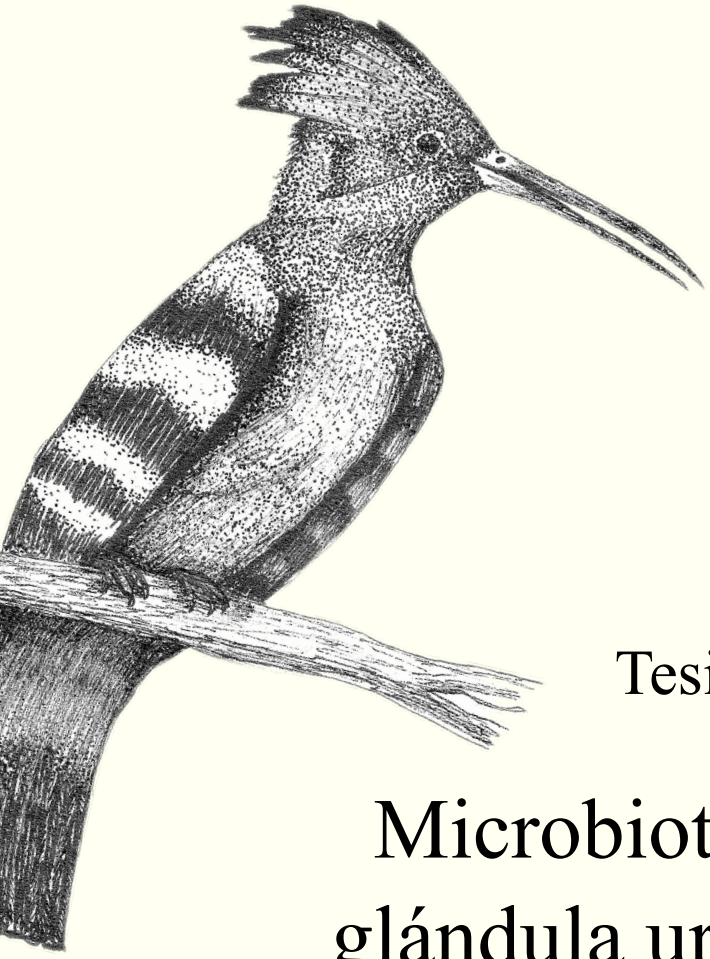

UNIVERSIDAD DE GRANADA

Tesis doctoral

Microbiota asociada a la glándula uropigial de aves; diversidad y funciones en escenarios de parasitismo y comunicación social

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DE GRANADA

Facultad de Ciencias

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TESIS DOCTORAL

2024

Editor: Universidad de Granada. Tesis Doctorales
Autor: Ester Martínez Renau
ISBN: 978-84-1195-217-0
URI: https://hdl.handle.net/10481/89880

Este trabajo ha sido posible gracias a la concesión de una “Ayudas para contratos predoctorales para la formación de doctores 2018” del Ministerio de Ciencia, Innovación y Universidades (PRE2018-085378), y a la financiación del Ministerio de Ciencia e Innovación / Agencia Estatal de Investigación y el Fondo Europeo de Desarrollo Regional con los proyectos (CGL2017-83103-P, PID2020-117429GB-C21 y PID2020-117429GB-C22. El trabajo de campo se ha podido realizar gracias a las prestaciones que el Ayuntamiento de Guadix ha puesto a nuestra disposición. Esta tesis ha sido desarrollada en el Departamento de Ecología Funcional y Evolutiva de la Estación Experimental de Zonas Áridas, perteneciente al Consejo Superior de Investigaciones Científicas (EEZA-CSIC).

Dibujo portada**: Libya González**

Agradecimientos

La sensación agridulce de cerrar esta etapa se hace más real al empezar a escribir estas líneas, quizás por eso he estado alargando este momento hasta el final, pero toca echar la vista atrás y agradecer a todas las personas que me habéis acompañado estos años.

Como no puede ser de otra forma, primer tengo que agradecer a quienes me han dado la oportunidad de estar aquí: mil gracias Juan y Platero por dejarme emprender este camino, tan duro como bonito, bajo vuestro cobijo. Juan, gracias por enseñarme tanto y por transmitir siempre la tranquilidad, la serenidad y los ánimos necesarios para seguir, incluso cuando yo no sabía dónde encontrarlos. Gracias por dejarme volar sin soltarme la mano. De la misma manera quiero agradecerte a ti, Platero, por todos los buenos consejos, el conocimiento transmitido y, sin duda, por trasladar y despertarme ese interés por las bacterias que no sabía que tenía. Gracias otra vez a los dos por conducirme, siempre desde el cariño, para que esta tesis haya salido a la luz.

No puedo dejar de agradecer y mencionar al resto del grupo que habéis amenizado estos años haciendo que el camino fuera mucho más llevadero. Gracias Cristina, fuiste la primera persona a la que conocí aquí y enseguida tuve la certeza de que en este grupo iba a estar a gusto. Gracias por acompañarme en mis primeros pasos en el campo, y enseñarme, con muchas risas de por medio, como muestrear un pájaro (ojalá haber aprendido también a organizarme como tú). Gracias Antonio y Libya por los buenos ratos de descanso, comidas, palique y desahogo. A Manolo y a Gustavo por las cervezas, los pájaros en mano y la buena compañía. A Estefanía por tu dedicación y tu esfuerzo, sabes que estos datos no serían posibles sin tu trabajo. Gracias Pirri por tus soluciones mágicas que nos han salvado más de una vez. Y a ti, Lola, apareciste casi de rebote el primer año de esta aventura y aquí sigues. Gracias por levantarme en cada caída y por convertirte, casi al instante, en una pieza fundamental de esta historia. Gracias a todos, de corazón, por haber hecho que una Valenciana se sienta como en casa.

A la gente de la EEZA, a Pilar por su ayuda en el laboratorio, a la gente del café, a Carmen por el cariño, y al resto de personal que, con una sonrisa, han hecho tan agradable la estancia en aquí. A Armando por las comidas ricas que han hecho que sobreviva estos últimos meses. A todo el personal del departamento de Microbiología y Zoología, y al Ayuntamiento de Guadix por toda la ayuda para el trabajo de campo.

A mis amigas de siempre, de Valencia, Madrid o Salamanca: gracias por seguir, pese a estar lejos de vosotras, siendo un pilar en mi vida. Gracias por escucharme, apoyarme y darme los mejores consejos. La vida con vosotras es mucho más divertida. Os debo muchísimas cervezas, ¡¡pero os prometo que os las devuelvo!! A las Albas y Cristinas de mi vida, a Núria, Elen, Marta, Sara, María, Marina, Natalia, Fátima. Gracias por tanto.

A la meua família, pare, mare, res d'açò seria possible sense que m'haguereu convençut, des de ben xicoteta, que puc aconseguir el que em propose. Gràcies, cada dia, per ser el millor suport i per donar-nos l'amor mes incondicional. Laura, gràcies per ser brúixola, i per ser, des de sempre, com una segona mare. També a vosaltres, iaia, uela i papi, per creure sempre en mi. I a tu, David, per estar, per dibuixar-me un somriure d'immediat en qualsevol moment, per ser sempre la llum en el túnel, i el millor company de vida que podria imaginar.

Índice

Resumen

Los animales han evolucionado en ambientes dominados por microorganismos, con los que mantienen relaciones tanto de parasitismo, como de comensalismo, e incluso de mutualismo, en las que ambos, el microorganismo simbionte y el hospedador se benefician de la interacción. Estos microorganismos mutualistas son, en muchas ocasiones, necesarios para la supervivencia y reproducción de los animales, facilitando o realizando funciones esenciales tales como la digestión de nutrientes, la síntesis de vitaminas, la activación y mantenimiento del sistema inmune, la producción de compuestos volátiles que median en la comunicación del individuo, la protección frente a patógenos, etc. Dado los beneficios derivados de adquirir y mantener una comunidad bacteriana adecuada, los caracteres de los animales que permitan ensamblar una microbiota óptima adaptada a su medio deberían estar sujetos a selección natural. Los animales pueden empezar a adquirir estas bacterias durante la etapa embrionaria, y seguir incorporando simbiontes a lo largo de su vida. Algunos simbiontes se transmiten verticalmente, es decir, de padres a hijos, generalmente en las primeras etapas de vida. Sin embargo, la adquisición de bacterias directamente del ambiente (transmisión horizontal) en el que se incluyen los individuos con los que se mantiene un contacto físico (ambiente social) es también importante para el ensamblaje de una microbiota adecuada, ya que permite que la microbiota se pueda adaptar a las características del ambiente dónde vive el animal de forma plástica.

Uno de los principales beneficios que los animales obtienen de la interacción con la microbiota simbionte que se desarrolla dentro o sobre su cuerpo es la defensa contra microorganismos patógenos. Algunas bacterias producen sustancias antibióticas que pueden impedir el crecimiento y desarrollo de microorganismos potencialmente patógenos, evitando así infecciones tanto en el propio individuo, como en su descendencia. En algunos animales estas bacterias pueden incluso desarrollarse o cultivarse en glándulas especializadas, como puede ser la glándula uropigial de las aves. La secreción uropigial contiene compuestos con propiedades antimicrobianas y, recientemente se ha visto en las abubillas (*Upupa epops*) que esta capacidad antimicrobiana es debida, al menos en parte, a las bacterias simbiontes que se desarrollan en su glándula. En las últimas décadas se han ido aislando bacterias asociadas a esta glándula o a su secreción en distintas especies de aves, lo que abre la posibilidad de que las relaciones mutualistas entre las bacterias simbiontes productoras de sustancias antimicrobianas asociadas a la glándula uropigial y sus hospedadores, esté más extendida

entre la filogenia de las aves de lo que conocíamos hasta el momento. Por tanto, si las características antimicrobianas de estas bacterias estuvieran mediando en la defensa de sus hospedadores contra microorganismos patógenos, cabría esperar que existiera una variación entre especies y que tanto la microbiota como sus características se ajustaran al riesgo de sufrir infecciones patógenas por sus hospedadores.

Las bacterias simbiontes en los animales, además, también tienen un rol importante en la comunicación tanto inter- como intraespecífica, por ejemplo, mediante la producción de compuestos volátiles que los receptores perciben mediante el olfato. Este es el caso de las bacterias asociadas a la glándula uropigial de diversas especies de aves, pero, en la abubilla, el papel de las bacterias de la glándula uropigial no se limita a una posible comunicación olfativa, sino que también desempeñan un papel crucial en la comunicación visual entre machos y hembras. La coloración de la secreción uropigial de las abubillas depende de la comunidad bacteriana que en ella se desarrolla, y las hembras utilizan esta secreción coloreada como sustancia cosmética para impregnar sus huevos y, de esa forma, señalizar a los machos su calidad y la de su descendencia. En general, la coloración cosmética mediada por la glándula uropigial de las aves se ha estudiado, principalmente, en contextos de selección sexual, con solo algún ejemplo en contextos de comunicación paterno-filial. Los pollos de las especies altriciales muestran sus bocas de coloraciones llamativas a sus padres durante el reparto de cebas, y así señalizan su calidad genética o necesidades a corto plazo. Esta coloración podría modificarse o potenciarse si los pollos utilizaran la secreción uropigial, que puede ser transparente o coloreada al ojo humano, como sustancia cosmética para maquillar sus bocas. Si lo hicieran, los pollos estarían transmitiendo, no solo la información proveniente de la coloración de sus bocas, sino también la información derivada de la coloración de su secreción uropigial. Si en el resto de especies de aves más allá de la abubilla la coloración de la secreción uropigial también dependiera, en parte, de la microbiota asociada a esta glándula, estas bacterias estarían desempeñando un papel crucial en la comunicación visual intraespecífica, más concretamente entre padres e hijos.

Los objetivos de esta tesis se encuadran en estos contextos de defensa contra microorganismos patógenos y de comunicación entre padres e hijos. En ella estudiamos el posible papel que pueden desempeñar las bacterias en algunas funciones ya conocidas de la glándula uropigial de las aves. La tesis consta de dos partes bien diferenciadas. En la primera, mediante un estudio interespecífico, exploramos la hipótesis de que las bacterias de la glándula uropigial intervienen en la defensa contra patógenos produciendo compuestos antimicrobianos que se suman a las defensas propias del animal. En esta parte nos

planteamos: (i) poner de manifiesto el efecto del ambiente social (contacto físico con otros individuos) en la comunidad de bacterias de la piel de la glándula uropigial de los pollos de urraca (*Pica pica*) y su parásito de cría, el críalo (*Clamator glandarius*); (ii) estudiar la comunidad bacteriana de la piel de la glándula y la secreción de 26 especies de aves, así como sus capacidades antimicrobianas; (iii) analizar las variaciones en las comunidades bacterianas y sus características entre especies con distintos hábitos de nidificación, factor que usamos como aproximación al riesgo de infección que experimentan las aves; y (iv) explorar la asociación entre las comunidades de la piel y de la secreción para esclarecer el origen de las bacterias productoras de sustancias antibióticas encontradas en la piel y las plumas de las aves. En la segunda parte de la tesis, mediante aproximaciones experimentales usando como especie modelo el estornino negro (*Sturnus unicolor*), estudiamos la hipótesis de que las bacterias, a través de la producción o modificación de la coloración de la secreción uropigial, pueden intervenir en los procesos de comunicación intraespecífica, concretamente entre padres e hijos. Para ello, (i) analizamos el componente genético y el componente ambiental que determinan la coloración de los rasgos de petición (boca, boqueras y piel) y de la secreción uropigial de los pollos de estornino mediante un experimento de intercambio de pollos entre nidos y otro de suplemento de antioxidantes en la dieta, (ii) exploramos la posibilidad de que los pollos de estornino utilicen la secreción uropigial para maquillar sus bocas y, así, modificar la información que transmiten a sus padres, así como (iii) la respuesta de los padres a las coloraciones, tanto de los rasgos de petición como de la secreción. Por último (iv) estudiamos la correlación entre la coloración de la secreción y su comunidad de bacterias, y, por tanto, el posible papel de las bacterias en la comunicación intrafamiliar.

Respecto al primer bloque temático, los resultados mostraron que las diferencias detectadas en la comunidad de bacterias de la piel de los pollos de urraca y críalo cuando ambas especies se desarrollan en nidos separados (sin la presencia de pollos de la otra especie), desparecen cuando ambas especies conviven conjuntamente en nidos de urraca. Además, observamos diferencias entre urracas que compartían o no nido con críalos. Estos resultados sugieren que el ambiente social en el que se desarrollan los pollos de estas especies es un factor importante en el ensamblaje de la comunidad bacteriana de su glándula, al menos en la primera etapa de vida de estos animales.

El estudio interespecífico de las comunidades bacterianas de la piel de la glándula uropigial y sus capacidades antimicrobianas, así como de la comunidad de su secreción, reveló diferencias entre especies y hábitos de nidificación. Las especies que anidan en agujeros, que son aquellas que sufren mayores presiones selectivas debidas al riesgo de

infección, presentaron mayores densidades bacterianas en la piel de sus glándulas, pero las capacidades antimicrobianas de las bacterias aisladas de esta comunidad fueron mayores que las aisladas de especies que anidaban en nidos abiertos y que, por tanto, experimentaban un menor riesgo de infección por bacterias patógenas. Además, las comunidades bacterianas tanto de la piel como de la secreción fueron distintas entre ambos tipos de especies, lo que sugiere que las características de las comunidades bacterianas simbiontes de la glándula uropigial de las aves están adaptadas al riesgo de infección que sufren.

También encontramos una asociación entre las comunidades bacterianas de la piel de la glándula y de la secreción, que fue más fuerte y patente en las especies que anidan en cavidades respecto a las especies de nidos abiertos. Esta asociación puede ser debida al comportamiento de acicalamiento que, mediante la recolecta y distribución de la secreción uropigial por los distintos tegumentos del ave, las aves pueden estar dispersando las bacterias que se desarrollan dentro de la glándula uropigial (en la secreción) por otras partes del cuerpo, lo que explicaría el patrón de anidamiento que encontramos. Si las aves están cultivando bacterias productoras de sustancias antimicrobianas en el interior de su glándula, un mayor acicalamiento aseguraría que estas bacterias alcancen los tegumentos donde actúan frente a microorganismos patógenos y, además, provocaría una mayor asociación entre las comunidades de la glándula y de los tegumentos acicalados en las especies con mayores riesgos de infección.

Respecto al segundo bloque temático, el estudio de la coloración de los caracteres de petición de los pollos de estornino negro evidenció que los pollos, mediante la coloración amarilla de sus rasgos de petición, señalizan a los padres tanto características genéticas como calidad fenotípica y estado oxidativo. El color de los rasgos de petición se ha explicado principalmente como consecuencia de características ambientales. Sin embargo, nuestros resultados experimentales demostraron la existencia de un componente genético en la coloración de la boca y la piel. En el color de las boqueras, sin embargo, solo encontramos evidencias del factor ambiental, y, de acuerdo con esto, éste fue el carácter más afectado por el suplemento de antioxidantes. Los padres, por tanto, reciben múltiples señales con las que evalúan las características genéticas y fenotípicas de su descendencia que utilizan para decidir a qué pollo alimentar.

Gracias a estos experimentos de intercambio de pollos y de suplemento de antioxidantes también pusimos de manifiesto que la coloración de la secreción uropigial, igual que la de los rasgos de petición, señalizaba tanto características genéticas como calidad

fenotípica y estado oxidativo. Además, mostramos que los pollos de estornino se acicalan desde edades tempranas en el nido, impregnando con secreción uropigial los caracteres de petición y, por tanto, modificando su coloración y la información que transmiten a través de ella. Curiosamente, el reparto de alimento que hacían los padres entre los pollos del mismo nido se asoció, no solo a la coloración de los rasgos de petición, sino también al color de su secreción. Por tanto, al usar la secreción como cosmético para maquillar las bocas y boqueras, los pollos estarían utilizándola en la comunicación con sus progenitores.

Finalmente, mediante la caracterización de la comunidad de bacterias de la secreción de los pollos de estornino vimos que ésta se correlacionaba con la coloración de la secreción. Además de correlaciones en los perfiles de composición de las comunidades, taxones concretos como fueron los géneros *Parabacteroides* y *Pseudogracilibacillus* se correlacionaron con el componente UV y amarillo-rojo de la secreción, sugiriendo que estos géneros, directamente a través de la producción o degradación de pigmentos, o indirectamente mediante interacciones con el resto de la comunidad bacteriana, pueden estar determinando la coloración de la secreción uropigial y, de esta manera, la información que los pollos de estornino transmiten al enseñar sus bocas y boqueras maquilladas con secreción. Por tanto, estos resultados sugieren que la comunidad de bacterias podría estar implicada en la comunicación entre padres e hijos.

En su conjunto, los resultados de esta tesis evidencian importantes funciones de la comunidad bacteriana asociada a la glándula uropigial de las aves, y apoyan la hipótesis general de que estas bacterias desempeñan un papel fundamental en las funciones que ya habían sido sugeridas para esta glándula. Esta microbiota, por un lado, protege a los individuos de infecciones por microorganismos patógenos mediante la producción de sustancias con propiedades antimicrobianas, y, por otro, interviene en la comunicación intraespecífica entre padres e hijos, mediante la modificación de la coloración de la secreción.

