

Rodríguez-Ruano, S. M. 2015. Diversidad bacteriana en la glándula uropigial de la abubilla: dinámica estacional y beneficios asociados.

Rodríguez-Ruano, S. M., Martín-Vivaldi, M., Martín-Platero, A. M., López-López, J. P., Peralta-Sánchez, J. M., Ruiz-Rodríguez, M., Soler, J. J., Valdivia, E. and Martínez-Bueno, M. 2015. The hoopoe's uropygial gland hosts a bacterial community influenced by the living conditions of the bird. - PLoS One 10: 1–18.

Rodríguez-Ruano, S. M., Martín-Vivaldi, M., Peralta-Sánchez, J. M., García-Martín, A. B., Martínez-García, Á., Soler, J. J., Valdivia, E. and Martínez-Bueno, M. 2018. Seasonal and Sexual Differences in the Microbiota of the Hoopoe Uropygial Secretion. - Genes (Basel). 9: 407.

Rognes, T., Flouri, T., Nichols, B., Quince, C. and Mahé, F. 2016. VSEARCH: a versatile open source tool for metagenomics. - PeerJ 4: e2584.

Rolig, A. S., Parthasarathy, R., Burns, A. R., Bohannan, B. J. M. and Guillemin, K. 2015. Individual Members of the Microbiota Disproportionately Modulate Host Innate Immune Responses. - Cell Host Microbe 18: 613–620.

Rollins-Smith, L. A., Ramsey, J. P., Pask, J. D., Reinert, L. K. and Woodhams, D. C. 2011. Amphibian Immune Defenses against Chytridiomycosis: Impacts of Changing Environments. - Integr. Comp. Biol. 51: 552–562.

Rosvall, K. A. 2011. Intrasexual competition in females: evidence for sexual selection? - Behav. Ecol. 22: 1131–1140.

Rubolini, D., Liker, A., Garamszegi, L. Z., Møller, A. P. and Saino, N. 2015. Using the birdtree.org website to obtain robust phylogenies for avian comparative studies: A primer. - Curr. Zool. 61: 959–965.

Ruiz-Castellano, C., Tomás, G., Ruiz-Rodríguez, M., Martín-Gálvez, D. and Soler, J. J. 2016. Nest material shapes eggs bacterial environment. - PLoS One 11: e0148894.

Ruiz-de-Castañeda, R., Burtt Jr, E. H., González-Braojos, S. and Moreno, J. 2012. Bacterial degradability of an intrafeather unmelanized ornament: a role for feather-

degrading bacteria in sexual selection? - Biol. J. Linn. Soc. 105: 409–419.

Ruiz-De-Castañeda, R., Burtt, E. H., González-Braojos, S. and Moreno, J. 2015. Bacterial degradability of white patches on primary feathers is associated with breeding date and parental effort in a migratory bird. - Ibis (Lond. 1859). 157: 871–876.

Ruiz-Rodríguez, M., Azcárate-García, M., Ruiz-Castellano, C., Tomás, G., Díaz-Lora, S., Pérez-Contreras, T. and Soler, J. J. Antimicrobial capacity is related to body colouration and reproductive success in female spotless starlings. - Under Rev. in press.

Ruiz-Rodríguez, M., Valdivia, E., Soler, J. J., Martín-Vivaldi, M., Martín-Platero, A. M. and Martínez-Bueno, M. 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. - J. Exp. Biol. 212: 3621–6.

Ruiz-Rodríguez, M., Valdivia, E., Martín-Vivaldi, M., Martín-Platero, A. M., Martínez-Bueno, M., Méndez, M., Peralta-Sánchez, J. M. and Soler, J. J. 2012. Antimicrobial activity and genetic profile of *enteroccoci* isolated from hoopoes uropygial gland. - PLoS One 7: 1–6.

Ruiz-Rodríguez, M., Martínez-Bueno, M., Martín-Vivaldi, M., Valdivia, E. and Soler, J. J. 2013. Bacteriocins with a broader antimicrobial spectrum prevail in enterococcal symbionts isolated from the hoopoe's uropygial gland. - FEMS Microbiol. Ecol. 85: 495–502.

Ruiz-Rodríguez, M., Soler, J. J., Martín-Vivaldi, M., Martín-Platero, A. M., Méndez, M., Peralta-Sánchez, J. M., Ananou, S., Valdivia, E. and Martínez-Buenob, M. 2014. Environmental factors shape the community of symbionts in the hoopoe uropygial gland more than genetic factors. - Appl. Environ. Microbiol. 80: 6714–6723.

Ruiz-Rodríguez, M., Tomás, G., Martín-Gálvez, D., Ruiz-Castellano, C. and Soler, J. J. 2015. Bacteria and the evolution of honest signals. The case of ornamental throat feathers in spotless starlings. - Funct. Ecol. 29: 701–709.

Ruiz-Rodríguez, M., Møller, A. P., Mousseau, T. A. and Soler, J. J. 2016. Defenses against keratinolytic bacteria in birds living in radioactively contaminated areas. - Sci. Nat. 103: 71.

Sachs, J. L., Skophammer, R. G. and Regus, J. U. 2011. Evolutionary transitions in bacterial symbiosis. - Proc. Natl. Acad. Sci. 108: 10800 LP – 10807.

Saino, N., Romano, M., Rubolini, D., Ambrosini, R., Caprioli, M., Milzani, A., Costanzo, A., Colombo, G., Canova, L. and Wakamatsu, K. 2013. Viability Is Associated with

Melanin-Based Coloration in the Barn Swallow (*Hirundo rustica*). - PLoS One 8: e60426.

Sanz, J. J. and García-Navas, V. 2009. Eggshell pigmentation pattern in relation to breeding performance of blue tits *Cyanistes caeruleus*. - J. Anim. Ecol. 78: 31–41.

Schwartzman, J. A., E. Koch, E. A. C. Heath-Heckman, L. Zhou, N. Kremer, M. J. McFall-Ngai, and E. G. Ruby. 2015. The chemistry of negotiation: Rhythmic, glycan-driven acidification in a symbiotic conversation. Proceedings of the National Academy of Sciences 112(2):566-571.

Scott, J. J., Oh, D.-C., Yuceer, M. C., Klepzig, K. D., Clardy, J. and Currie, C. R. 2008. Bacterial Protection of Beetle-Fungus Mutualism. - Science (80-.). 322: 63.

Searcy, W. A. and Nowicki, S. 2005. The evolution of animal communication. - Princeton Univ. Press, Princeton, NJ.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S. and Huttenhower, C. 2011. Metagenomic biomarker discovery and explanation. - Genome Biol. 12: R60.

Senar, J. C. and Pascual, J. 1997. Tarsus lenght predictor body size. - Ardea 85: 269–274.

Sender, R., Fuchs, S. and Milo, R. 2016. Revised Estimates for the Number of Human and Bacteria Cells in the Body. - PLOS Biol. 14: e1002533.

Sergio, F. and Newton, I. 2003. Occupancy as a measure of habitat quality. - J Anim Ecol. 72: 857–865.

Shapira, M. 2016. Gut Microbiotas and Host Evolution: Scaling Up Symbiosis. - Trends Ecol. Evol. 31: 539–549.

Sharon, G., Segal, D., M.Ringo, H., Hefetz, A., Zilber-Rosenberg, I. and Rosenberg, E. 2010. Commensal bacteria play a role in mating preference of Drosophila melanogaster. - PNAS 107: 20051–20056.

Shawkey, M. D., Pillai, S. R. and Hill, G. E. 2003. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. - J. Avian Biol. 34: 345–349.

Shawkey, M. D., Pillai, S. R. and Hill, G. E. 2009. Do feather-degrading bacteria affect sexually selected plumage color? - Naturwissenschaften 96: 123–128.

Sheldon, B. C. 2000. Differential allocation: Tests, mechanisms and implications. - Trends Ecol. Evol. 15: 397–402.

Shephard, K. L. 1994. Functions for fish mucus. - Rev. Fish Biol. Fish. 4: 401–429.