Abstract

Animals have evolved in a world dominated by microorganisms, engaging in relationships ranging from parasitism and commensalism to mutualism, where both the symbiotic microorganism and the host benefit from the interaction. These microorganisms often play important roles in the survival and reproductive success of animals, by facilitating or carrying out essential functions such as nutrient digestion, synthesizing vitamins, activating and maintaining the immune system, producing volatile compounds involved in animal communication, defending against pathogen infections, etc. Due to the benefits derived from acquiring and maintaining a suitable bacterial community, animal characteristics that allow the assembly of a bacterial community adapted to the environment should be under selection. Animals initiate the acquisition of their microbiota during the embryonic stage, continuing to acquire symbionts throughout their lives. Some bacterial symbionts are vertically transmitted from parents to offspring, typically during early life stages. However, acquiring symbionts directly from the environment in which animals develop (horizontal transmission), including individuals with whom they maintain physical contact (social environment) is also important for assembling an appropriate microbiota, since it allows the microbiota to adjust to the specific environmental characteristics in which the animal lives.

One of the main benefits that animals obtain from the interaction with symbiotic microbiota, either within or on their body, is the defence against pathogenic microorganisms. Some bacteria produce antibiotic substances that impede the growth and development of potentially harmful microorganisms, thereby preventing infection in the individual or their offspring. In some animals, these bacteria thrive in specialized glands, such as the uropygial gland of birds. Specific compounds of the uropygial secretion have antimicrobial properties, and, recently, it was discovered in the Euorpean hoopoe (*Upupa epops*) that bacterial symbionts developing in their uropygial glands contribute, at least partially, to these antimicrobial properties. Over the past few decades, several bacterial strains have been isolated from the gland or its secretion in different bird species, which opens de possibility that the mutualistic interactions among gland-associated bacterial symbionts, producing antimicrobial substances, and their host might be more widespread than previously thought. Therefore, if the antimicrobial properties of these bacteria contribute to the host defences against pathogenic microorganisms, we would expect interspecific variations and an

adjustment in the associated microbiota based on the risk of pathogenic infections experienced by their host.

Symbiotic bacteria in animals also play an important role in both inter- and intraspecific communication, for instance, by producing volatile compounds perceived by receptors through smell. This is the case of several bird species, however, in the hoopoe the role of uropygial gland bacteria extends beyond potential olfactory communication; they also play a crucial role in visual communication between males and females. The colouration of the hoopoe's uropygial secretion depends on the bacterial community that develops within the gland, and females use this coloured secretion as a cosmetic substance to stain their eggs, thus signalling the quality of both themselves and their offspring to males. In general, cosmetic colouration mediated by the uropygial gland of birds has been studied mainly in contexts of sexual selection, with only few examples in contexts of parent-offspring communication. Nestlings of altricial bird species display brightly coloured mouths to their parents when begging, signalling their genetic quality or short-term needs. This colouration could be modified or enhanced if nestlings used the uropygial secretion, which can be transparent or coloured to the human eye, as a cosmetic substance to make up their mouths. If so, nestlings would convey not only information from the colouration of their mouths but also information from the colouration of their uropygial secretion. If in bird species beyond the hoopoe the colouration of the uropygial secretion also depended, partially, on the associated microbiota, these bacteria would play a crucial role in intraspecific visual communication, specifically between parents and offspring.

The aims of this thesis are framed within these contexts of defence against pathogenic microorganisms and parent-offspring communication. We studied the possible role that bacteria might play in some known functions of the uropygial gland of birds. The thesis consists of two distinct parts. In the first part, through an interspecific study, we explore the hypothesis that uropygial gland bacteria contribute to defence against pathogens by producing antimicrobial compounds that complement the animal's own defences. In this part, we aimed to: (i) highlight the effect of the social environment (physical contact with other individuals) on the bacterial community of the uropygial gland skin of magpie (*Pica pica*) nestlings and their brood parasite, the great spotted cuckoo (*Clamator glandarius*), (ii) study the bacterial community of the gland skin and secretion of 26 bird species, as well as their antimicrobial capabilities, (iii) analyse the variability in bacterial communities and their characteristics among species with different nesting habits, used here as a proxy of the infection risk experienced by each species, and (iv) to explore the association between the

bacterial communities of gland skin and secretion in order to clarify the origin of antibiotic-producing bacteria found in the skin and feathers of birds. In the second part of the thesis, through an experimental approach using the spotless starling (*Sturnus unicolor*) as a model species, we studied the hypothesis that bacteria, by producing or altering the uropygial secretion colouration, may be involved in intraspecific communication processes, specifically between parents and their offspring. Then, (i) we studied both the genetic and environmental components determining the colouration of begging related traits (mouth, flanges, and skin) and the uropygial secretion of starling nestlings by performing a cross-fostering experiment and a diet supplementation experiment, (ii) we explored the possibility that starling nestlings used the uropygial secretion to stain their mouths, thus modifying the information they transmit to their parents, as well as (iii) the parents' response to the colouration of both the begging traits and the secretion. Finally (iv) we studied the correlation between the colouration of the secretion and its bacterial community, and thus the possible role of bacteria in the communication within the family.

Regarding the first part, the results showed that the detected differences in the bacterial community associated to the gland skin of magpie and great spotted cuckoo nestlings developing in separate nests (without the presence of nestlings from the other species), disappeared when both species cohabited in magpie nests. Moreover, we detected differences among magpie nestlings that did and did not share the nest with great spotted cuckoos. These results suggest that the social environment in which these nestlings develop is an important factor in assembling the bacterial community of their glands, at least in the early life stages of these animals.

The interspecific study of the bacterial communities of uropygial gland skin and their antimicrobial capacities, as well as the secretion, revealed differences between species and nesting habits. Cavity nester species, which are those experiencing higher selective pressures due to infection risk, had higher bacterial densities on their gland skin, but also higher antimicrobial capabilities of isolated bacteria compared to those strains isolated from non-cavity nester species, which experience a lower risk of infection by pathogenic bacteria. Additionally, both the bacterial communities of the skin and the secretion differed between both types of nesting habits, suggesting that the characteristics of symbiotic bacterial communities of birds' uropygial gland are adjusted to the infection risk that they experience.

We also found an association between the bacterial communities of the gland skin and the secretion, which was stronger in cavity nester species than in non-cavity nesters. This

association might be the result of preening, since birds collect and spread their uropygial secretion through different bird integuments, potentially dispersing the bacteria developing within the uropygial gland to other parts of the body, which might explain the detected nested pattern. If birds were cultivating antibiotic-producing bacteria within their gland, increased preening rates would ensure that these bacteria reach the teguments where they act against pathogenic microorganisms. It would therefore lead to a stronger association between the microbiotas associated to the gland and the preened integument in species with higher infection risks.

Regarding the second part, the study of the colouration of the begging related traits of spotless starling nestlings showed that starling nestlings, through the yellow colouration of their gapes, signal to their parents both genetic characteristics and phenotypic quality, as well as oxidative status. The colour of the begging related traits has been mainly explained as a consequence of environmental characteristics. However, our experimental results demonstrated the existence of a genetic component in the colouration of the mouth and skin. For the flange colouration, however, we found evidence only for the environmental factor, and, furthermore, this was the trait most affected by the antioxidant supplement. Therefore, parents receive multiple signals to evaluate the genetic and phenotypic characteristics of their offspring, which they use to decide which nestling to feed.

Through the cross-fostering and antioxidant supplementation experiments, we also highlighted that uropygial secretion colouration, similar to that of the begging related traits, signals both genetic characteristics and phenotypic quality, as well as oxidative status. Furthermore, we demonstrated that starling nestlings use the uropygial secretion from early stages in the nest, coating their begging related traits with uropygial secretion and thus modifying their colouration and the information they transmit through it. Interestingly, the parental food allocation among nestlings in the same nest was associated not only with the colouration of the begging related traits but also with the colour of their uropygial secretion. Therefore, by making up their mouths and flanges with uropygial secretion, nestlings might be using it to communicate with their parents.

Finally, through the characterization of the bacterial community of the uropygial secretion of starling nestlings, we observed that it correlated with the secretion's colouration. Besides correlations in community composition profiles, specific taxa such as the genera *Parabacteroides* and *Pseudogracilibacillus* correlated with the UV and yellow-red components of the secretion colour. This suggests that these genera, either directly through

the production or degradation of pigments, or indirectly through interactions with the rest of the bacterial community, might influence the colouration of the uropygial secretion and thus the information conveyed by starling nestlings when displaying their mouths and flanges stained with uropygial secretion. Consequently, these results suggest that the bacterial community might be playing a role in parent-offspring communication.

Overall, the findings of this thesis demonstrate the importance of the bacterial community associated with the uropygial gland of birds and support the general hypothesis that these bacteria have a fundamental role in the functions previously suggested for this gland. This microbiota, on one hand, protect birds from infections caused by pathogenic microorganisms by producing substances with antimicrobial properties; and on the other hand, mediate in the intraspecific communication between parents and offspring by modifying the colouration of the secretion.

Introducción general

Vivimos en un mundo dominado por microorganismos que, en las recientes décadas, han captado el interés de ecólogos evolutivos interesados en el estudio de las interacciones entre organismos simbiontes y sus hospedadores. Especialmente las bacterias se encuentran en casi cualquier superficie y múltiples tejidos de los animales. Aunque las interacciones parásitas en las que éstos microorganismos producen enfermedades al hospedador son las más conocidas, existen numerosos ejemplos en los que estos microorganismos contribuyen o se encargan de funciones esenciales para la vida de otros seres vivos, por ejemplo, síntesis de vitaminas, digestión de nutrientes, protección frente a patógenos, etc. (Bosch & McFall-Ngai 2011; Flint *et al.* 2012; Archie & Tung 2015; Schwarzer *et al.* 2016; Sherwin *et al.* 2019), aumentando así las posibilidades de supervivencia y el éxito reproductor del hospedador. Por su parte, los animales proporcionan un ambiente adecuado donde los microorganismos beneficiosos se desarrollan, tratándose así de una relación *mutualista* en la que ambas partes (microorganismo y hospedador) salen beneficiadas. Es por tanto probable que procesos de selección natural hayan favorecido animales que consigan ensamblar una comunidad bacteriana adaptada al medio donde viven y que les confiera beneficios que les permitan realizar sus funciones vitales con éxito. Además, debido a que el establecimiento de una comunidad de bacterias especie-específica adecuada a edades tempranas del desarrollo va a implicar una mayor supervivencia a largo plazo (Velando *et al.* 2021), entender los mecanismos que explican cómo se establecen estas relaciones, así como los efectos en la eficacia biológica de sus hospedadores es esencial para comprender la evolución de los organismos en general, y la de su relación con microorganismos simbiontes en particular.

Los animales pueden adquirir estos simbiontes por transmisión vertical, es decir, la transferencia de bacterias de padres a hijos. Generalmente, los simbiontes que dependen completamente de su hospedador se transmiten por esta vía, dando lugar a procesos coevolutivos estrechos entre el hospedador y el microorganismo (Moran, McCutcheon & Nakabachi 2008). Los microorganismos pueden pasar de padres a hijos por distintas vías y en distintos momentos durante el desarrollo. Por ejemplo, existen evidencias de que, al contrario de lo que se pensaba, los embriones de ratones, lagartos y distintas especies de aves se desarrollan en un ambiente no estéril, ya que las madres inoculan bacterias en los embriones (Jiménez *et al.* 2008; Funkhouser & Bordenstein 2013; Trevelline *et al.* 2018). Para una revisión crítica de esta posibilidad ver Perez-Muñoz *et al.* (2017). Además, los

animales vivíparos adquieren parte de su microbiota proveniente de la madre durante el parto (Dominguez-Bello *et al.* 2010; Kohl *et al.* 2017). Una vez nacidos, los juveniles también pueden adquirir esta microbiota por transmisión vertical a través de la ingestión de fluidos o desechos corporales cargados de bacterias beneficiosas que le proporcionan sus progenitores, como a través de la lactancia en mamíferos (Fernández *et al.* 2013; Van den Elsen *et al.* 2019), la regurgitación en aves, escarabajos o arañas (Wang & Rozen 2017; Ding *et al.* 2020; Rose *et al.* 2023) o la coprofagia en insectos, mamíferos y algunas aves (Nalepa, Bignell & Bandi 2001; Cooper 2004; Combes *et al.* 2014; Bo *et al.* 2020; Blyton *et al.* 2022).

Por otro lado, los animales pueden adquirir los microorganismos simbiontes por transmisión horizontal. Durante la primera etapa de la vida de un animal, éste entra en contacto con las bacterias del ambiente que le rodea. Algunos de estos simbiontes son también beneficiosos, y existen adaptaciones en los animales que les permiten adquirirlos repetidamente en cada generación (Ruiz-Rodríguez *et al.* 2014). La transmisión horizontal también podría conllevar beneficios para el hospedador, ya que podría adquirir el simbionte más óptimo que le confiera ventajas adaptativas según las características del ambiente (Scheuring & Yu 2012). Los individuos podrían adquirir a lo largo de su vida estas bacterias de su entorno (Kikuchi *et al.* 2012; van Veelen, Salles & Tieleman 2017; Baker *et al.* 2019; Campos-Cerda & Bohannan 2020), incluyendo aquellos individuos con los que mantienen contacto, ya sean familiares, parejas o miembros del mismo grupo social (Troyer 1984; Kulkarni & Heeb 2007; Tung *et al.* 2015; Perofsky *et al.* 2017; Dowd & Renson 2018; Dill-McFarland *et al.* 2019). Por tanto, conocer el modo de transmisión y ensamblaje de una comunidad de bacterias óptima para cada especie, generalmente adaptada a las características ecológicas del ambiente en el que vive, es crucial para entender las relaciones mutualistas entre simbionte y hospedador, así como la historia evolutiva que les ha llevado a establecer el vínculo que nos encontramos en el presente. En esta tesis se aborda la importancia del ambiente social en el ensamblaje de comunidades microbianas, así como posibles funciones de bacterias mutualistas.

1. Importancia del ambiente social en el ensamblaje de la comunidad bacteriana

El ambiente social se puede describir como las características de los individuos con los que otro individuo focal mantiene contacto físico estrecho. Estas interacciones, generalmente, homogenizan las comunidades de bacterias de los individuos que interactúan (Tung *et al.* 2015; Grieneisen *et al.* 2017; Perofsky *et al.* 2017; Dill-McFarland *et al.* 2019), haciendo que estas sean más similares entre sí que con individuos con los que no mantienen contacto físico.

Es por esto que la transmisión horizontal de simbiontes debida a las interacciones con miembros de una familia o un grupo social parece un factor importante para determinar la composición de la microbiota. Existen pocos estudios experimentales que exploren el efecto de este factor, dado que la mayoría de las evidencias que apoyan la asociación entre las interacciones sociales y la composición de la comunidad de bacterias son generalmente fruto de correlacionales (Song *et al.* 2013; Amato *et al.* 2017; Antwis *et al.* 2018; Raulo *et al.* 2018; Dill-McFarland *et al.* 2019). Sin embargo, en estos estudios correlacionales es difícil separar el efecto del contacto físico entre individuos de otros factores asociados, tales como las relaciones genéticas y familiares entre los individuos estudiados, la dieta que consumen, los recursos que utilizan, el espacio físico que comparten, etc. Todos estos factores podrían inducir la similitud entre comunidades microbianas de distintos individuos incluso en ausencia de contacto físico entre ellos. En el **Capítulo I** de esta tesis, intentamos mostrar la importancia del ambiente social (contacto entre individuos) explicando características de la microbiota de los animales. Para ello aprovechamos el sistema de parásito de cría-hospedador que forman el críalo europeo (*Clamator glandarius*) y la urraca (*Pica pica*), para explorar el efecto del contacto físico estrecho (pollos de distinta especie en un mismo nido), mientras se controla por posibles factores de confusión como los genéticos, ambiente del nido, alimentación, o características de los padres, entre otros.

2. Funciones de las bacterias simbiontes mutualistas

Las bacterias simbiontes desempeñan funciones cruciales para su hospedador desde los primeros estadios de desarrollo de los animales. Intervienen, por ejemplo, en su etapa embrionaria (Stappenbeck, Hooper & Gordon 2002; Bates *et al.* 2006), así como en su crecimiento y maduración (Fraune & Bosch 2010; Heijtz *et al.* 2011; Cryan & Dinan 2012; Schwarzer *et al.* 2016). A lo largo de la vida del animal, estas bacterias son fundamentales, por ejemplo, favoreciendo procesos fisiológicos como la nutrición (Backhed *et al.* 2004; Flint *et al.* 2012; Krajmalnik-Brown *et al.* 2012) o la activación y mantenimiento del sistema inmunitario (Umesaki *et al.* 1995; Weiss, Maltz & Aksoy 2012; Belkaid & Hand 2014), mediando en la defensa del hospedador contra patógenos (Soler *et al.* 2010; Martín-Vivaldi *et al.* 2014; Hoyt *et al.* 2015; Flechas *et al.* 2019; Iacob, Iacob & Luminos 2019; Bodawatta *et al.* 2020), o incluso influyendo en el comportamiento (Cryan & Dinan 2012; Archie & Tung 2015; Sherwin *et al.* 2019) o la comunicación química (Wada-Katsumata *et al.* 2015; Maraci, Engel & Caspers 2018; Grieves *et al*. 2019; Mazorra-Alonso, Tomás & Soler 2021). En esta tesis nos vamos a centrar en las funciones de defensa contra infecciones patógenas y

de comunicación, específicamente en la comunicación visual entre padres e hijos. En los siguientes apartados paso a introducir el papel de estos microorganismos en ambos contextos.

a. Defensa contra infecciones por bacterias patógenas y el papel de la microbiota simbionte

Los animales tienen diferentes mecanismos para hacer frente a la colonización de microorganismos capaces de inducir enfermedades. Estos mecanismos pueden ser fisiológicos o comportamentales. La primera línea de defensa sería su sistema inmune, que a grandes rasgos cuenta con dos tipos de procesos: la inmunidad innata y la adquirida. La primera es común a animales y plantas, es inespecífica al patógeno, y se encarga de detectar la infección y activar los mecanismos requeridos para hacerle frente (Nürnberger *et al.* 2004). La inmunidad adquirida o adaptativa, que es propia de animales vertebrados y es activada por la inmunidad innata (Pancer & Cooper 2006), es específica para el patógeno que coloniza, y está mediada por los linfocitos T y B, encargados de producir anticuerpos frente a los patógenos. Además de las respuestas fisiológicas, los animales exhiben distintos comportamientos para evitar la infección o disminuir los efectos de posibles patógenos. Para reducir la probabilidad de infección y evitar la colonización de microorganismos infecciosos, los animales se acicalan y desparasitan, tanto a ellos mismos como a sus congéneres (Mooring, Blumstein & Stoner 2004; Akinyi *et al.* 2013; Bush & Clayton 2018). También evitan las infecciones higienizando el ambiente en el que viven o se reproducen (Schmid-Holmes *et al.* 2001; Guigueno & Sealy 2012; Sun & Zhou 2013; Diez, Lejeune & Detrain 2014; Ibáñez-Álamo, Rubio & Soler 2017). Una vez infectados, algunos animales pueden llegar a auto-medicarse con el fin de paliar los efectos de la enfermedad y ayudar a erradicar el patógeno de su cuerpo (de Roode, Lefèvre & Hunter 2013). A estos mecanismos de defensa se están añadiendo recientemente evidencias del uso de bacterias simbiontes. Productos antimicrobianos derivados del metabolismo de bacterias simbiontes evitan infecciones patógenas en los hospedadores (Suzuki & Nagano 2006; Ruiz-Rodríguez *et al.* 2009; Hoyt *et al.* 2015; Flechas *et al.* 2019; Bodawatta *et al.* 2020), en sus huevos o descendencia (Gil-Turnes, Hay & Fenical 1989; Kaltenpoth *et al.* 2005; Ruiz-Castellano 2016; Heise *et al.* 2019), o en cultivos de los que estos animales dependen para su supervivencia (Currie *et al.* 1999; Scott *et al.* 2008). Las bacterias productoras de sustancias antibióticas pueden crecer en la superficie de plantas o materiales que los animales utilizan, por ejemplo, para revestir los nidos, rebajando así la carga parasitaria del nido e incrementando la probabilidad de supervivencia de su descendencia (Peralta-Sánchez *et al.* 2010; Peralta-Sánchez *et al.* 2014; Ruiz-Castellano *et al.* 2016; Ruiz-Castellano *et al.* 2019). Este tipo de bacterias también

pueden establecerse y desarrollarse en el propio cuerpo del animal, tanto en el tracto digestivo (Pickard *et al.* 2017; Heise *et al.* 2019), como en los tegumentos exteriores (piel, plumas, pelo) (Brucker *et al.* 2008; Harris *et al.* 2009; Javůrková *et al.* 2019; Rojas-Gätjens *et al.* 2022), o en glándulas especializadas. En insectos, por ejemplo, éste tipo de bacterias pueden establecerse y crecer en glándulas exocrinas localizadas en las antenas o en el exosqueleto, como es el caso de la avispa *Philanthus triangulum* (Kaltenpoth et al. 2006) y de las hormigas cultivadoras de hongos (Currie *et al.* 2006), respectivamente. Entre los vertebrados, el taxón más conocido y estudiado por cultivar bacterias productoras de antimicrobianos en glándulas exocrinas son las aves.

La gran mayoría de las especies de aves presentan una única glándula exocrina ubicada en la parte baja del dorso, por encima de la cola, llamada glándula uropigial. Esta glándula está formada, generalmente, por dos lóbulos que contienen el tejido secretor activo que produce la secreción uropigial. Los lóbulos desembocan en una papila donde la secreción se almacena y sale a la superficie a través de uno o más poros que, con frecuencia, están rodeados por un penacho de pequeñas plumas (Jacob & Ziswiler 1982). La secreción que se produce es aceitosa y está compuesta mayormente por lípidos, como monoésteres y diésteres de alcoholes alifáticos y ácidos grasos, y en menor medida por triglicéridos, esteroles, e hidrocarburos (Jacob & Ziswiler 1982). Tanto la morfología de la glándula como la composición de su secreción varían bastante entre especies (Jacob & Ziswiler 1982), y dentro de una misma especie, por ejemplo, dependiendo del sexo (Martín-Vivaldi *et al.* 2009; Amo *et al.* 2012; Grieves, Bernards & MacDougall-Shackleton 2019), de la edad (Martín-Vivaldi *et al.* 2009; Amo *et al.* 2012; Grieves, Bernards & MacDougall-Shackleton 2019), o de la época del año (Reneerkens, Piersma & Damste 2002; Martín-Vivaldi *et al.* 2009).

Las aves colectan la secreción uropigial con su pico, comprimiendo la glándula hasta sacar la secreción que esparcen por las distintas partes de su cuerpo durante el acicalamiento. Las funciones que más comúnmente se han atribuido a la secreción uropigial son el mantenimiento (higiene y protección contra abrasión física), la flexibilización y la impermeabilización del plumaje. También se han descrito algunos compuestos de la secreción con propiedades antimicrobianas (Jacob & Ziswiler 1982) frente a bacterias queratinolíticas (degradadoras de plumas) o microorganismos infecciosos (Bandyopadhyay & Bahttacharyya 1996; Shawkey, Pillai & Hill 2003; Møller, Czirjak & Heeb 2009; Giraudeau *et al.* 2010; Ruiz-Rodríguez *et al.* 2015). Curiosamente, en los años 2000 se aislaron diversas cepas bacterianas de la glándula uropigial de dos especies de upupiformes, la abubilla arbórea (*Phoeniculus purpureus*; Law-Brown & Meyers 2003) y la abubilla

europea (*Upupa epops*; Martín-Platero *et al.* 2006; Soler *et al.* 2008; Martín-Vivaldi *et al.* 2010; Rodríguez-Ruano *et al.* 2015), y se lanzó la hipótesis de que estas bacterias podrían ser las responsables de las propiedades antimicrobianas de la secreción, y que, por tanto, podían tener un rol importante en la ecología de este orden de aves.

La comunidad de bacterias asociada a la glándula uropigial de la abubilla europea ha sido, con diferencia, la más estudiada. Las bacterias que alberga la glándula de esta especie son las responsables de: (i) el color de la secreción uropigial de hembras y pollos (Martín-Vivaldi *et al.* 2009), (ii) la composición de sus volátiles (Martín-Vivaldi *et al.* 2010) y, (iii) al menos, de parte de las propiedades antimicrobianas de la secreción (Martín-Vivaldi *et al.* 2010). Estas bacterias productoras de sustancias antimicrobianas, al esparcirse junto con la secreción por las distintas partes del cuerpo del ave, previenen la degradación de las plumas por bacterias queratinolíticas (Soler *et al.* 2008; Ruiz-Rodríguez *et al.* 2009; Soler *et al.* 2010; Ruiz-Rodríguez *et al.* 2013), aumentando así la probabilidad de supervivencia de los individuos que las albergan. Las hembras no solo acicalan sus plumas con esta secreción, sino que, en el momento de la incubación, también la esparcen por la superficie de los huevos, que presenta unas criptas donde se establecen las bacterias simbiontes provenientes de su secreción, aumentando así el éxito de eclosión de las nidadas y el éxito de vuelo de los pollos (Soler *et al.* 2012; Martín-Vivaldi *et al.* 2014).

Desde estos primeros estudios, el número de evidencias de que la glándula uropigial de las aves alberga bacterias simbiontes no para de crecer. En los últimos años se han aislado o secuenciado bacterias de la glándula uropigial de especies como el pavo salvaje (*Meleagris gallopavo*; Braun *et al.* 2016), el junco pizarroso (*Junco hyemalis*; Whittaker & Theis 2016), el pico picapinos (Dendrocopos major; Braun *et al.* 2018a), la lechuza común americana (Tyto furcata; Braun *et al.* 2018c; Braun *et al.* 2019), el ganso del Nilo (Alopochen aegyptiacus; Braun *et al.* 2018b), el carbonero (*Parus major*; Bodawatta *et al.* 2020), el gorrión común (*Passer domesticus*; Videvall *et al.* 2021), el gorrión cantor (*Melospiza melodia*; Grieves *et al.* 2021), o diversas especies de calaos (Díaz-Lora 2020). Todos estos estudios apuntan a que las relaciones mutualistas entre la glándula uropigial de las aves y las bacterias simbiontes está más extendida en la filogenia de los hospedadores de lo que inicialmente se pensaba. Además, también se han encontrado bacterias productoras de sustancias antibióticas en las plumas de distintas especies de aves (Javůrková *et al.* 2019), que se cubren regularmente de secreción uropigial al acicalarse. Todas estas evidencias, abren la posibilidad de que, no solo en las abubillas sino también en el resto de especies de aves, las bacterias productoras de sustancias antibióticas encontradas en las plumas estuvieran

creciendo y desarrollándose en la glándula uropigial, y/o que la secreción uropigial, al esparcirse en otros tegumentos, estuviera actuando como sustrato nutricional que las bacterias mutualistas usarían para crecer. Sea cuál sea el mecanismo, estas bacterias productoras de antibióticos estarían protegiendo a sus hospedadores de bacterias degradadoras de plumas o de bacterias patógenas capaces de provocar enfermedades en las aves.

Las defensas mediadas por las bacterias simbiontes de la glándula se sumarían a las defensas derivadas del sistema inmunitario del propio hospedador. Distintas características del sistema inmune de las aves se relacionan con el riesgo de infección que sufren las especies por sus características ecológicas. Por ejemplo, las especies coloniales sufren mayor riesgo de parasitismo, infección y transmisión de patógenos (Møller *et al.* 2001), y como consecuencia evolutiva, presentan mayores niveles de respuesta inmunitaria mediada por linfocitos T y B (Møller *et al.* 2001). Por otro lado, las especies migradoras y aquellas que anidan en cavidades también experimentan mayores riesgos de parasitismo e infección por microorganismos patógenos, ya que la migración expone a estos individuos a una mayor diversidad de patógenos (Møller & Erritzøe 1998), mientras que las especies que anidan en cavidades se ven obligadas a reutilizar los nidos que previamente han usado otros individuos de la misma u otra especie debido a la escasez de agujeros en la naturaleza (Møller & Erritzøe 1996). La exposición a estos factores de riesgo resulta en una mayor inversión de estas especies en el desarrollo y funcionamiento del sistema inmune y, en consecuencia, presentan por ejemplo mayores tamaños de bazo (relacionado con la inmunidad tanto humoral como celular) y de la bolsa de Fabricio (relacionada con la síntesis de anticuerpos) (Møller & Erritzøe 1996; Møller & Erritzøe 1998).

Si existieran mecanismos que permitieran a los animales el cultivo de bacterias productoras de sustancias antimicrobianas que participaran en las defensas frente a enfermedades, se esperaría que en especies con mayor riesgo de infección aparecieran cepas con mayor capacidad antimicrobiana. Así, la comunidad de bacterias de la glándula uropigial debería variar entre especies según sus características ecológicas y el grado de presión selectiva debido al riesgo de infección por microorganismos patógenos al que se ven sometidas. De la misma manera, las propiedades antimicrobianas de las bacterias que conforman estas comunidades deberían ser mayores en estas mismas especies. Estas predicciones las exploramos en los **Capítulos II y III**, donde, caracterizando la microbiota de la glándula uropigial y sus propiedades antimicrobianas, exploramos cómo estos caracteres varían entre especies que anidan en agujeros y las que anidan en nidos abiertos,

parámetro que utilizamos como indicador de presiones selectivas debido a riesgo de infección por microorganismos patógenos (Møller & Erritzøe 1996).

Las comunidades de bacterias establecidas en las distintas partes del cuerpo de un individuo no están aisladas ni entre ellas ni con el ambiente. De hecho, el éxito de la relación mutualista entre las aves y las bacterias productoras de antibióticos que alberga su glándula uropigial radica, en parte, en que estas bacterias consigan alcanzar los tejidos donde desempeñan su papel de protección frente a patógenos. En 2016 se describió en abubillas un patrón anidado de las bacterias desde la secreción de la glándula hasta el pico, la placa incubatriz y finalmente la cáscara de los huevos, sugiriendo que esta jerarquía era el resultado de la colonización de distintas estructuras del cuerpo de las hembras por parte de las bacterias productoras de antibióticos de la secreción (Soler *et al.* 2016). Por tanto, estudiar esta asociación entre comunidades de bacterias de distintas localizaciones y la comunidad de bacterias de la secreción uropigial en otras especies distintas a la abubilla, añadiría más evidencias a la hipótesis de que las bacterias productoras de sustancias antibióticas encontradas en distintas partes del cuerpo de las aves provienen, en parte, de la glándula uropigial. Si se cumpliera este patrón, las aves podrían estar cultivando en su glándula bacterias beneficiosas con propiedades antimicrobianas que se ajustarían a las presiones selectivas ejercidas por microorganismos patógenos y, gracias al comportamiento de acicalamiento, estas bacterias beneficiosas se podrían establecer en los tegumentos donde actuarían defendiendo al hospedador contra posibles infecciones. Además, si las bacterias productoras de sustancias antibióticas encontradas en otras estructuras como plumas o huevos provienen de la glándula uropigial, cabría esperar que la asociación entre ambas comunidades fuera más fuerte en especies con más necesidades de defensa. En el **Capítulo III** de esta tesis exploramos la asociación entre las comunidades de bacterias de la secreción de la glándula uropigial y de la piel de esta misma glándula, así como la predicción de que esta asociación sería más fuerte en las especies con mayor riesgo de infección por organismos patógenos.

b. Coloración y papel de las bacterias en contextos de comunicación entre padres e hijos

Los animales utilizan distintos medios para transmitir información sobre su estado fisiológico, sus características genéticas o incluso sobre comportamientos que pueden o van a realizar. Por su parte, los receptores de esta información modulan su comportamiento de acuerdo a la información recibida. Los caracteres involucrados en la comunicación se denominan *señales* cuando han evolucionado específicamente para enviar información al receptor, y la respuesta de éste beneficia a ambas partes (Maynard-Smith & Harper 2003).

Además, esta información puede ser usada por receptores a los que no va específicamente dirigida. En este caso, los receptores serían los únicos beneficiados y se denominarían *pistas* o *información social no intencionada* (Danchin *et al.* 2004). Las señales y pistas se pueden transmitir por varios canales como el acústico, el olfativo o químico y el visual, este último, a través del movimiento o la coloración.

Existen varios tipos de coloración según su origen siendo la coloración pigmentaria y la coloración estructural las más estudiadas. La coloración pigmentaria es la resultante de la acumulación de substancias químicas (pigmentos) que reflejan la luz en unas longitudes de onda concretas que determinan el color de la estructura (Blount *et al.* 2003; Logan, Burn & Jackson 2006; McGraw 2006; McGraw & Hill 2006), mientras que la coloración estructural es el resultado del reflejo de la luz en nanoestructuras biológicas presentes en el tegumento en cuestión (Shawkey & D'Alba 2017). Sin embargo, el color base de estas estructuras se puede modificar, intencionada- o inintencionadamente con distintos comportamientos del animal. En aves, por ejemplo, el desgaste y rotura de las plumas puede también hacer que el color se desvanezca. Este desgaste puede ser debido a varias causas: (i) a la abrasión mecánica (por rozamiento con elementos del ambiente), (ii) a los agentes meteorológicos (sol, lluvia, viento, radiación UV etc.; Møller & Erritzøe 1992; Willoughby, Murphy & Gorton 2002; Montgomerie 2006) o (iii) a la acción de microorganismos (Burtt & Ichida 2004; Montgomerie 2006; Shawkey, Pillai & Hill 2009). La coloración de las plumas u otras partes del cuerpo de las aves también puede variar al ser teñidas con substancias provenientes del ambiente (como el caso del quebrantahuesos *Gypaetus barbatus*; Negro *et al.* 1999), o producidas por el propio animal, como la secreción uropigial. A este tipo de coloración se le conoce como coloración cosmética. La secreción uropigial puede ser transparente o coloreada al ojo humano, y, al esparcirse entre las plumas u otras estructuras durante el acicalamiento, puede producir un efecto de brillo o un cambio en las longitudes de onda que se reflejan. Por tanto, el acicalamiento con secreción uropigial produciría una modificación en la coloración de distintos tegumentos y en la información que se transmite a través de ella. Curiosamente, los factores ambientales que afectan a la condición corporal y la calidad fenotípica del individuo, también afectan a la composición de la secreción y a la tasa de acicalamiento (Delhey, Peters & Kempenaers 2007; Piault *et al.* 2008). Además, el sistema inmunitario está relacionado con el tamaño de la glándula (Moreno-Rueda 2015; Moreno-Rueda 2016), un rasgo que, a su vez, determina la cantidad de secreción que se produce (Pap *et al.* 2010). Esto sugiere que, tanto la producción de secreción uropigial (Piault *et al.* 2008; Magallanes *et al.* 2016) como el acicalamiento (Goldstein 1988; Walther & Clayton 2005), conllevan un coste

para el individuo, y por tanto, la coloración cosmética mediada por la glándula uropigial se podría considerar una señal honesta.

La coloración cosmética mediada por la secreción uropigial se ha descrito en diferentes especies de aves (Nesbitt 1975; Negro *et al.* 1999; Kemp 2001; Piault *et al.* 2008; Lopez-Rull, Pagan & Macias Garcia 2010). En algunas incluso se ha sugerido la presencia de carotenos en estas secreciones (Montgomerie 2006), aunque solo en los flamencos (*Phoenicopterus roseus*) se ha demostrado empíricamente (Amat *et al.* 2011; Amat *et al.* 2018). En abubillas se ha visto que la comunidad de bacterias que alberga la glándula uropigial tiene un rol importante en el color marrón de la secreción que presentan los pollos y las hembras durante la época de reproducción (Martín-Vivaldi *et al.* 2009). En esta especie, las hembras durante el invierno, y los machos a lo largo de todo el año, presentan una secreción blanquecina con una carga bacteriana mucho menor a la encontrada en la secreción marrón de pollos y hembras en primavera (Martín-Vivaldi *et al.* 2009; Rodríguez-Ruano *et al.* 2018). Curiosamente, al inyectar antibióticos en la glándula de hembras y pollos, tanto los compuestos volátiles como la coloración cambia significativamente (Martín-Vivaldi *et al.* 2009; Martín-Vivaldi *et al.* 2010), lo que sugiere que las bacterias están, de alguna manera, interviniendo en estas propiedades. Además, las hembras, al cubrir las cáscaras de los huevos con esta secreción marrón cargada de bacterias productoras de sustancias antibióticas (Martín-Vivaldi *et al.* 2014; Soler *et al.* 2014), señalizan al macho las características de su secreción (y por tanto de ellas mismas). Los machos evalúan el color de los huevos, y ajustan la tasa de cebas en respuesta a la información recibida (Díaz-Lora *et al.* 2020; Díaz-Lora *et al.* 2021). Todos estos resultados apuntan a que las bacterias de la secreción uropigial de las abubillas, a través del uso de la coloración cosmética por parte de las hembras para teñir sus huevos, estarían mediando en la comunicación intraespecífica entre miembros de una misma pareja.

Generalmente, el uso de la secreción uropigial como cosmético se ha descrito y estudiado para contextos de selección sexual y elección de pareja (Lopez-Rull, Pagan & Macias Garcia 2010; Amat *et al.* 2011; Díaz-Lora *et al.* 2020; Díaz-Lora *et al.* 2021). Sin embargo, también se ha estudiado en otros contextos como la comunicación entre padres e hijos (Piault *et al.* 2008). La comunicación visual a través de la coloración tiene también una relevancia importante en este tipo de comunicación. Los pollos de muchas especies presentan una coloración llamativa (rojos, amarillos, blancos o incluso patrones punteados) en los rasgos de petición, como la boca y las boqueras, que muestran a los padres en el momento del reparto de cebas para llamar su atención. Existen distintas evidencias de que esta

coloración señaliza calidad fenotípica (señales de calidad; Grafen 1990; De Ayala *et al.* 2007; Jacob & Heeb 2013) o necesidad (señales de necesidad; Godfray 1991; Kilner & Johnstone 1997; Johnstone & Godfray 2002). No obstante, la información emitida por los pollos a través del color podría ser modificada en el caso de que los pollos, igual que los adultos, se esparcieran la secreción uropigial por estos rasgos. De esta manera, los pollos señalizarían a los padres, no solo la información del color de sus bocas y boqueras, sino también la información de la secreción utilizada como cosmético. En los **Capítulos IV y V** estudiamos qué tipo de información mandan los pollos de estornino negro (*Sturnus unicolor*) a sus padres a través de la coloración amarilla de sus rasgos de petición, así como la posibilidad de que los pollos de estornino utilicen su secreción uropigial, de un color amarillo muy parecido al de sus bocas, para maquillarlas, modificando así la información percibida por los padres. En el **Capítulo VI** exploramos la posibilidad de que la comunidad de bacterias de la secreción de los pollos de estornino tenga un rol importante en la producción o modulación de su coloración. Hasta donde sabemos, ésta sería la primera evidencia del rol de las bacterias de la secreción uropigial en la comunicación visual intraespecífica en un contexto de comunicación paterno-filial.