Sherwin, E., Bordenstein, S. R., Quinn, J. L., Dinan, T. G. and Cryan, J. F. 2019.

Microbiota and the social brain. - Science (80-.). 366: eaar2016.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. and Ishikawa, H. 2000. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera sp.* APS. - Nature 407: 81–86.

Sibley, C. G., and J. E. Ahlquist. 1990. Phylogeny and classification of birds. Yale University Press, New Haven.

Siefferman, L., Navara, K. J. and Hill, G. E. 2006. Egg coloration is correlated with female condition in eastern bluebirds (*Sialia sialis*). - Behav. Ecol. Sociobiol. 59: 651–656.

Singleton, D. R. and Harper, R. G. 1996. Bacteria in Old House Wren nests. - J F. Ornithol 69: 71–74.

Smith, H. G. and Montgomerie, R. 1991. Nestling American robins compete with siblings by begging. - Behav. Ecol. Sociobiol. 29: 307–312.

Soini, H. A., Whittaker, D. J., Wiesler, D., Ketterson, E. D. and Novotny, M. V. 2013. Chemosignaling diversity in songbirds: Chromatographic profiling of preen oil volatiles in different species. - J. Chromatogr. A 1317: 186–192.

Soler, J. J. and Møller, A. P. 1996. A comparative analysis of the evolution of variation in appearance of eggs of European passerines in relation to brood parasitism. - Behav. Ecol. 7: 89–94.

Soler, J. J., Moreno, J., Aviles, J. M. and Møller, A. P. 2005. Blue and green egg-color intensity is associated with parental effort and mating system in passerines: support for the sexual selection hypothesis. - Evolution (N. Y). 59: 636–644.

Soler, J. J., Martín-Vivaldi, M., Ruiz-Rodríguez, M., Valdivia, E., Martín-Platero, A. M., Martínez-Bueno, M., Peralta-Sánchez, J. M. and Méndez, M. 2008a. Symbiotic association between hoopoes and antibiotic-producing bacteria that live in their uropygial gland. - Funct. Ecol. 22: 864–871.

Soler, J. J., Navarro, C., Contreras, T. P., Avilés, J. M. and Cuervo, J. J. 2008b. Sexually Selected Egg Coloration in Spotless Starlings. - Am. Nat. 171: 183–194.

Soler, J. J., Martín-vivaldi, M., Peralta-sánchez, J. M. and Ruiz-rodríguez, M. 2010. Antibiotic-Producing Bacteria as a Possible Defence of Birds against Pathogenic Microorganisms. - Open Ornithol. J. 3: 93–100.

Soler, J. J., Martín-Vivaldi, M., Peralta-Sánchez, J. M., Arco, L. and Juárez-García-Pelayo, N. 2014. Hoopoes color their eggs with antimicrobial uropygial secretions. -

Naturwissenschaften 101: 697–705.

Soler, J. J., Martínez-García, Á., Rodríguez-Ruano, S. M., Martínez-Bueno, M., Martín-Platero, A. M., Peralta-Sánchez, J. M. and Martín-Vivaldi, M. 2016. Nestedness of hoopoes' bacterial communities: symbionts from the uropygial gland to the eggshell. - Biol. J. Linn. Soc. 118: 763–773.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martín-Vivaldi, M., Martínez-de la Puente, J., Ruiz-Rodríguez, M. and Tomás, G. 2017. Telomere length and dynamics of spotless starling nestlings depend on nest-building materials used by parents. - Anim. Behav. 126: 89–100.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martínez-de la Puente, J., Ruiz-Rodríguez, M. and Tomás, G. 2018. Egg colouration predicts brood size, telomere length and body condition of spotless starling fledglings. - J. Avian Biol. 49: 1–12.

Soler, J. J., Morales, J., Cuervo, J. J. and Moreno, J. 2019. Conspicuousness of passerine females is associated with the nest-building behaviour of males. - Biol. J. Linn. Soc. 126: 824–835.

Solís, J. C. and de Lope, F. 1995. Nest and Egg Crypsis in the Ground-Nesting Stone Curlew *Burhinus oedicnemus*. - J. Avian Biol. 26: 135–138.

Sorgo, A., F. Gaill, J. P. Lechaire, C. Arndt, and M. Bright. 2002. Glycogen storage in the *Riftia pachyptila* trophosome: contribution of host and symbionts. Marine Ecology-Progress Series 231:115-120.

Spicer, M. E., Pruitt, J. N. and Keiser, C. N. 2019. Spiders, microbes and sex: Bacterial exposure on copulatory organs alters mating behaviour in funnel-web spiders. - Ethology 125: 677–685.

Spor, A., Koren, O. and Ley, R. 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. - Nat. Rev. Microbiol. 9: 279–290.

Stanback, M. T. and Rockwell, E. K. 2003. Nest-site fidelity in Eastern Bluebirds (Sialia sialis) depends on the quality of alternative cavities. - Auk 120: 1029–1032.

Statsoft, I. 2006. STATISTICA (data analysis software system), version 7. Available at www.statsoft.com.

Steinert, M., Hentschel, U. and Hacker, J. 2000. Symbiosis and pathogenesis: evolution of the microbe-host interaction. - Naturwissenschaften 87: 1—11.

Stoddard, M. C. and Prum, R. O. 2008. Evolution of avian plumage color in a tetrahedral colour space: a phylogenetic analysis of New World buntings. - Am. Nat. 171: 755–

776.

Stoddard, M. C. and Osorio, D. 2019. Animal Coloration Patterns: Linking Spatial Vision to Quantitative Analysis. - Am. Nat. 193: 000–000.

Stoddard, M. C., Fayet, A. L., Kilner, R. M. and Hinde, C. A. 2012. Egg speckling patterns do not advertise offspring quality or influence male provisioning in great tits. - PLoS One 7: e40211.

Suedmeyer, W. K., McCaw, D. and Turnquist, S. 2001. Attempted Photodynamic Therapy of Squamous Cell Carcinoma in the Casque of a Great Hornbill (*Buceros bicornis*). - J. Avian Med. Surg. 15: 44–49.

Sumasgutner, P., Vasko, V., Varjonen, R. and Korpimäki, E. 2014. Public information revealed by pellets in nest sites is more important than ecto-parasite avoidance in the settlement decisions of Eurasian kestrels. - Behav. Ecol. Sociobiol. 68: 2023–2034.

Surmacki, A. and Nowakowski, J. K. 2007. Soil and preen waxes influence the expression of carotenoid-based plumage coloration. - Naturwissenschaften 94: 829–835.

Suzuki, T. A. 2017. Links between Natural Variation in the Microbiome and Host Fitness in Wild Mammals. - Integr. Comp. Biol. 57: 756–769.

Swynnerton, C. F. M. 1916. On the coloration of the mouths and eggs of birds. II. On the coloration of eggs. - Ibis (Lond. 1859). 4: 529–606.

Swynnerton, C. F. M. 1918. Rejections by birds of eggs unlike their own: with remarks on some of the cuckoo problems. - Ibis (Lond. 1859).: 127–154.

Team, R. D. C. 2019. R: A Language and Environment for Statistical Computing (RDC Team, Ed.). - R Found. Stat. Comput. in press.

Theis, K. R., Venkataraman, A., Dycus, J. A., Koonter, K. D., Schmitt-Matzen, E. N., Wagner, A. P., Holekamp, K. E. and Schmidt, T. M. 2013. Symbiotic bacteria appear to mediate hyena social odors. - Proc. Natl. Acad. Sci. 110: 19832–19837.

Tomás, G., Merino, S., Moreno, J., Sanz, J. J., Morales, J. and García-Fraile, S. 2006. Nest Weight and Female Health in the Blue Tit (*Cyanistes Caeruleus*). - Auk 123: 1013–1021.

Tomás, G., Merino, S., Moreno, J. and Morales, J. 2007. Consequences of nest reuse for parasite burden and female health and condition in blue tits, *Cyanistes caeruleus*. - Anim. Behav. 73: 805–814.