Hipótesis y objetivos

La glándula uropigial de las aves juega un papel importante en contextos de parasitismo (defensa frente a patógenos y enfermedades) y comunicación (entre padres e hijos), y la hipótesis general que tratamos de explorar en esta tesis es que la microbiota asociada a esta glándula es participe de esas funciones. Esta hipótesis asume la existencia de una comunidad de bacterias simbionte estable en la glándula uropigial de las aves, que hasta la fecha solo se ha podido demostrar en unas pocas especies. Por tanto, para explorar esa hipótesis, es necesario caracterizar las comunidades de bacterias asociadas a la glándula uropigial de las aves y estudiar su implicación en procesos de defensa contra patógenos, y en la comunicación intrafamiliar. La comprobación de esta hipótesis se desarrolla a lo largo de los 6 capítulos de la tesis, abordando los siguientes objetivos específicos:

- **Objetivo 1**: Explorar el efecto del ambiente social (características de los individuos con los que se mantiene contacto físico directo) en el establecimiento y estructura de la microbiota de la piel de la glándula uropigial.

- **Objetivo 2**: Estudiar las propiedades antimicrobianas de las bacterias aisladas de la piel de la glándula uropigial de distintas especies y cómo éstas varían entre especies dependiendo de las presiones selectivas debidas al riesgo de infección patógena que sufren cada una de ellas.

- **Objetivo 3**: Caracterizar la comunidad bacteriana tanto de la piel de la glándula como de su secreción en diversas especies de aves y estudiar cómo varían entre especies en relación al riesgo de infección por bacterias patógenas al que están expuestas.

- **Objetivo 4**: Estudiar la relación entre la comunidad de bacterias de la piel de la glándula uropigial y de la secreción de la glándula con el fin de dilucidar el origen de las bacterias productoras de sustancias antibióticas que se encuentran en la piel y en las plumas de las aves.

- **Objetivo 5**: Separar el componente genético y ambiental de la coloración amarilla tanto de los rasgos de petición como de la secreción uropigial de los pollos de estornino negro.

- **Objetivo 6**: Explorar la influencia del alimento extra en la coloración amarilla tanto de los rasgos de petición como de la secreción uropigial de los pollos de estornino negro.

- **Objetivo 7**: Estudiar la posibilidad de que los pollos de estornino utilicen su secreción uropigial para maquillar los rasgos de petición y cómo la coloración de estos rasgos determina la tasa de cebas recibida por cada pollo de parte de los padres.

- **Objetivo 8**: Explorar el posible papel de la comunidad de bacterias de la glándula uropigial de los pollos de estornino en la coloración de su secreción.

Para llevar a cabo estos objetivos, en esta tesis se han realizado tanto aproximaciones correlacionales como experimentales que nos han permitido, mediante estudios intra- e interespecíficos, responder a los objetivos propuestos. La tesis se compone de 6 capítulos que voy a explicar brevemente a continuación:

Capítulo I: En este capítulo, aprovechando el sistema de parasitismo de cría formado por el críalo (*Clamator glandarius*) y, su hospedador, la urraca (*Pica pica*), abordamos la importancia de la transmisión horizontal de los simbiontes en las comunidades externas de la piel (**Objetivo 1**), y predecimos que la microbiota de la piel variará dependiendo del ambiente social del nido (convivencia o no con hetero-específicos).

Capítulo II: Realizamos un estudio interespecífico en el que, por medio de ensayos de antagonismo, caracterizamos las capacidades antagónicas de bacterias aisladas de la piel de la glándula uropigial (**Objetivo 2**). Se predice que especies con mayores presiones selectivas tendrán bacterias simbiontes con mayores capacidades antagónicas. Se estudian especies que anidan en agujeros y en nidos abiertos, y se asume que las presiones selectivas debidas a parásitos y enfermedades son mayores en las primeras (Møller & Erritzøe 1996; Møller & Erritzøe 1998; Møller *et al.* 2001).

Capítulo III: En este capítulo, caracterizando la microbiota de la piel de la glándula y de la secreción uropigial de distintas especies, llevamos a cabo el **Objetivo 3** en el que, asumiendo que las propiedades antimicrobianas de los aislados de la microbiota de la piel de glándula varían según el riesgo de infección, predecimos que la riqueza y diversidad, así como la composición de la comunidad bacteriana completa también variará según este factor. Además, en este capítulo también exploramos la asociación entre ambas comunidades de bacterias (**Objetivo 4**). Puesto que ya se ha visto en abubillas que la glándula uropigial alberga una comunidad de bacterias rica en cepas productoras de sustancias antimicrobianas,

y que éstas se dispersan desde la glándula hasta los huevos, pasando por el pico y la placa de incubación (Soler *et al.* 2016), se predice que en otras especies, las bacterias con propiedades antimicrobianas encontradas fuera de la glándula también provienen de la comunidad de bacterias simbiontes establecida dentro de la glándula.

Capítulo IV: En este capítulo se utilizan dos aproximaciones experimentales para estudiar la coloración de los rasgos de petición (boca, boqueras y piel) de los pollos de estornino negro en un contexto de comunicación intraespecífica. Realizamos un experimento de intercambio de pollos para separar y estimar la importancia del componente genético del componente ambiental determinando la coloración de estos rasgos (**Objetivo 5**). Para explorar con mayor detalle la influencia del componente ambiental, realizamos un segundo experimento de suplemento de antioxidantes (**Objetivo 6**). Se predice que, aunque la coloración de estos caracteres debe depender principalmente del ambiente, también debe existir un componente genético que explique la diferencia de coloración entre las distintas especies. Además, se predice que el alimento extra con antioxidantes afectará a la condición fenotípica del pollo y también así, a la coloración de los distintos caracteres.

Capítulo V: En este capítulo se abordan los **Objetivos 5** y **6** respecto a la coloración de la secreción uropigial, usando las mismas aproximaciones experimentales que en el **Capítulo IV**. Para este capítulo se predice que la coloración de la secreción dependerá también tanto de las características genéticas como de las ambientales. Además, si los pollos de estornino, al igual que los adultos, utilizan su secreción uropigial desde edades tempranas en el nido para acicalarse, cabría la posibilidad de que la secreción esté modificando la coloración de los rasgos de petición. Si encontráramos evidencias de este acicalamiento, podríamos concluir que el color de la secreción influye en la información que los pollos muestran a los padres en el reparto de cebas (**Objetivo 7**). En relación a este objetivo, predecimos que los padres variarán la tasa de cebas dependiendo de la coloración de los rasgos de petición, pero también de la coloración de la secreción de cada uno de los pollos.

Capítulo VI: En el último capítulo de la tesis estudiamos la relación entre la coloración de la secreción y su comunidad de bacterias, en un contexto de comunicación entre padres e hijos (**Objetivo 8**). Predecimos que la coloración de la secreción la determina, al menos en parte, la comunidad de bacterias de la glándula. Por tanto, asumiendo que los pollos de estornino esparcen su secreción por los rasgos de petición desde edades tempranas en el nido cambiando su coloración, el apoyo a esta predicción implicaría que la comunidad de bacterias estaría mediando en la comunicación entre padres e hijos.

Material y métodos generales

Especies de estudio

Urraca y Críalo (Capítulo I)

En nuestra zona de estudio existe una población bien establecida de urracas (*Pica pica*), que anidan en árboles (encinas, almendros y pinos, principalmente). Esta especie pertenece a la familia Corvidae (Orden: Passeriformes). Es un ave de tamaño medio, monógama, sedentaria y bastante social fuera de la temporada de cría (Martínez & Salvador Milla 2016). El cortejo y construcción del nido en nuestra área de estudio ocurre entre los meses de marzo y abril, mientras que la puesta tiene lugar entre los meses de abril y mayo, poniendo las hembras un huevo al día con una puesta media en nuestra población de 7 huevos (Soler, Martinez & Soler 1996). Parte de la población de urracas de esta zona es frecuentemente parasitada por el críalo. El críalo es un parásito de cría obligado perteneciente a la familia Cuculidae (Orden: Cuculiformes), cuyo principal hospedador en la península es la urraca. Es un ave de tamaño mediano y migradora. Las hembras de esta especie depositan un huevo cada dos días en distintos nidos de urraca con puestas ya comenzadas, pudiendo depositar hasta 15 huevos en un periodo de 6 a 10 semanas (Soler, Salvador Milla & Carrascal 2016). El período de incubación de los críalos es 5-6 días más corto que el de las urracas, y, por tanto, eclosionan antes. De esta manera, cuando el pollo de urraca eclosiona, sus hermanos heteroespecíficos son suficientemente grandes para acaparar la atención de los padres y llevarse la mayoría de las cebas (Soler *et al.* 1995), lo que resulta en la muerte de prácticamente todos los pollos de urraca (Soler, Martinez & Soler 1996).

Otras especies (Capítulos II y III)

Los **Capítulos II** y **III** constan de estudios interespecíficos en los que comparamos varios aspectos de la microbiota uropigial entre especies de aves con características ecológicas distintas, como son aquéllas que anidan en agujeros (especies de nidos cerrados) y las que construyen nidos nuevos cada año (especies de nidos abiertos). Para ello, hemos tratado de muestrear el mayor número de especies posibles y únicamente hemos considerado en los distintos análisis las especies para las que conseguimos un mínimo de 3 individuos adultos o de 3 nidos con pollos (independientemente del número de pollos que hubiera en el nido). Hemos muestreado un total de 26 especies de aves para los estudios interespecíficos, de las

cuales 13 son especies que anidan en agujeros y 13 son especies que anidan en nidos abiertos. Estas especies pertenecen a un total de 6 órdenes y 14 familias (Tabla 1).

Tabla 1. Tamaños de muestra de cada especie, grupo de edad (adulto o pollo) y tipo de muestra, recolectada para los Capítulos II y III.

					Capítulo II	**Capítulo III**	
Orden	**Familia**	**Especie**	**Habito nidificación**	**Edad**	**Piel glándula**	**Piel glándula**	**Secreción glándula**
Bucerotiformes	Upupidae	*Upupa epops*	Cavidad	Adultos	38	41	
				Pollos	67	67	
Columbiformes	Columbidae	*Columba oenas*	Cavidad	Adultos			
				Pollos	12	12	21
		Columba livia	Abierto	Adultos			
				Pollos			6
		Columba palumbus	Abierto	Adultos			
				Pollos	5	6	9
Coraciiformes	Coraciidae	*Coracias garrulus*	Cavidad	Adultos	3	4	7
				Pollos	15	17	17
Passeriformes	Corvidae	*Corvus monedula*	Cavidad	Adultos			
				Pollos	12	12	22
		Pyrrhocorax pyrrhocorax	Cavidad	Adultos			
				Pollos	11	11	23
		Corvus corone	Abierto	Adultos			
				Pollos	5		4
	Paridae	*Parus major*	Cavidad	Adultos	3	3	10
				Pollos	12	12	13
	Passeridae	*Passer domesticus*	Cavidad	Adultos	11	11	10
				Pollos	16	15	33
		Passer montanus	Cavidad	Adultos			4
				Pollos			
		Petronia petronia	Cavidad	Adultos	5	5	10
				Pollos			25
	Sturnidae	*Sturnus unicolor*	Cavidad	Adultos	9	11	
				Pollos	17	17	
	Acrocephalidae	*Acrocephalus scirpaceus*	Abierto	Adultos	7	6	13
				Pollos			
	Fringillidae	*Carduelis carduelis*	Abierto	Adultos		4	
				Pollos			
		Chloris chloris	Abierto	Adultos	3	4	9
				Pollos			
		Serinus serinus	Abierto	Adultos	11	11	13
				Pollos	6	8	10
	Cettiidae	*Cettia cetti*	Abierto	Adultos	4	4	8
				Pollos			
	Muscicapidae	*Luscinia megarhynchos*	Abierto	Adultos			5
				Pollos			
		Muscicapa striata	Abierto	Adultos	4	5	11
				Pollos			
	Sylviidae	*Sylvia atricapilla*	Abierto	Adultos			6
				Pollos			
		Curruca melanocephala	Abierto	Adultos			11
				Pollos			
Piciformes	Picidae	*Picus viridis*	Cavidad	Adultos			
				Pollos	8	9	
Strigiformes	Strigidae	*Athene noctua*	Cavidad	Adultos	6	8	9
				Pollos	14	13	18
		Otus scops	Cavidad	Adultos	9	8	11
				Pollos	16	15	11
		Asio otus	Abierto	Adultos			
				Pollos			3

Estornino negro (Capítulos IV, V y VI)

El estornino negro es un ave de mediano tamaño que pertenece a la familia Sturnidae (Orden: Passeriformes). Es una especie sedentaria y colonial, nidifica en cavidades y presenta dimorfismo sexual, que se hace más patente durante la reproducción. Mientras los machos tienen la base del pico azul en época de reproducción, las hembras lo presentan de un color rosa pálido. Quizás el rasgo más distintivo son las plumas ornamentales de la garganta, más largas y estrechas en machos respecto a las hembras (Veiga & Polo 2011). El cortejo en nuestra población de estudio comienza en los meses de febrero-marzo, y ambos sexos participan en la construcción del nido. Las hembras ponen un huevo al día y el tamaño de puesta común en nuestra zona de estudio es de 4-5 huevos, empezando a incubarlos con la puesta del penúltimo huevo, lo que lleva al último pollo a eclosionar un día más tarde que sus hermanos.

Los pollos de estornino negro presentan unas características y llamativas bocas de color amarillo intenso (Fig. 1A y B), que enseñan a los padres durante la petición de alimento. Este color amarillo intenso, además, coincide con el amarillo de la secreción de estos pollos en la primera mitad de estancia en el nido (Fig. 1B y C)

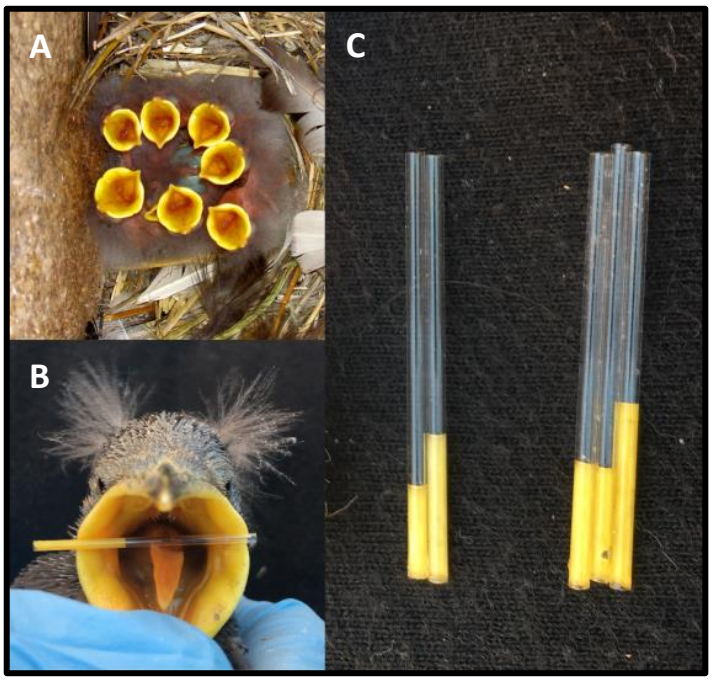

Figura 1. A y B muestran el color de la boca y las boqueras de los pollos de estornino de alrededor de 4 días (A) y de 14 días (B). B muestra la similitud entre las coloraciones de la secreción y de la boca de los pollos de estornino. C muestra el color amarillo de la secreción en los capilares con los que se recolecta. Autores: imagen A, Cristina Ruiz Castellano; imágenes B y C, Juan José soler.

Área de estudio

El trabajo de campo se realizó durante las temporadas de reproducción (meses de marzo a julio) de los años 2018, 2019 y 2021 en dos localizaciones distintas. Generalmente se muestreó en la comarca de la Hoya de Guadix (37°15′N; 3°01′W), en la provincia de Granada. Esta zona forma parte de una meseta de alrededor de 1000 m de altitud con clima semiárido y distintos hábitats como (i) dehesas de encinas (*Quercus ilex* y *Quercus rotundifolia*), (ii) pinares de pino resinero (*Pinus pinaster*) y pino carrasco (*Pinus halepensis*) y (iii) cultivos tanto de secano como de regadío de almendros (*Prunus dulcis*), olivos (*Olea europea*), otros frutales y distintas hortalizas (Fig. 2). Distribuidas por los distintos hábitats hay colocadas varios centenares de cajas nido (Fig. 2) desde hace más de 15 años, con las que cada primavera se monitorean las poblaciones de distintas especies de aves con el fin de realizar diversos estudios de ecología evolutiva. Las cajas nido se encuentran instaladas en troncos de árboles, paredes de construcciones abandonadas, o enterradas entre piedras. Tienen unas dimensiones internas de 180 mm de ancho x 210 mm de profundidad y 350 mm de alto, con una distancia de la base al agujero de la entrada de 240 mm. Los nidos de urraca y de otras especies, como la corneja (*Corvus corone*) o el búho chico (*Asio otus*), se detectaron y monitorearon en el entorno de la Hoya de Guadix. Además, se muestreó también en la Charca de Suárez, un humedal de 13,5 ha que consta de diversas charcas de agua subterránea que se complementan con el aporte del rio Guadalfeo. Este humedal está situado a escasos metros de la costa en la población de Motril, Granada (36° 43' 18.707"N, 3° 32' 30.836"W). El muestreo en estas charcas se realizó utilizando redes de niebla para capturar especies de nidos abiertos.