Tomás, G., Martín-Gálvez, D., Ruiz-Castellano, C., Ruiz-Rodríguez, M., Peralta-Sánchez, J. M., Martín-Vivaldi, M. and Soler, J. J. 2018. Ectoparasite Activity During

Incubation Increases Microbial Growth on Avian Eggs. - Microb. Ecol. 76: 555–564.

Trail, P. W. 2007. African hornbills: keystone species threatened by habitat loss, hunting and international trade. - Ostrich 78: 609–613.

Trivers, R. L. 1972. Parental investment and sexual selection. - In: Campbell, B. (ed), Sexual selection and the descent of man 1871-1971. pp. 136–179.

Tschumi, M., Schaub, M. and Arlettaz, R. 2014. Territory occupancy and parental quality as proxies for spatial prioritization of conservation areas. - PLoS One 9: e97679.

Uchida, Y. 1970. On the color change in Japanese crested ibis. A new type of cosmetic coloration in birds. - Misc Rep Yamashina Inst Ornithol 6: 54–72. In Japanese, with English summary.

Umesaki, Y., Setoyama, H., Matsumoto, S., Imaoka, A. and Itoh, K. 1999. Differential Roles of Segmented Filamentous Bacteria and Clostridia in Development of the Intestinal Immune System. - Infect. Immun. 67: 3504 LP – 3511.

Underwood, T. and Sealy, S. 2002. Adaptive significance of egg coloration. - In: Deeming DC (ed), Avian incubation: behaviour, environment, and evolution. Oxford Uni. pp. 280–298.

van Dongen, W. F., White, J., Brandl, H. B., Moodley, Y., Merkling, T., Leclaire, S., Blanchard, P., Danchin, É., Hatch, S. A. and Wagner, R. H. 2013. Age-related differences in the cloacal microbiota of a wild bird species. - BMC Ecol. 13: 11.

van Veelen, H. P. J., Falcao Salles, J. and Tieleman, B. I. 2017. Multi-level comparisons of cloacal, skin, feather and nest-associated microbiota suggest considerable influence of horizontal acquisition on the microbiota assembly of sympatric woodlarks and skylarks. - Microbiome 5: 156.

van Veelen, H. P. J., Salles, J. F. and Tieleman, B. I. 2018. Microbiome assembly of avian eggshells and their potential as transgenerational carriers of maternal microbiota. - ISME J. 12: 1375–1388.

van Wijk, R. E., Schaub, M. and Bauer, S. 2017. Dependencies in the timing of activities weaken over the annual cycle in a long-distance migratory bird. - Behav. Ecol. Sociobiol. 71: 73.

van Wijk, R. E., Schaub, M., Hahn, S., Juárez-García-Pelayo, N., Schäfer, B., Viktora, L., Martín-Vivaldi, M., Zischewski, M. and Bauer, S. 2018. Diverse migration strategies in hoopoes (*Upupa epops*) lead to weak spatial but strong temporal connectivity. - Sci. Nat. 105: 42.

Victoria, J. K. 1972. Clutch characteristics and egg discriminative ability of African village weaverbird Ploceus cucullatus. - Ibis (Lond. 1859). 114: 367–376.

Videler, H., Geertjes, G. J. and Videler, J. J. 1999. Biochemical characteristics and antibiotic properties of the mucous envelope of the queen parrotfish. - J. Fish Biol. 54: 1124–1127.

Viseshakul, N., Charoennitikul, W., Kitamura, S., Kemp, A., Thong-Aree, S., Surapunpitak, Y., Poonswad, P. and Ponglikitmongkol, M. 2011. A phylogeny of frugivorous hornbills linked to the evolution of Indian plants within Asian rainforests. - J. Evol. Biol. 24: 1533–1545.

Wallace AR 1889. Darwinism: an exposition of the theory of natural selection, with some of its applications.

Walters, L. A. and Getty, T. 2010. Are brighter eggs better? Egg color and parental investment by House Wrens. - J. F. Ornithol. 81: 155–166.

Wang, B., Wan, L., Wang, A. and Li, L. M. 2017. An adaptive decorrelation method removes Illumina DNA base-calling errors caused by crosstalk between adjacent clusters. - Sci. Rep. 7: 41348.