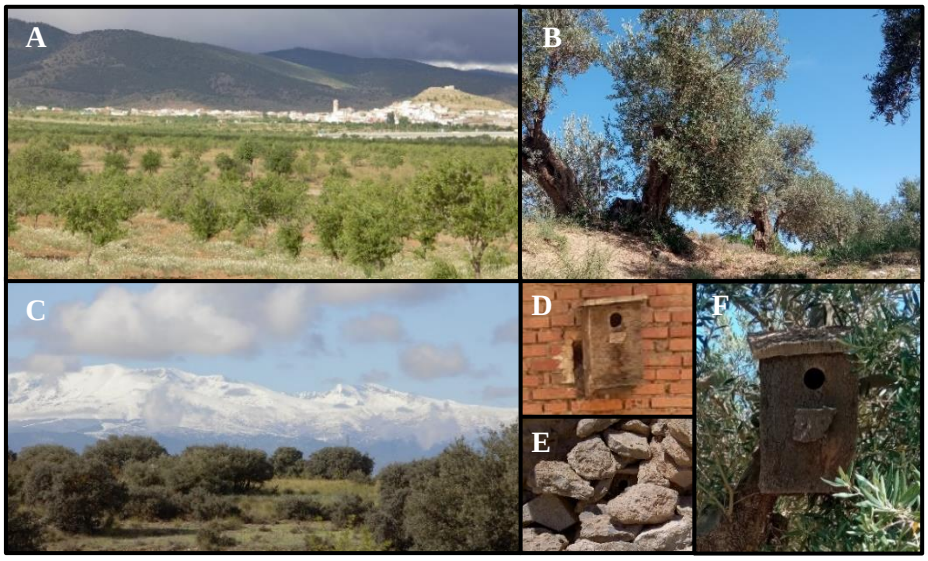

Figura 2. Fotos de los distintos hábitats y detalles de las cajas nido. A. Campo de almendros. B. Campo de olivos. C. Dehesa de encinas. D. Detalle de caja nido instalada en construcción abandonada. E. Caja nido enterrada y F. Detalle de caja nido en un olivo. Imágenes cedidas por: Juan Soler (A y C), María Dolores Barón (B y F), Cristina Ruiz (E), Gustavo Tomás (D).

Seguimiento y diseño experimental

Estudio del ambiente social en urracas y críalos

Desde mitad de marzo de 2018 se realizó una búsqueda exhaustiva de nidos de urraca, intentando encontrarlos con la puesta de los primeros huevos, lo que nos permitió inferir la fecha de puesta y la fecha de eclosión tanto de las urracas como de los críalos. Los pollos de urraca no suelen sobrevivir en los nidos parasitados por críalos, por lo que llevamos a cabo un experimento de intercambio de huevos en el que intercambiamos huevos de críalo entre nidos de urraca, intentando que los críalos eclosionaran el mismo día que las urracas, o uno o dos días más tarde. En total conseguimos 10 nidos de urraca en los que convivieron las dos especies y 15 nidos en los que solo sobrevivió una las especies (11 y 4 nidos en los que había solo urracas o solo críalos respectivamente). De esta manera, maximizamos el número de nidos heteroespecíficos dónde se desarrollaron individuos de las dos especies hasta la fase de abandono del nido. Puesto que ambas especies se desarrollan a ritmos distintos, la toma de muestras para cada especie se realizó a distintas edades. Teniendo en cuenta un desarrollo de plumas similar, se muestreó a los críalos aproximadamente 15 días después de la eclosión, mientras que a las urracas se les muestreó 17 días después de la eclosión. Los adultos de estas especies no se muestrearon.

Estudio interespecífico

A partir de mediados de marzo de los años 2018, 2019 y 2021 se comenzó la revisión de cajas nido, que se repetía cada 7-8 días con el fin de detectar las puestas. Una vez detectadas, se calculó la fecha de puesta y la fecha de eclosión para planificar los muestreos de adultos y pollos. Los adultos (hembras generalmente) se capturaron los últimos días del período de incubación o primeros días de eclosión, cuando se maximiza la probabilidad de captura con un mínimo riesgo de abandono. La captura de las hembras se realizó generalmente bloqueando el agujero de entrada de la caja-nido y capturando los individuos por la parte superior de la caja nido (construida específicamente para facilitar este proceso). Para capturar los machos de las especies que anidan en las cajas nido, se instalaron redes japonesas rodeando el acceso a las cajas. Los pollos de estas especies se capturaron de manera similar a las hembras durante la última etapa de los pollos en el nido. La edad exacta de muestreo de los pollos varió según la especie debido al distinto desarrollo de cada una. Puesto que capturar directamente del nido a los individuos de las especies que anidan en nidos abiertos no resulta tan simple, en mayo de 2019 y 2021, instalamos redes japonesas tanto en la Hoya de Guadix como en la Charca de Suarez para capturar adultos y volantones recién salidos de los nidos.

En todos los casos se tomaron las medidas biométricas y se realizó el muestreo de la microbiota de la piel de la glándula y de la secreción en el momento de la captura.

Estudio de caracteres relacionados con la comunicación padres hijos en el estornino negro

Al igual que en el estudio interespecífico, a partir de mediados de marzo de 2019 se comenzaron las revisiones de las cajas nido que se repitieron una vez cada tres días para detectar nidos durante la puesta que nos permitiera inferir la fecha de puesta del primer huevo y por tanto la fecha de eclosión de los pollos. Posteriormente, los nidos se visitaron diariamente y, el día siguiente de la puesta del último huevo, se midió el color de los huevos con un espectrofotómetro. 12 días después de la puesta del primer huevo, se visitaron los nidos cada día hasta detectar la eclosión. Realizamos dos experimentos, uno de intercambio de huevos y otro de alimento extra y, el día de la eclosión, se asignó de manera aleatoria un tipo de experimento a cada nido. Los pollos de cada nido se marcaron individualmente cortándoles el plumón de distintas partes de la cabeza y alas.

El experimento de intercambio de huevos consistió en intercambiar dos pollos recién nacidos (día 1 = día de la eclosión) entre dos nidos con fechas similares de eclosión y tamaños similares de puesta siempre que fuera posible. Las muestras de secreción y las medidas biométricas y de coloración se tomaron el día 10, mientras que el día 14 se tomaron las muestras de sangre para la medición de carotenos en plasma. En el experimento de alimento extra se le administró, a la mitad de los pollos de la nidada, una dosis dependiente de la edad de Vitamina E (α-Tocoferol) diluido en aceite de maíz, mientras que la otra mitad se trató como control y se le administró aceite de maíz en la misma cantidad. Cada dos días se les suministró la dosis correspondiente hasta el día 12. El día 10 se tomaron las medidas biométricas, las muestras de secreción y las medidas de la coloración, mientras que el día 14 se tomaron las muestras de sangre.

Toma de muestras

Muestreo de la microbiota

- Piel de la glándula

  En el estudio interespecífico, siempre se muestreó primero la piel de la glándula, para evitar contaminaciones durante el manejo. Utilizando guantes desinfectados con alcohol de 70º, se apartaron las plumas que rodean la glándula uropigial y se frotó la piel de los lóbulos de la glándula (Fig. 3A) con un hisopo estéril (APTACA, ref. 2160, Canelli, Italy) humedecido en tampón fosfato (1X PBS, 0,2 M). El hisopo se almacenó

en un microtubo estéril que contenía 1 ml de tampón fosfato. Este microtubo se mantuvo en frío usando neveras portátiles, y, ese mismo día, después de la siembra para la estima de densidad bacteriana (ver más abajo), se almacenó a -18º C hasta su análisis en el laboratorio.

Figura 3. Detalle del muestreo de la comunidad de bacterias de la piel de la glándula uropigial de una abubilla (A) y de la secreción uropigial de un pollo de mochuelo (B).

- Secreción uropigial

  Después del muestreo de la piel de la glándula, se limpió toda la zona de la glándula y su apertura con un algodón humedecido con alcohol de 70º. Acto seguido, se recolectó la secreción colocando un microcapilar estéril (32 mm, 10 µl) en la apertura de la glándula (Fig. 3B), y se masajeó la glándula hasta sacar toda la secreción. Una vez se vació la glándula, el capilar con la secreción se almacenó en un microtubo estéril vacío y se mantuvo en frío hasta llegar al laboratorio donde se almacenó a -18º C hasta su análisis.

Medidas biométricas y muestreo de sangre

Para cada individuo, medimos la longitud del tarso con un calibre digital (precisión de 0,01 mm), la longitud del ala con una regla (precisión de 1 mm) y el peso con una balanza (Pesola de 0-100 con precisión de 1 gr o de 0-300 con precisión de 2 gr según la especie). Para los pollos de estornino se usó una balanza digital con una precisión de 0,01 g, puesto que en estos experimentos los pollos se muestrearon a una edad más temprana que en el resto de especies. Además, medimos el ancho, largo y alto de la glándula uropigial con un calibre digital (precisión de 0,01 mm) siguiendo a Martín-Vivaldi *et al.* (2009) (Fig. 4) y calculamos el volumen de la misma como el producto de las tres medidas.

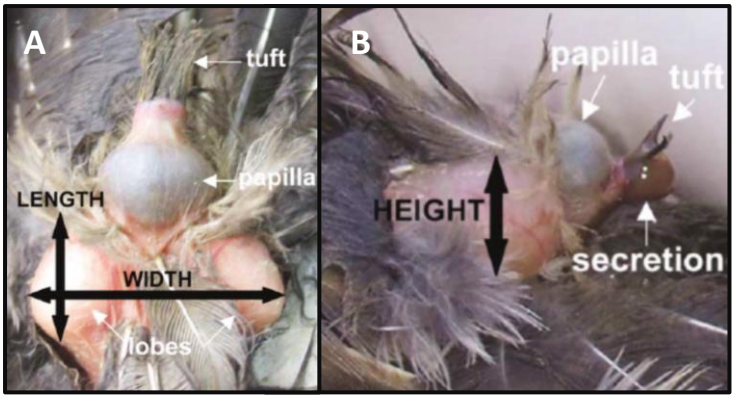

Figure 4. Glándula uropigial de la abubilla. A. Visión dorsal de la glándula uropigial de la abubilla llena de secreción donde se distinguen los lóbulos y la papila. B. Visión lateral de la glándula. Tanto las distintas partes de la glándula y como las medidas que se realizaron están marcadas con flechas. Modificado de Martín-Vivaldi *et al.* (2009)

Las muestras de sangre para la estimación de carotenos en plasma se obtuvieron por punción de la vena braquial con una aguja estéril y colectando la sangre en capilares heparinizados (2-3 capilares por individuo, medidas del capilar: 75 mm^3/75 µl) que se vaciaron en microtubos de 1,5 ml. Estas muestras se conservaron en frío usando neveras portátiles y, el mismo día, se centrifugaron (5 min a 18.000 x g RCF) para separar el plasma de la fracción celular. Este plasma se almacenó a -18º C por un máximo de una semana y luego se almacenó a -80º C hasta el análisis en el laboratorio.

Medidas de color (Capítulos IV-VI)

Todas las estimas de color de los huevos y pollos de estornino negro se realizaron dentro de una bolsa negra en condiciones de oscuridad. Usando el espectrofotómetro Ocean Optics S2000 conectado a una lámpara halógena de deuterio (D2-W) a través de una fibra óptica (QR-400-7-UV-vis), se tomaron medidas de reflectancia en intervalos de 1 nm entre las longitudes de onda de 300 y 700 nm. Antes de la medición de los huevos o pollos de cada nido, se calibró el espectrofotómetro con un blanco (Ocean Optics WS-2) y un negro (ausencia total de luz dentro de la bolsa negra donde se tomaron las medidas) de referencia para estandarizar las medidas. Se tomaron 3 repeticiones de cada medida: superficie de los huevos, boca de los pollos (zona del paladar), boqueras de los pollos (zona ensanchada del labio), piel (zona del pecho sin plumón) y secreción (que se esparció en papel secante y se midió el color de la mancha). Para los análisis se utilizaron valores medios después de comprobar la alta repetibilidad de las medidas.

Grabación de nidos (Capítulo V)

Para el **Capítulo V** se grabó el interior de los nidos de estornino para detectar si los pollos utilizaban la secreción uropigial y para estimar la tasa de cebas recibida por cada pollo del nido. Para ello, entre los días 7 y 11 instalamos una mini-cámara de vídeo en la tapa de la caja (parte superior interna de la caja). Las cámaras se dejaron puestas durante un mínimo de 3 horas, e intentando que abarcara las primeras horas de la mañana donde la tasa de cebas es mayor. Los vídeos se analizaron durante dos horas contando a partir de la primera ceba grabada.

Métodos de laboratorio

Estima de la densidad bacteriana y de las propiedades antimicrobianas

El mismo día de los muestreos, se sembraron las muestras recogidas con hisopos en placas de TSA (Triptic Soy Agar, Scharlau, Barcelona, Spain), un medio generalista en el que crecen bacterias mesófilas. Para ello, se agitó con un vórtex el microtubo con el tampón fosfato y el hisopo, con el fin de suspender las células fijadas al hisopo, y se sembraron 100 µl de la suspensión en placas Petri. Cada muestra se sembró en diluciones seriadas en solución salina hasta llegar a la dilución 10^{-4}. Estas placas se incubaron en la estufa por 24 h a 37º C. Una vez incubadas, se estimó la densidad bacteriana contando las unidades formadoras de colonias (UFC) y se estandarizaron por cm^2 de piel muestreada (superficie de la glándula).

Una vez crecidas las colonias, se aislaron 5 morfológicamente distintas para hacer los ensayos de antagonismo por el método de la doble capa. Para asegurar un cultivo puro, cada aislado se sembró en estrías con el asa de siembra en placas Petri, y se incubaron 24h a 37º C. De ese cultivo puro, se picaron 9 colonias en sendas placas de Petri con medio TSA y se incubó otras 24 h a 37º C. A estas placas se les añadió 7 ml de agar blando BHI (Scharlau Chemie S.A., Barcelona) en sobrefusión (45 º C) e inoculado con 100 µl de cultivo de una de las bacterias indicadoras (incubada una noche a 37º C). Una vez cubiertas, las placas se incubaron a 37º C por 24 h, y los halos de inhibición aparecidos alrededor de las colonias se midieron con una regla (Fig. 5). Las 9 bacterias indicadoras usadas en este ensayo incluyen bacterias conocidas como patógenos en aves: *Bacillus licheniformis* D13, *Enterococcus faecalis* S47, *Escherichia coli* CECT774, *Listeria inocua* CECT340, *Micrococcus luteus* 241, *Mycobacterium* sp., *Pseudomonas putida*, *Salmonella choleraesuis* CECT443, y *Staphylococcus aureus* CECT240.

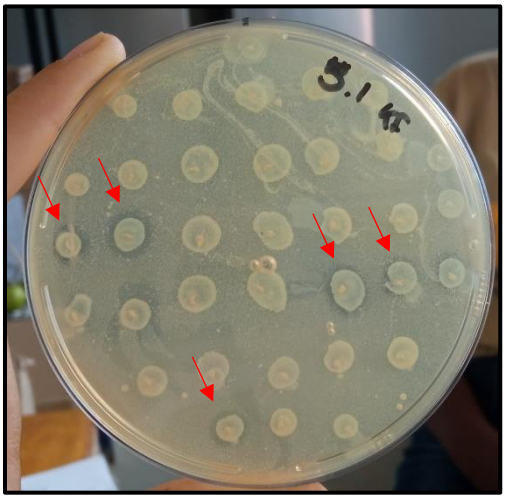

Figura 5. Detalle de una placa de TSA con un ensayo de antagonismo. Los halos de inhibición (marcados con flechas rojas) se aprecian alrededor de algunas de las colonias crecidas. Imagen cedida por María Dolores Barón.

Estima de la concentración de carotenos en plasma

Para medir la concentración de carotenos en plasma se realizó un ensayo midiendo la absorbancia de concentraciones conocidas de luteína (CAYM10010811-1, VWR) con un espectrofotómetro (Sunrise-basic Tecan, 16039400). Para ello, primero se realizaron las curvas patrón (R^2 = 0,999) midiendo la absorbancia a 450 nm de distintas concentraciones de luteína (de 0 a 200 µg x mL^{-1}). Para la estima de la concentración en las muestras se añadieron 135 µl de etanol a 15 µl de plasma previamente descongelado. Esta mezcla se agitó con el vórtex y se centrifugó a 4º C, 1.500 x g RCF y 10 min. Luego se midió la absorbancia de esta mezcla a 450 nm y, utilizando la curva patrón, se extrapolaron los resultados a concentraciones de carotenos en plasma.

Extracción del ADN bacteriano y secuenciación del gen ribosómico 16S

- Piel de la glándula

  La extracción del ADN bacteriano de los hisopos de la piel de la glándula se realizó mediante el uso del kit de extracción FavorPrep™ Blood Genomic DNA Extraction Mini Kit (Favorgen Biotech Crop., Taipei, Taiwan). Con las siguientes modificaciones: las muestras se sonicaron a 120 Hz por dos minutos para separar las células bacterianas del hisopo. Después, se retiró el hisopo y se centrifugó la muestra a 13.000 rpm por 5 min, se descartó el sobrenadante y se le añadió 180 µl de TES (25 mM Tris-HCl, pH 8, 10 mM EDTA y 10% sacarosa), 10 mg/ml de lisozima y 4 µl de

RNasa (10 mg/mL). Con esta mezcla, se siguieron los pasos especificados en el protocolo del kit de extracción.

- Secreción uropigial

  Dada la baja concentración de ADN en las muestras de secreción y la composición lipídica de la misma, la comunidad de bacterias de la secreción se extrajo usando un protocolo de lisis directa. Se siguió el protocolo descrito por Boom *et al.* (2000) con las siguientes modificaciones: primero se añadieron 30 µl de solución de lisis al microtubo que contenía el microcapilar de secreción y se agitó vigorosamente con el vórtex para maximizar la salida de la secreción del capilar. Se incubó 10 minutos a 75º C y se añadieron 30 µl de la solución de neutralización.

Para verificar la presencia de material genómico de las extracciones de ADN de la piel de glándula y de la secreción se utilizaron 5 µl para realizar una PCR de comprobación. Esta PCR se realizó siguiendo el protocolo descrito en Comeau *et al.* (2011) usando los cebadores B969F (ACGCGHNRAACCTTACC) y BA1406R (ACGGGCRGTGWGTRCAA) para la región V6-V8 del gen 16S rRNA. Para visualizar el producto de PCR se realizó una electroforesis en gel de agarosa al 1%. Una vez verificada la presencia de ADN se construyeron las librerías para Illumina con los mismos cebadores y se secuenciaron en lecturas pareadas 2x275 en las instalaciones del Instituto de Parasitología y Biomedicina "López-Neyra" (IPBLN, Granada, Spain).

Procesamiento de datos y análisis estadísticos

Estimas y variables de coloración

El software AVICOL v.6 (Gomez 2006) se usó para procesar las medidas de color y estimar las variables de coloración. Primero se corrigieron los valores negativos de reflectancia que se establecieron en 0, y después se corrigió el ruido usando un suavizado triangular. Con estas medidas corregidas, se estimó el componente acromático del color ((i) el brillo) como la proporción de reflectancia en el intervalo de longitudes de onda 300-700. Como componentes cromáticos se estimaron (ii) el tono como la longitud de onda a la que la reflectancia es máxima, y (iii) la saturación como la proporción de reflectancia para un rango de longitudes de onda determinado. Dado que el espectro de reflectancia del amarillo de la boca, las boqueras y la secreción de los pollos de estornino presenta dos picos claros, uno en la zona del UV (300-400 nm) y otro en la zona del amarillo-rojo (600-700) (Fig. 6), el tono y la saturación se estimaron para estos dos rangos por separado. Además, se estimó el tono total como la longitud de onda del espectro donde la pendiente de la reflectancia era máxima,

el croma de los carotenos como la reflectancia a 700 nm menos la reflectancia a 450 nm (Cuthill 2006; Isaksson *et al.* 2008; Charmantier *et al.* 2017).