Warnecke, L., Turner, J. M., Bollinger, T. K., Lorch, J. M., Misra, V., Cryan, P. M., Wibbelt, G., Blehert, D. S. and Willis, C. K. R. 2012. Inoculation of bats with European Geomyces destructans supports the novel pathogen hypothesis for the origin of white-nose syndrome. - Proc. Natl. Acad. Sci. 109: 6999–7003.

Weiß, B. M., Kücklich, M., Thomsen, R., Henkel, S., Jänig, S., Kulik, L., Birkemeyer, C. and Widdig, A. 2018. Chemical composition of axillary odorants reflects social and individual attributes in rhesus macaques. - Behav. Ecol. Sociobiol. 72: 65.

Werren, J. H. 2012. Symbionts provide pesticide detoxification. - Proc. Natl. Acad. Sci. 109: 8364–8365.

Werren, J. H., Baldo, L. and Clark, M. E. 2008. Wolbachia: master manipulators of invertebrate biology. - Nat. Rev. Microbiol. 6: 741–751.

Wesolowski, T. and Maziarz, M. 2012. Dark tree cavities – a challenge for hole nesting birds? - J. Avian Biol. 43: 454–460.

Westcott, S. L. and Schloss, P. D. 2015. De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. - PeerJ 3: e1487.

Whittaker, D. J. and Theis, K. R. 2016. Bacterial Communities Associated with Junco

Preen Glands : Preliminary Ramifications for Chemical Signaling. - In: Schulte, B. A. et al. (ed), Chemical Signals in Vertebrates 13. Springer International Publishing, pp. 105–117.

Whittaker, D. J., Soini, H. A., Atwell, J. W., Hollars, C., Novotny, M. V and Ketterson, E. D. 2010. Songbird chemosignals: volatile compounds in preen gland secretions vary among individuals, sexes, and populations. - Behav. Ecol. 21: 608–614.

Whittaker, D. J., Richmond, K. M., Miller, A. K., Kiley, R., Bergeon Burns, C., Atwell, J. W. and Ketterson, E. D. 2011. Intraspecific preen oil odor preferences in dark-eyed juncos (*Junco hyemalis*). - Behav. Ecol. 22: 1256–1263.

Whittaker, D. J., Gerlach, N. M., Slowinski, S. P., Corcoran, K. P., Winters, A. D., Soini, H. A., Novotny, M. V., Ketterson, E. D. and Theis, K. R. 2016. Social Environment Has a Primary Influence on the Microbial and Odor Profiles of a Chemically Signaling Songbird. - Front. Ecol. Evol. 4: 1–15.

Whittaker, D. J., Slowinski, S. P., Greenberg, J. M., Alian, O., Winters, A. D., Ahmad, M. M., Burrell, M. J. E., Soini, H. A., Novotny, M. V., Ketterson, E. D. and Theis, K. R. 2019. Experimental evidence that symbiotic bacteria produce chemical cues in a songbird. - J. Exp. Biol.: jeb.202978.

Widder, E. A. 2010. Bioluminescence in the Ocean: Origins of Biological, Chemical, and Ecological Diversity. Science 328(5979):704. doi: 10.1126/science.1174269

Wright, E. S. and Vetsigian, K. H. 2016. Quality filtering of Illumina index reads mitigates sample cross-talk. - BMC Genomics 17: 876.

Zahavi, A. 1975. Mate selection-A selection for a handicap. - J. Theor. Biol. 53: 205–214.

Zahavi, A. and Zahavi, A. 1997. The handicap principle; a missing piece of Darwin's puzzle.

Zamzow, J. P. and Losey, G. S. 2002. Ultraviolet radiation absorbance by coral reef fish mucus: Photo-protection and visual communication. - Environ. Biol. Fishes 63: 41–47.

Zinter, M. S., Mayday, M. Y., Ryckman, K. K., Jelliffe-Pawlowski, L. L. and DeRisi, J. L. 2019. Towards precision quantification of contamination in metagenomic sequencing experiments. - Microbiome 7: 62.