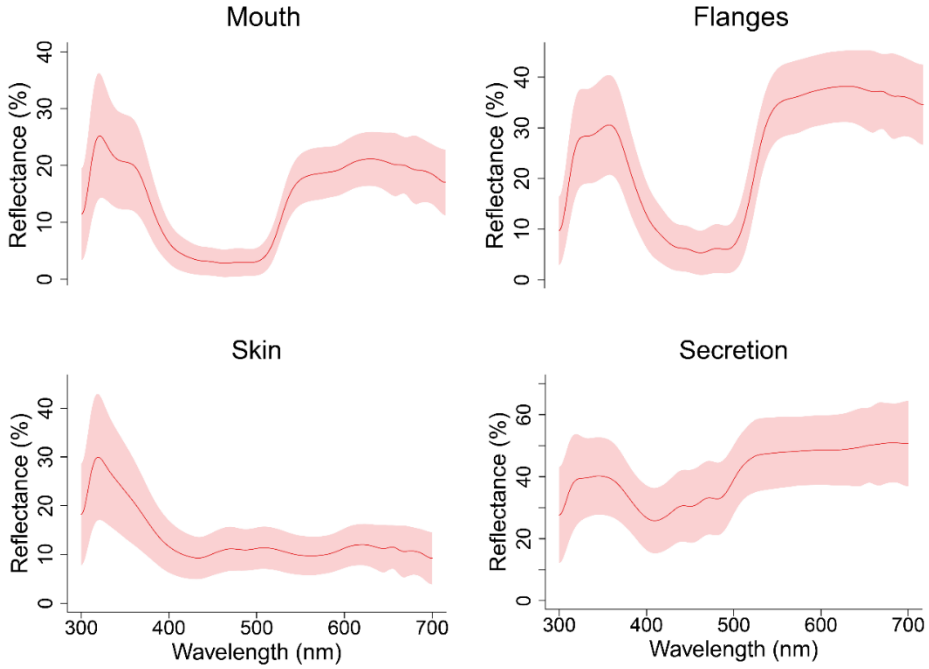

Figura 6. Espectros de reflectancia (±95CI) de la boca, las boqueras, la piel y la secreción de pollos de estornino

Análisis de secuencias

Las secuencias de la comunidad de bacterias se procesaron primeramente con el software QIIME2 (Bolyen *et al.* 2019). Se utilizó el algoritmo DADA2 (Callahan *et al.* 2016) para el truncado de los cebadores y de las zonas con poca calidad de secuenciación (extremo 3' con un índice Phred < 20). Una vez solapadas los pares de lecturas, se realizó la asignación taxonómica utilizando la base de datos Silva 138 (Quast *et al.* 2012) y se eliminaron todas aquéllas que pertenecían a mitocondrias, cloroplastos, hongos o arqueas. También se eliminaron las secuencias que no estaban clasificadas a nivel de filo, así como las muestras que presentaron un número de lecturas inferior al nivel de rarefacción seleccionado en el correspondiente trabajo. Las secuencias resultantes se alinearon y, con ellas, se construyó la tabla de ASVs (Amplicon Sequence Variant). Usando el paquete Decontam en R (Davis *et al.* 2018; R-Core-Team 2020) se identificaron posibles contaminantes comparando las abundancias de los ASV en las muestras respecto a los controles. Se eliminaron de la tabla ASV las secuencias identificadas como contaminantes y se construyó el árbol filogenético usando el algoritmo *align-to-tree-mafft-fasttree* en QIIME2.

Índices de alfa y beta diversidad

La tabla ASV, la información taxonómica y el árbol filogenético se exportaron a R para continuar el análisis usando, entre otros, el paquete "phyloseq" (McMurdie & Holmes 2013). Para calcular los índices de alfa diversidad primeramente se rarificó la tabla de ASVs a la mínima profundidad de secuenciación, es decir, al número mínimo de lecturas detectadas en las muestras. Una vez rarificada la tabla ASV se calcularon el índice de riqueza de Chao1, el índice de diversidad de Shannon y el índice de diversidad filogenética de Faith (PD).

En cuanto a la diversidad beta se calculó de manera distinta en los **Capítulos I** y **III**, y en el **Capítulo VI**. En los **Capítulos I y III** se calcularon las matrices de distancias para la beta diversidad a partir de la tabla ASV rarificada. Los índices usados para calcular las distancias fueron Bray-Curtis, Jaccard y UniFrac, tanto ponderado como sin ponderar. Bray-Curtis y Jaccard no tienen en cuenta la filogenia de las bacterias, y se diferencian entre sí porque Jaccard únicamente tiene en cuenta la presencia/ausencia de un ASV (y por tanto equipara las bacterias más y menos abundantes a simplemente "presentes"), mientras que Bray-Curtis tiene en cuenta la presencia de cada ASV (y por tanto da más peso e importancia a las bacterias más abundantes). Los índices UniFrac son similares a estos, pero sí tienen en cuenta la filogenia de las bacterias. Mientras UniFrac ponderado también da importancia a la abundancia, UniFrac no ponderado solo cuenta la presencia de los ASVs.

En los últimos años, se está haciendo mucho hincapié en la importancia de considerar la naturaleza composicional para la que la transformación ideal se basa en transformaciones logarítmicas de las abundancias de las tablas ASVs (sin rarificar) centradas (Gloor *et al.* 2017). Para el **Capítulo VI** decidimos seguir esta nueva metodología y, para ello, realizamos las transformaciones CLR (Center-Log Ratio) y PhILR (Phylogenetic Isometric Log-Ratio). Ésta última tiene en cuenta la filogenia de las bacterias. Una vez transformadas las tablas, se calcularon las distancias Euclideas (que aplicadas a la matriz CLR se denominan distancias *Aitchison*) con las que se construyeron las matrices de distancia.

Tanto los índices de alfa como de beta diversidad se han calculado en el entorno de R Studio con la versión de R v4.0.2 (R-Core-Team 2020).

Modelos estadísticos usados

- **ANOVAs mixtas**: Estos modelos se caracterizan por incluir factores fijos y aleatorios como variables explicativas. Dependiendo de la estructura de los datos, los modelos incluyen interacciones entre factores fijos y aleatorios o anidamientos entre ellos. A

lo largo de la tesis se pueden encontrar con los nombres General Lineal Models (GLMs), hierarchized ANOVAs, o mixed model ANOVAs. Se han usado en todos los capítulos para explorar el efecto de diversas variables (edad, especie, tipo de nido, tratamiento, etc.) controlado por otros factores de interés y sus interacciones. Generalmente, el factor que se ha incluido como aleatorio es la identidad del nido, para controlar la no-independencia de los datos de los pollos de un mismo nido. Estos análisis se han llevado a cabo con el software TIBCO Statistica™ (versión 14).

- **PERMANOVAs**: Estos modelos se utilizan para explorar el efecto de uno o más factores (variables independientes) en una matriz de distancias (variable dependiente). Por ello, se han utilizado para ver la influencia de factores como la edad, la especie, el tipo de nido, el tratamiento etc., en las matrices de distancias de la beta diversidad. Estos análisis se han usado en los **Capítulos I, III y VI** para la beta diversidad de la microbiota, y en el **Capítulo II** para el perfil de antagonismo. Estos modelos se han llevado a cabo con el software PRIMER v7.0.17 (PRIMER-e).
- **Mantel test**: Estos modelos se utilizan para explorar las asociaciones entre matrices de distancias. En la tesis hemos utilizado estos modelos en el **Capítulo III** para explorar la relación entre la comunidad de bacterias y la filogenia de las aves (filosimbiosis), y para analizar la relación entre la comunidad de bacterias de la piel de la glándula uropigial y la de su secreción. Además, en el **Capítulo VI** se han usado para ver la relación entre matrices de distancias construidas a partir de las variables de color y la comunidad de bacterias. Estos modelos se han llevado a cabo en el entorno de R v4.0.2.
- **MCMCglmm:** Son modelos generales lineales mixtos que usan técnicas de Monte Carlo basadas en cadenas Markov desde un enfoque bayesiano. Estos modelos permiten incluir una matriz de distancias como factor aleatorio y, por tanto, cuando son distancias filogenéticas, permiten controlar los análisis por los efectos de la filogenia. Se han usado en los estudios interespecíficos (**Capítulos II** y **III)** para controlar el efecto de las relaciones filogenéticas entre las distintas especies de aves en la alfa diversidad de las propiedades antagónicas (**Capítulo II**) y de las comunidades de bacterias (**Capítulo III**). En el caso del **Capítulo II**, se descargaron 100 árboles de birdtree.org (Jetz *et al.* 2012) y se construyó un árbol consenso que se usó para construir la matriz de distancias usadas en los análisis. Para el **Capítulo III** se construyó el árbol filogenético con información de 3 genes mitocondriales y usando análisis bayesianos con el algoritmo MCMC. Los modelos MCMCglmm

están basados en estadística bayesiana y, por tanto, se requiere incluir información de unos parámetros que, basados en conocimiento previo, delimitan la probabilidad de un evento (*priors*). En ningún caso teníamos información previa que nos permitiera delimitar la probabilidad y, por tanto, en todos los casos los modelos se ajustaron con un *prior* no informativo. Estos análisis se han llevado a cabo en R v4.0.2 (R-Core-Team 2020).

- **sPLS (sparse Partial Least Squares)**. El análisis sPLS se encuadra dentro de los métodos de selección de modelos óptimos explicativos que tienen en cuenta la multicolinealidad de las variables. Estos modelos ajustan una regresión linear entre dos conjuntos de datos (p.ej. abundancia de taxones bacterianos y distintas variables de coloración) de grandes dimensiones en los que interesa estudiar su asociación y poner de manifiesto los componentes de cada set de datos responsables de la misma. En contraste con el PLS (partial least square), el sPLS es capaz de realizar la selección de variables utilizado simultáneamente cada conjunto de datos como dependiente o independiente, maximizando la covarianza entre variables (latentes) con las que se conforman los componentes latentes. En esta tesis, este tipo de análisis, se ha usado en el **Capítulo VI** para extraer géneros de bacterias (matriz independiente), relacionadas con variables de color (matriz dependiente). Los análisis se han llevado a cabo en R v4.0.2 (R-Core-Team 2020) y, para visualizar la red resultante de estos análisis se ha usado el paquete "igraph" v1.3.4 (Csardi & Nepusz 2006).
- **Selección del mejor modelo AIC**: Se ha utilizado el criterio de Akaike (AIC) para la selección de modelos tanto en el **Capítulo V**, donde se explora la asociación entre la coloración y la concentración de carotenos en plasma, y en el **Capítulo VI**, para explorar la relación entre géneros específicos de bacterias y coloración. Estos análisis se han realizado con el software TIBCO Statistica™ (versión 14).
- **ANOVAs de medidas repetidas**: Estos modelos permiten explorar posibles factores que explican las diferencias entre dos medidas tomadas a un mismo objeto. Aunque esos efectos también se pueden modelar en modelos mixtos, hemos preferido usar modelos de medidas repetidas por ser más simples y fáciles de interpretar. Se han usado en el **Capítulo VI** para explorar las diferencias en la alfa diversidad de las comunidades bacterianas de los pollos de estornino muestreadas a distintas edades (10 y 14 días después de la eclosión) en los mismos individuos. Estos análisis se han realizado con el software TIBCO Statistica™ (versión 14).

- **Análisis de abundancia diferencial de bacterias**: Con estos análisis se ha tratado de encontrar géneros de bacterias que presentan abundancias significativamente distintas entre grupos. Se han usado en el **Capítulo I** para explorar los géneros de bacterias que son más o menos abundantes según el ambiente social en el que se han desarrollado los individuos muestreados. Estos análisis se han llevado a cabo con R v4.0.2 (R-Core-Team 2020) utilizando el paquete "microeco" (Liu *et al.* 2021).
- **Test multivariante de homogeneidad de varianzas (betadisper)**: Estos análisis exploran la diferencia entre las distancias medias al centroide entre los distintos niveles de un factor. Se han usado en los **Capítulos I** y **III** para poner de manifiesto posibles diferencias en la dispersión de la comunidad microbiana entre los distintos niveles del ambiente social (**Capítulo I**) y del tipo de nido utilizado por las distintas especies (**Capítulo III**). Estos análisis se han realizado en el entorno de R v4.0.2 (R-Core-Team 2020) utilizando el paquete "vegan" (Oksanen *et al.* 2022).

Capítulos

Parte 1. Caracterización de la microbiota de la glándula uropigial y su función como defensa contra infecciones patógenas

I. **The social environment influences the microbiota and pathogenic bacterial communities on the skin of developing birds.** Martínez-Renau E, Martín-Platero AM, Bodawatta KH, Martín-Vivaldi M, Martínez-Bueno M, Poulsen M, Soler JJ. *Animal Microbiome* (submitted).

II. **Microbial infection risk predicts antimicrobial potential of avian symbionts.** Martínez-Renau E, Mazorra-Alonso M, Ruiz-Castellano C, Martín-Vivaldi M, Martín-Platero AM, Barón MD, Soler JJ. *Frontiers in Microbiology* (2022). 13:1010961. doi: https://doi.org/10.3389/fmicb.2022.1010961

III. **Pathogen risks of nesting wild birds drives their skin and uropygial microbiome** Martínez-Renau E, Bodawatta KH, Martín-Platero AM, Martín-Vivaldi M, Barón MD, Ruiz-Castellano C, Martínez-Bueno M, Jønsson KA, Michael P, Soler JJ.

Parte 2. Relación de la comunidad de bacterias y la coloración de la secreción uropigial del estornino negro, y el papel de ambos en la comunicación entre padres e hijos.

IV. **Coloration of spotless starling nestlings shows genetic and environmentally determined characteristics while begging for food.** Martínez-Renau E, Ruiz-Castellano C, Azcárate-García M, Barón MD, Soler JJ. *Functional Ecology* (2020). 35 (2), 499-510. doi**:** https://doi.org/10.1111/1365-2435.13711

V. **Made-up mouths with preen oil reveal genetic and phenotypic conditions of starling nestlings.** Soler JJ, Martínez-Renau E, Azcárate-García M, Ruiz-Castellano C, Martín J, Martín-Vivaldi M. *Behavioral Ecology* (2021). 33 (3), 494-503. doi: https://doi.org/10.1093/beheco/arac024

VI. **Colouration of begging related traits in spotless starling *Sturnus unicolor* nestlings: a possible role of bacteria in parent-offspring communication.** Martínez-Renau E, Martín-Platero AM, Barón MD, García-Núñez AJ, Martín-Vivaldi M, Martínez-Bueno M, Ruiz-Castellano C, Tomás G, Soler JJ

CAPÍTULO I

The social environment influences the microbiota and pathogenic bacterial communities on the skin of developing birds

Abstract

Animal bacterial symbionts are established early in life, either through vertical transmission and/or by horizontal transmission from both the physical (i.e., nests) and the social (physical contact with con- or hetero-specifics) environment. The social environment can influence the acquisition of mutualistic and pathogenic bacteria, with consequences on the stability of symbiotic communities. However, segregating the effects of the shared physical environment from those of the social interactions is challenging, limiting our current knowledge on the role of the social environment in structuring bacterial communities in wild animals. Here, we take advantage of the avian brood-parasite system of Eurasian magpies (*Pica pica*) and great spotted cuckoos (*Clamator glandarius*) to explore how the interspecific social environment (magpie nestlings developing with or without hetero-specifics) affects bacterial communities on uropygial gland skin. We demonstrated interspecific differences in bacterial community compositions in members of the two species when growing up in monospecific nests. However, the bacterial community of magpies in hetero-specific nests was richer, more diverse, and more similar to their cuckoo nest-mates than when growing up in monospecific nests. These patterns were alike for the subset of microbes that could be considered core, but when looking at the subset of potentially pathogenic bacterial genera, we detected a negative influence of the presence of cuckoos on the relative abundance of these genera on magpies. Our findings suggest that social interactions during early life shapes the transmission and assembly of bacterial communities, but that their magnitude depends on the life history strategy of the host.

Keywords: *avian skin microbiome, bacterial community, brood parasitism, social transmission, Eurasian magpie, great spotted cuckoo*

Introduction

Animal hosts maintain diverse and complex microbial communities in both internal and external body regions. These symbiotic microbiotas play important roles related to host evolution and ecology (McFall-Ngai *et al.* 2013; Colston & Jackson 2016; Parfrey, Moreau & Russell 2018; Bodawatta *et al.* 2022) through facilitating a myriad of essential functions related to development (Bates *et al.* 2006; Heijtz *et al.* 2011; Schwarzer *et al.* 2016), nutrition (Backhed *et al.* 2004; Krajmalnik-Brown *et al.* 2012), immunity (Umesaki *et al.* 1995; Riley & Wertz 2002; Weiss, Maltz & Aksoy 2012; Mazorra-Alonso, Tomás & Soler 2021), and even chemical communication (Ezenwa & Williams 2014; Maraci, Engel & Caspers 2018; Mazorra-Alonso, Tomás & Soler 2021). The microbiota associated with animals also includes potential pathogens that may infect hosts and/or alter community compositions, both of which carry potential negative consequences for host health and fitness (Stephens & Murray 2001; Mitchell 2011; Aujoulat *et al.* 2012). Assemblies of these microbial communities can be influenced by a plethora of evolutionary and ecological factors, such as host phylogeny (Ruiz-Rodríguez *et al.* 2018; Knowles, Eccles & Baltrūnaitė 2019; Lee *et al.* 2020), diet (Ley *et al.* 2008; Singh *et al.* 2017; Bodawatta *et al.* 2021), environment and geography (Roggenbuck *et al.* 2014; Avena *et al.* 2016; Assis *et al.* 2020), and social interactions (i.e., direct physical contact with conspecifics) (Proudfoot, Weary & von Keyserlingk 2012; Tung *et al.* 2015; Grieneisen *et al.* 2017; Perofsky *et al.* 2017; Dowd & Renson 2018; Dill-McFarland *et al.* 2019). However, the impact of these factors differs by animal hosts and depends on whether the microbial communities are internal (i.e., gut) or external (i.e., skin) (Valles-Colomer *et al.* 2023). Assemblies of external microbiota, such as on the skin, feathers, or hair, are particularly vulnerable to colonization by microorganisms from the environment or from con- or hetero-specifics that focal individuals interact with (social transmission).

The social environment (i.e., environmental characteristics of interacting individuals) has been suggested to promote similarities in bacterial communities between interacting animals (Tung *et al.* 2015; Grieneisen *et al.* 2017; Perofsky *et al.* 2017; Dill-McFarland *et al.* 2019). Thus, it should be important in driving similarities in microbiome-derived physiological and behavioural characteristics of hosts, as well as explaining susceptibility to parasitism (McFall-Ngai *et al.* 2013; Sherwin *et al.* 2019). Most evidence for effects of the social environment comes from experimental approaches in a few captive animal models, or from correlational studies in gut [in humans (Dill-McFarland *et al.* 2019); non-human primates (Tung *et al.* 2015; Amato *et al.* 2017; Perofsky *et al.* 2017; Dowd & Renson 2018;

Raulo *et al.* 2018; Sarkar *et al.* 2020); other mammals (Antwis *et al.* 2018; Raulo *et al.* 2021); birds (Kulkarni & Heeb 2007; Maraci *et al.* 2022); and arthropods (Kwong & Moran 2016; Onchuru *et al.* 2018)], but also in skin [in humans (Song *et al.* 2013; Lax *et al.* 2014; Ross, Doxey & Neufeld 2017); amphibians (Xu *et al.* 2020); and birds (Whittaker *et al.* 2016; Engel *et al.* 2020)] microbiomes. Despite evidence supporting associations between social interactions and the microbiota, we are just starting to understand how social interactions structure microbial communities. This is in part because of the confounding effects of sharing environments without interactions and the role of host genetics (Dowd & Renson 2018). Within social groups, individuals are likely to share early life environmental conditions, physiological stress, similar resources, diet, and/or genetic relatedness (family groups). Similarities in microbial communities among individuals would be predicted in such cases even in the absence of social interactions. Thus, to understand the role of social interactions, it is essential to disentangle these effects, and studying non-genetically related individuals interacting with con- or hetero-specifics in identical environmental conditions may serve to do so.

In the present study, we take advantage of the brood parasite – host system formed by great spotted cuckoos (*Clamator glandarius*) (hereafter cuckoos) and Eurasian magpies (*Pica pica*) (hereafter magpies). Cuckoos are obligated brood parasites that in Europe mainly lay their eggs in magpie nests, where magpie adults incubate the eggs and take care of the cuckoo chicks during the nestling and fledgling periods (Soler & Soler 2000). Adult cuckoos thus do not have contacts with their own nestlings, restricting microbial transfers from parents to offspring to the pre-laying phase. When the eggs hatch, and parasitic nestlings do not outcompete host nestlings, host adults rear their own nestlings along with parasite nestlings (Soler & Soler 2000). In these cases, the skin and feathers of parasitic and host nestlings are in close contact, which allows exploring similarities in bacterial communities of natural and foster siblings that cannot be explained by relatedness. During the nesting phase, nestlings of both species share similar environmental conditions, including those related to parental care. However, magpie nestlings that develop together with cuckoos differ in the social environment from those that grow in monospecific nests and, thus, consistent differences in the microbiota of host nestlings that do or do not share nests with cuckoos can be interpreted as the results of social interactions with hetero-specifics.

Capitalizing on this natural system, we conducted a cross-fostering experiment, where we manipulated the hetero-specific social environments to disentangle the effect of physical and social environments on assemblies of uropygial gland skin microbiomes (Fig. 1). The

cross-fostering approach allowed us to avoid possible biases due to cuckoos choosing nests of particular environmental characteristics (Soler *et al.* 1995; Soler *et al.* 2014) and to maximize the number of magpie nests with individuals of both species. Our experimental design allows testing effects of exposing individuals of the same social group (here members of the same species: magpies) to members of a different social group (cuckoos) in the same environment (magpie nests and parents). We characterized the bacterial community of the uropygial gland skin of magpies and cuckoos developing in con- or hetero-specific broods via amplicon sequencing of the bacterial 16S rRNA gene. We focused on the uropygial gland skin, as this gland produces a secretion that birds spread onto their feathers and skin while preening (Jacob & Ziswiler 1982), and from which several bacterial strains have been isolated (Law-Brown & Meyers 2003; Soler *et al.* 2008; Braun *et al.* 2016; Whittaker & Theis 2016; Braun *et al.* 2018a; Braun *et al.* 2018b; Braun *et al.* 2018c; Bodawatta *et al.* 2020; Videvall *et al.* 2021). The uropygial gland secretion has a species-specific chemical composition (Jacob & Ziswiler 1982) which, in contact with skin and other body parts, may act as a substrate that improves the establishment and growth of species-specific bacterial communities (Javůrková *et al.* 2019; Martínez-Renau *et al.* 2022). First, we hypothesised that the skin microbiota of chicks from magpie nests with only magpies or cuckoos growing (monospecific nests) would vary interspecifically, because of the impact of host intrinsic characteristics on structuring the skin microbiota (Fig. 1A). Secondly, assuming transmission of microbes between nest-mates in a shared social and physical environment, we expected that chicks growing in hetero-specific nests would show reduced interspecific differences (Fig. 1B). Third, given the expected effects of social transmission, we hypothesised that microbial communities would differ between magpie chicks that did or did not grow up with cuckoos (Fig. 1C). We explored these predictions in the skin bacterial community as a whole, but also in subsets of the core microbiota and of potential pathogens.

Methods

Study area and fieldwork

Fieldwork was performed during the breeding season of 2018 in the Hoya de Guadix (37°15′N; 3°01′W), a semiarid high-altitude plateau in southern Spain, where a magpie population frequently parasitized by cuckoos is established (Soler *et al.* 2001; Soler *et al.* 2013). The vegetation is sparse, with disperse holm-oak trees (*Quercus rotundifolia*), grove

of almond trees (*Prunus dulcis*) and pines (*Pinus halepensis*), where magpies usually build their nests.

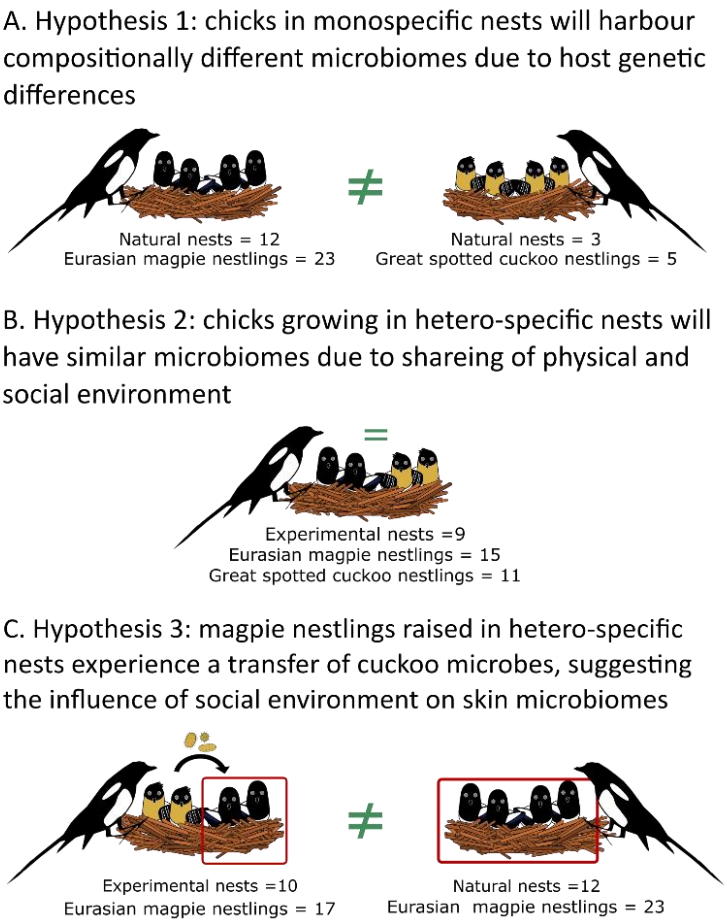

Figure 1. Experimental approach scheme, including the types of magpie nests in the study as well as sample sizes of magpies and cuckoos in each type of nests. The predicted similarities (=) and differences (≠) in microbial composition between nestlings are indicated with green labels.

Since mid-March, we intensively searched for magpie nests, which allowed us to infer the start of laying (hereafter laying date) and, thus, the expected hatching dates of cuckoo and magpie eggs. Cuckoos might choose to parasitize host nests with characteristics that maximize their reproductive success (Soler *et al.* 1995), which might result in biased samples when exclusively using natural parasitized nests, and, thus, highlight the importance of adopting an experimental approach. Moreover, magpie nestlings usually starve in naturally parasitic nests because cuckoo eggs hatch four or more days ahead of those of magpie, which are outcompeted by older cuckoo chicks (Martín-Gálvez & Soler 2017). To avoid possible bias of naturally parasitized nests, and to maximize the probability that magpies and cuckoo nestlings develop together in the same magpie nests, we cross-fostered cuckoo eggs between

magpie nests whenever possible, synchronizing their expected hatching date of cuckoo eggs to the same or one or two days later than that of the magpie eggs. This approach allowed us to maximize the number of hetero-specific nests where cuckoo and magpie nestlings developed together (see Lee *et al.* 2020 for a similar experimental approach). Magpie monospecific nests were simply non-parasitized nests, whereas cuckoo monospecific nests were parasitized magpie nests where natural death of magpie nestlings occurred (Fig. 1). Cuckoo and magpie nestlings develop at different rates (Soler & Soler 1991) and, thus, they were sampled at different age. We collected bacterial samples from 56 nestlings when cuckoo and magpies had similar feather development, approximately 15 and 17 days old respectively. Briefly, we sampled skin microbiota of nestlings by rubbing the surface skin of the uropygial gland, including the opening, with a sterile cotton swab wetted in sterile Phosphate Buffer Saline (PBS, 0.2 M). The swab with the bacterial sample was kept in a sterile microfuge vial with 1 mL of sterile PBS and stored at -18 °C until DNA extraction. At the time of sampling, we also measured tarsus length with a digital calliper (accuracy 0.01 mm), wing length with a ruler (accuracy 1 mm), body mass with a digital scale (accuracy 0.01 g), and gland dimensions (length, width and height) with a digital calliper (accuracy 0.01 mm) as described previously (Martín-Vivaldi *et al.* 2009).

DNA extraction and MiSeq amplicon sequencing

DNA from the sampled bacterial communities was extracted using the FavorPrep™ Blood Genomic DNA Extraction Mini Kit (Favorgen Biotech Crop., Taipei, Taiwan), with the following protocol: first, we sonicated the sample for 2 minutes at 120 Hz trying to release the bacterial cells from the swab. We then removed the swab and centrifuged the PBS with the bacteria at 13,000 rpm for 5 min. Afterwards, we discarded the supernatant, and added 180 µl of TES (25 mM Tris-HCl, pH 8, 10 mM EDTA and 10% sucrose), 10 mg/ml of lysozyme and 10 mg/ml of RNasa to the pellet. Subsequent steps were performed following the FavorPrep™ protocol. Successful DNA extraction was verified with 1% agarose gel electrophoresis.

Libraries for Illumina sequencing were constructed with the bacterial V6-V8 region of the 16S rRNA by the primers B969F (ACGCGHNRAACCTTACC) and BA1406R (ACGGGCRGTGWGTRCAA) (Comeau *et al.* 2011). Library construction and sequencing in the MiSeq (Illumina) platform was carried out at the Institute of Parasitology and Biomedicine "López-Neyra" facilities (IPBLN, Granada, Spain).

MiSeq sequencing analyses

We first processed the amplicon sequences in QIIME2 v2020.6 (Bolyen *et al.* 2019), using default parameters unless stated otherwise. Primer trimming and sequence quality filtering were performed using DADA2 (Callahan *et al.* 2016), and all sequences were clustered into ASVs (Amplicon Sequence Variants) and assigned to taxonomy using the Silva 138 database (Quast *et al.* 2012). Due to the primers' specificity for Bacteria, non-bacterial sequences, and sequences identified as mitochondrial or chloroplast, were removed from the ASV table. Contaminant sequences were identified from field (open swabs without sample) and laboratory (extraction and sequencing blanks) negative controls with the "Decontam" package in R (Davis *et al.* 2018; R-Core-Team 2020) using the prevalence method and a threshold of 0.4. Only samples over 5,000 reads were considered for further analysis. Sequences were aligned and a rooted bacterial phylogeny was generated using the method *align-to-tree-mafft-fasttree* in QIIME2. The ASV table was rarefied to the minimum sampling depth (14,877 sequences) using the method *rarefy_even_depth* in the "phyloseq" package (McMurdie & Holmes 2013).

We also identified potential avian pathogens and the core microbiome for each species among the detected ASVs. For the potential avian pathogens, we first ran the FAPROTAX script in python (Louca, Parfrey & Doebeli 2016), which converts prokaryotic abundance tables (ASV tables) into putative functional abundance profiles. The ASVs considered by FAPROTAX as animal pathogens were searched in the literature to certify avian pathogenicity. Besides, we also used the Pathogen Host Interaction database (PHI-base) (Urban *et al.* 2020) and the review published by Benskin et al. in 2009 (Benskin *et al.* 2009) to search for genera that includes potential known pathogenic bacteria of birds. We use those datasets to build a new ASV table that included potential pathogenic ASVs belonging to genus with available information (Additional table 1). We also calculated the core microbiome using a relative abundance of 0.0001% in at least 50% of the samples in the "phylosmith" package (Smith 2019) in R v4.0.2 (R-Core-Team 2020). We did so separately for each species and type of social environment considered (i.e., only magpies, only cuckoos, magpies that grew with cuckoos and cuckoos that developed together with magpies). Then, we created a subset of the ASV table pruning out taxa that did not belong to the core microbiome for each species.

Statistical analyses

Alpha diversity indexes and beta diversity distance matrixes were calculated in R v4.0.2 (R-Core-Team 2020). Alpha diversity was calculated as Chao1 index and Shannon index using "microbiome" package (Lahti & Shetty 2017), while Faith's phylogenetic diversity (PD) was computed using "picante" package (Kembel *et al.* 2010). Beta diversity matrices were calculated using Bray-Curtis, Jaccard, weighted UniFrac and unweighted UniFrac distances, and PCoA plots were generated with Bray-Curtis distances and visualized using "phyloseq" package (McMurdie & Holmes 2013).

Factors expected to influence alpha and beta diversity indexes were respectively explored in mixed model ANOVAs and PERMANOVAs. The effects of species identity (hereafter, ID) were explored with information from nests where only cuckoo or only magpie nestlings developed (monospecific nests). The models included species ID as fixed factor and the nest ID (nested within, species ID) as the random factor. The effects of species ID were also explored in nests where magpie and cuckoo nestlings develop together (hetero-specific nests), but in this case, the statistical model included specie ID as the fixed factor and nest ID and the interaction of nest ID with species ID as random factors. The effect of social environment was analysed by comparing magpie nestlings that grew in monospecific nests with those that develop together with cuckoos in hetero-specific nests. These models included the social environment (mono- or hetero-specific magpie nests) as the fixed factor and nest ID (nested within social environment) as the random factor. Brood size did not significantly explain alpha diversity indexes (Additional table 2) and, thus, was not included as covariable in the statistical models.

We tested which bacterial genera had significant differential abundances among the four types of social environment. We did this by using the *trans_diff* function from "microeco" package (Liu *et al.* 2021) in R v4.0.2 (R-Core-Team 2020) with the Wilcoxon Rank Sum method and False Discovery Rate (FDR) adjusted p-values. We conducted differential abundance analyses with the whole ASV table. Finally, we used *betadisper* function in the "vegan" package (Oksanen *et al.* 2022) using spatial median and adjusted biases to analyse the homogeneity of variances among magpies sharing and not sharing nests with cuckoos. The effects of species ID and social environment on alpha and beta diversities of subsets that included potentially pathogenic bacteria, or the core microbiome, were explored in statistical models identical to those described above. ANOVAs were conducted

in STATISTICA v.12 (Statsoft 2015), while PERMANOVAs were performed with Primer7 v.7.0.17 (PRIMER-e).

Results

We successfully sequenced 56 nestling samples (40 magpies and 16 cuckoos), from which we obtained 1,950,249 sequences classified into 7,825 ASVs that belonged to 21 bacterial phyla. Before rarefaction, each sample had an average of 34,825.88 (SD ± 10,692.12) sequences. Rarefaction was set to the minimum sample depth of the dataset (14,877 sequences) and led to 7,758 ASVs (Additional table 3). The whole data set was dominated by Firmicutes (40.1%), Proteobacteria (22.7%), Actinobacteria (18%) and Bacteroidetes (14.2%). Firmicutes dominated in both species, but there were species-specific differences despite the high individual variation. In cuckoos, Bacteroidetes was the second most abundant phylum, followed by Proteobacteria and Actinobacteria, while Proteobacteria was the second most common phylum in magpies, followed by Actinobacteria and Bacteroidetes (Fig. 2A). Moreover, although some bacterial groups appeared in both cuckoo and magpie samples (Fig. 2A), the most abundant genera differed between bird species. *Clostridium* (4.8%), *Enterococcus* (3.8%), *Acinetobacter* (3.3%), and *Pseudomonas* (3.2%) were the most abundant genera in magpie samples, while *Bacteroides* (12%), *Clostridium* (7.2%), *Parabacteroides* (6.4%), and *Lachnoclostridium* (4.6%) were the most abundant bacteria in cuckoo samples (Fig. 2A; Additional table 4).

Microbial diversity

Considering the whole set of ASVs, alpha diversity indexes of magpie and cuckoo samples did not differ significantly (Table 1). That was the case independently of comparing samples from mono- or hetero-specific nests (Table 1). However, alpha diversity of magpie samples from hetero-specific nests was significantly higher than that of magpie samples from monospecific nests (Table 1, Fig. 2B). Interestingly, magpies growing up in hetero-specific nests shared more ASVs with cuckoos than with magpies or cuckoos from monospecific nests (Fig. 2C).

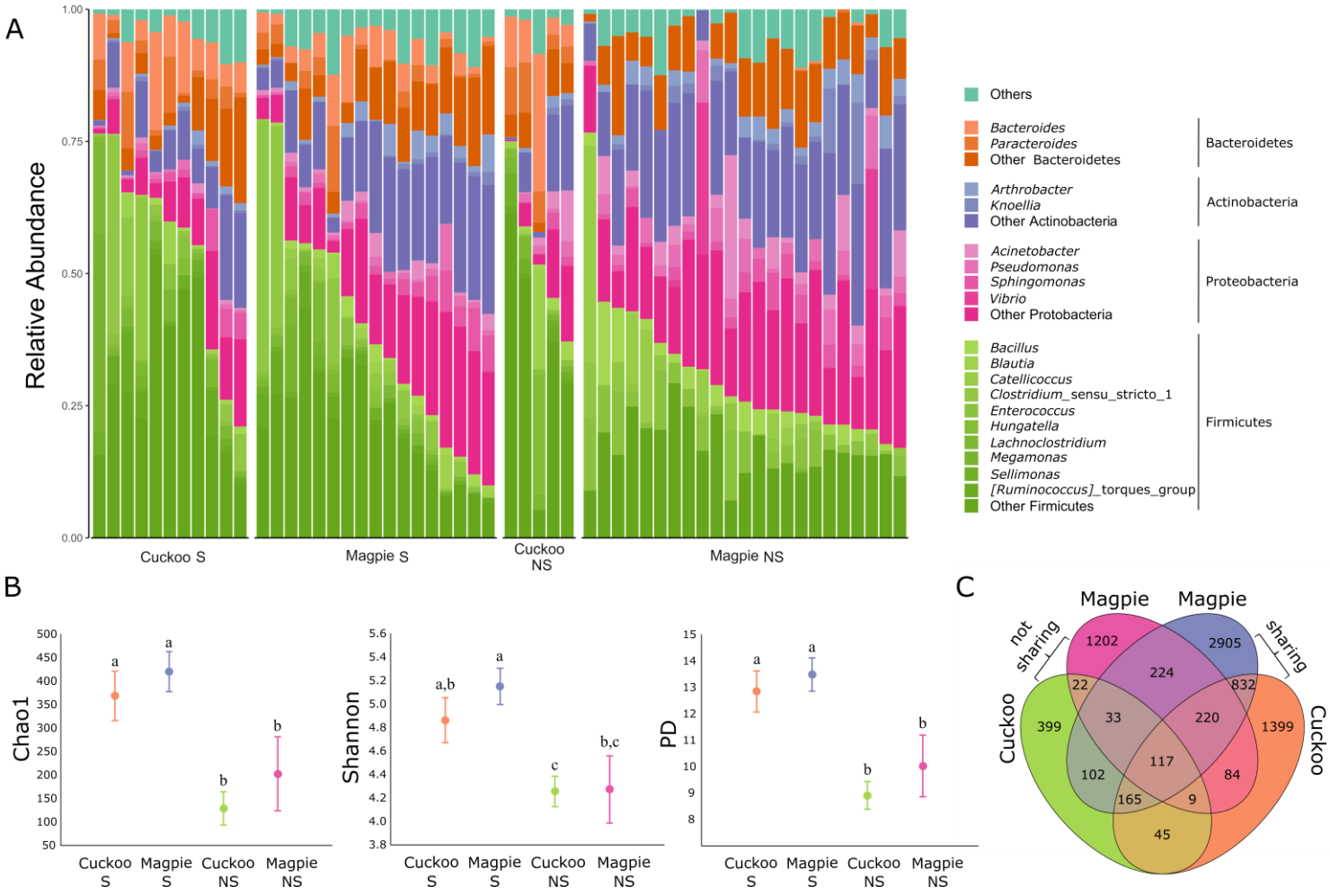

Figure 2. (A) Microbial composition at the phylum and genus levels of uropygial gland skin of great spotted cuckoos (*Clamator glandarius*) and Eurasian magpies (*Pica pica*) from monospecific (M) or hetero-specific (H) nests. (B) Least square means (±95% CI) of alpha diversity indexes (Chao1, Shannon index and Faith's phylogenetic distance (PD)) estimated for magpies and cuckoos from monospecific (M) or hetero-specific (H) nests. (C) Venn diagram showing the number of shared ASVs per group is also shown.

Table 1. Results from mixed model ANOVAs exploring the effects of species ID in either, mono- or hetero-specific nests, as well as the effect on social environment on the alpha diversity indexes (Chao1, Shannon index and Faith's phylogenetic diversity (PD)) on bacterial communities of the uropygial gland skin of magpies and great spotted cuckoos. We analysed the effects of species ID and social environment on diversity of the bacterial community whole, and of the core microbiome and potentially pathogenic ASVs subsets. Results with associated p-value lower than 0.05 are shown in bold.

		All ASVs			**Potentially pathogenic ASVs**			**Core microbiome**		
		F	*df*	p	F	*df*	p	F	df	p
Chao1	*Species effect (monospecific nests)*									
	Species ID	3.71	1, 15.1	0.073	4.17	1, 14.1	0.060	**29.17**	**1, 13.5**	**<0.001**
	Nest (Species ID)	2.40	13, 13	0.064	**4.53**	**13, 13**	**0.005**	**9.91**	**13, 13**	**<0.001**
	Species effect (hetero-specific nests)									
	Species ID	0.59	1, 8.2	0.592	2.79	1, 8.2	0.132	0.03	1, 8.4	0.871
	Nest	3.16	8, 8	0.062	**8.80**	**8, 8**	**0.003**	2.79	8, 8	0.084
	Species ID*Nest	**4.71**	**8, 8**	**0.021**	**3.63**	**8, 8**	**0.043**	2.06	8, 8	0.164
	Effect social environment magpies									
	Social environment	**26.45**	**1, 20.7**	**<0.001**	2.49	1, 20.4	0.130	**32.59**	**1, 21**	**<0.001**
	Nest (Social environment)	**5.09**	**20, 18**	**<0.001**	**8.27**	**20, 18**	**<0.001**	**3.40**	**20, 18**	**0.006**
Shannon	*Species effect (monospecific nests)*									
	Species ID	0.002	1, 13.9	0.962	2.91	1, 14.8	0.109	**11.9**	**1, 14.6**	**0.004**
	Nest (Species ID)	**5.57**	**13, 13**	**0.002**	**2.86**	**13, 13**	**0.034**	**3.17**	**13, 13**	**0.023**
	Species effect (hetero-specific nests)									
	Species ID	1.74	1, 8.3	0.223	0.67	1, 8.2	0.435	0.72	1, 8.4	0.419
	Nest	**4.98**	**8, 8**	**0.018**	2.08	8, 8	0.160	**5.42**	**8, 8**	**0.014**
	Species ID*Nest	3.32	8, 8	0.055	**5.96**	**8, 8**	**0.010**	2.42	8, 8	0.106
	Effect social environment magpies									
	Social environment	**15.75**	**1, 20.5**	**<0.001**	0.60	1, 20.7	0.445	**5.24**	**1, 20.9**	**0.033**
	Nest (Social environment)	**6.01**	**20, 18**	**<0.001**	**4.97**	**20, 18**	**<0.001**	**3.91**	**20, 18**	**0.003**
PD	*Species effect (monospecific nests)*									
	Species ID	1.00	1, 14.2	0.334	0.21	1, 16.5	0.650	**28.46**	**1, 14**	**<0.001**
	Nest (Species ID)	**4.28**	**13, 13**	**0.006**	1.45	13, 13	0.256	**5.07**	**13, 13**	**0.003**
	Species effect (hetero-specific nests)									
	Species ID	0.57	1, 8.5	0.470	0.27	1, 8.5	0.615	0.06	1, 8.35	0.818
	Nest	**3.79**	**8, 8**	**0.039**	1.19	8, 8	0.407	**4.42**	**8, 8**	**0.025**
	Species ID*Nest	1.89	8, 8	0.193	1.95	8, 8	0.183	2.57	8, 8	0.102
	Effect social environment magpies									
	Social environment	**32.38**	**1, 20.8**	**<0.001**	1.56	1, 21.3	0.226	**37.9**	**1, 20.7**	**<0.001**
	Nest (Social environment)	**4.60**	**20, 18**	**<0.001**	**2.66**	**20, 18**	**0.021**	**5.24**	**20, 18**	**<0.001**

When considering the beta diversity of cuckoo and magpie samples from monospecific nests, the bacterial communities differed significantly between the two species [except for weighted UniFrac distances (Table 2)], and their 95% confidence interval ellipses in PCoA plots hardly overlap (Fig. 3A). This effect disappeared when comparing samples of magpies and cuckoo nestlings that were raised in the same nest (Table 2), as revealed by overlapping points and 95% confidence intervals (Fig. 3A). Furthermore, regardless of the distance matrix used, the social environment influences the composition of the bacterial community of magpies (Table 2, Fig. 3). However, the individual variation in magpie microbiomes was not associated with social environment (Fig. 3C) when considering Bray-Curtis, Jaccard, or weighted UniFrac distance matrixes (betadisper test; $F_{1,38} < 2.64$, $p >$

0.109), but it was associated with social environment when considering Unweighted UniFrac (betadisper test; $F_{1,38} = 23.21$, $p < 0.001$).

Thirty bacterial genera were differentially abundant between groups (Additional table 5). Thirteen, 17 and 21 genera were respectively more abundant in cuckoos not sharing, cuckoos sharing, and magpies sharing than in magpies from monospecific nests. Magpies from monospecific nests had higher relative abundances of *Enterococcus* than magpies from hetero-specific nests, and higher relative abundances of *Escherichia – Shigella* and *Pseudomonas* than magpies and cuckoos from hetero-specific nests (Fig. 3D, Additional table 5). Interestingly, magpies growing in hetero-specific nests did not present any differentially abundant genera compared with cuckoos, either from mono- or hetero-specific nests.

Core microbiome

Core microbes present in relative abundance of at least 0.0001% and 50% of samples for a species comprised 232 ASVs from 75 genera in six phyla (Additional table 6). For these cores, we detected interspecific differences in alpha and beta diversity between nestlings of the two species from monospecific nests, with cuckoo nestlings having higher Chao1, Shannon diversity and PD than magpie nestlings (Tables 1 and 2). Furthermore, and in agreement with the results for all ASVs, bacterial richness, diversity, and community composition of birds growing in hetero-specific nests did not differ between species (Table 1 and 2). In addition, differences in social environment for magpie nestlings were also apparent when only exploring the core microbiome (Tables 1 and 2).

Diversity and composition of potentially pathogenic ASVs

Alpha diversity of potential pathogenic taxa did not differ significantly among magpies and cuckoos when considering either mono- or hetero-specific nests (Table 1). Similarly, sharing the nest with hetero-specifics did not affect the alpha diversity indexes of magpie samples (Table 1). When looking at beta diversity of communities of potential pathogens, we found statistically significant interspecific differences when comparing monospecific nests and considering Bray-Curtis or Jaccard distance matrices (Table 2). However, this effect disappeared when comparing cuckoo and magpie nestlings that grew up within the same nests (Table 2). Moreover, beta diversity of potential pathogenic bacteria of magpies differed according to social environment when considering Bray-Curtis and Jaccard distance matrixes (Table 2). Furthermore, *Pseudomonas*, *Escherichia – Shigella* and *Enterococcus* were significantly more abundant in magpies that grew up in mono-specific nests (Fig. 3D).

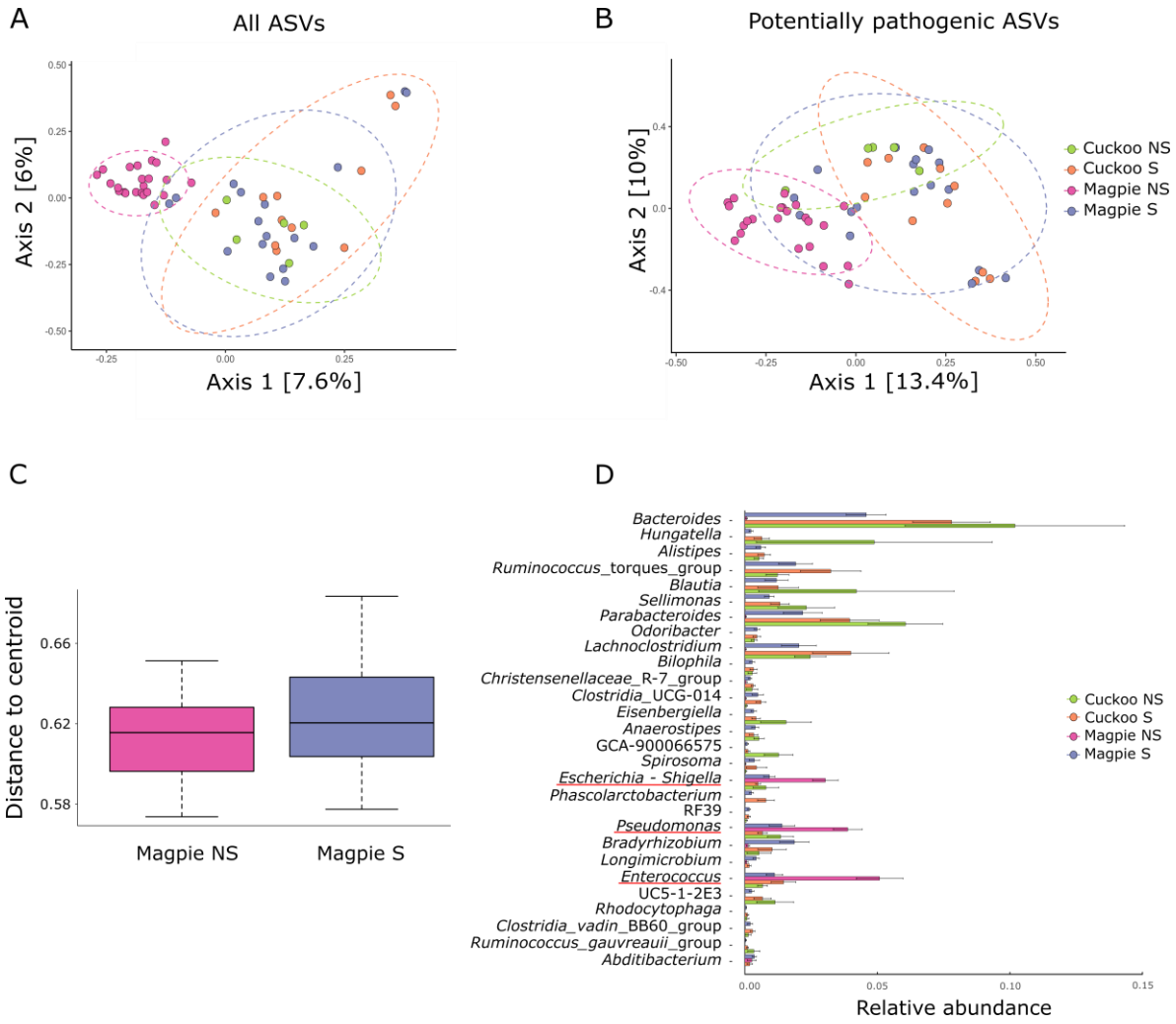

Figure 3. PCoA plots using Bray-Curtis distance matrixes showing the relative position of magpies and great spotted cuckoos from monospecific (M) or hetero-specific (H) nests using (A) the whole set of ASVs and (B) only potentially pathogenic ASVs. Ellipses are 95% confidence interval. (C) Box plots showing group dispersions with Bray-Curtis distance matrix (betadisper analyses) of Magpies from monospecific (M) or hetero-specific (H) nests. (D) Differential abundance of genera from the whole dataset of ASVs differing significantly among species (magpies or great spotted cuckoos) and/or social environments (nestlings from monospecific (M) or hetero-specific (H) nests). The list of bacterial species derived from Random Forest analyses with alpha = 0.01. Underlined in red are the potentially pathogenic genera.

Table 2. Results from PERMANOVAs analysing beta diversity matrixes of the bacterial community whole or of the core microbiome and potentially pathogenic ASVs subsets as dependent variables. The analyses explored the effect of species ID (taking into account mono- and hetero-specific nests), and the effect of social environment on the microbial communities of magpie and great spotted cuckoo nestlings. The number of permutations was set to 9999. Results with associated p-value lower than 0.05 are shown in bold.

		Bray-curtis			**Jaccard**			**Unweighted UniFrac**			**Weighted UniFrac**		
		Pseudo-F	*df*	p	Pseudo-F	*df*	p	Pseudo-F	*df*	p	Pseudo-F	*df*	p
All ASVs	*Species effect (monospecific nests)*												
	Species ID	**1.90**	**1, 16.3**	**0.002**	**1.51**	**1, 16.9**	**0.009**	**2.35**	**1, 16.4**	**0.009**	2.62	1, 15.2	0.091
	Nest (Species ID)	**1.55**	**13, 13**	**<0.001**	**1.30**	**13, 13**	**<0.001**	**1.47**	**13, 13**	**<0.001**	2.33	13, 13	0.099
	Species effect (hetero-specific nests)												
	Species ID	1.06	1, 9	0.430	1.02	1, 9	0.485	1.21	1, 9.1	0.282	2.96	1, 9.3	0.089
	Nest	**2.78**	**8, 8**	**<0.001**	**1.99**	**8, 8**	**<0.001**	**1.74**	**8, 8**	**0.001**	1.45	8, 8	0.271
	Species ID*Nest	0.91	8, 8	0.730	0.96	8, 8	0.652	0.85	8, 8	0.850	0.69	8, 8	0.729
	Effect social environment magpies												
	Social environment	**2.19**	**1, 22.2**	**<0.001**	**2.46**	**1, 22.5**	**<0.001**	**4.20**	**1, 22.6**	**<0.001**	**5.47**	**1, 21.8**	**0.006**
	Nest (Social environment)	**1.57**	**20, 18**	**<0.001**	**1.38**	**20, 18**	**<0.001**	**1.35**	**20, 18**	**<0.001**	1.99	20, 18	0.116
Potentially pathogenic ASVs	*Species effect (monospecific nests)*												
	Species ID	**2.76**	**1, 16.6**	**0.009**	**2.00**	**1, 17.1**	**0.009**	0.72	1, 16.4	0.404	0.86	1, 16.7	0.365
	Nest (Species ID)	**1.40**	**13, 13**	**0.004**	**1.22**	**13, 13**	**0.008**	1.50	13, 13	0.184	1.37	13, 13	0.276
	Species effect (hetero-specific nests)												
	Species ID	0.93	1, 8.8	0.526	0.96	1, 8.9	0.538	0.43	1, 8.6	0.589	2.83	1, 9	0.119
	Nest	**2.94**	**8, 8**	**<0.001**	**2.13**	**8, 8**	**<0.001**	1.88	8, 8	0.169	**4.31**	**8, 8**	**0.006**
	Species ID*Nest	1.08	8, 8	0.371	1.01	8, 8	0.487	1.49	8, 8	0.298	0.88	8, 8	0.584
	Effect social environment magpies												
	Social environment	**3.41**	**1, 22.1**	**<0.001**	**2.46**	**1, 22.5**	**<0.001**	2.15	1, 21.6	0.150	3.11	1, 21.1	0.075
	Nest (Social environment)	**1.66**	**20, 18**	**<0.001**	**1.38**	**20, 18**	**<0.001**	**2.15**	**20, 18**	**0.026**	**3.20**	**20, 18**	**0.004**
Core microbiome	*Species effect (monospecific nests)*												
	Species ID	**3.83**	**1, 15.4**	**0.002**	**2.60**	**1, 16.1**	**0.002**	**6.80**	**1, 15.3**	**0.002**	**13.00**	**1, 14.4**	**0.003**
	Nest (Species ID)	**2.14**	**13, 13**	**<0.001**	**1.65**	**13, 13**	**<0.001**	**2.20**	**13, 13**	**<0.001**	**3.59**	**13, 13**	**<0.001**
	Species effect (hetero-specific nests)												
	Species ID	1.20	1, 8.8	0.311	1.07	1, 8.8	0.419	0.95	1, 9	0.478	2.64	1, 8.5	0.103
	Nest	**4.55**	**8, 8**	**<0.001**	**3.08**	**8, 8**	**<0.001**	**3.12**	**8, 8**	**<0.001**	**7.87**	**8, 8**	**<0.001**
	Species ID*Nest	1.10	8, 8	0.356	1.09	8, 8	0.303	0.89	8, 8	0.650	1.93	8, 8	0.079
	Effect social environment magpies												
	Social environment	**4.24**	**1, 21.6**	**<0.001**	**2.97**	**1, 22.0**	**<0.001**	**12.62**	**1, 21.8**	**<0.001**	**3.50**	**1, 20.9**	**0.027**
	Nest (Social environment)	**2.22**	**20, 18**	**<0.001**	**1.71**	**20, 18**	**<0.001**	**1.99**	**20, 18**	**<0.001**	**4.15**	**20, 18**	**<0.001**

Discussion

By capitalising on the natural associations between magpie hosts and cuckoo brood parasites, we document the influence of social environment on microbial assemblages of the uropygial gland skin of developing magpie host and parasitic cuckoo chicks. As expected, we detected species-specific diversity, composition, and richness in both complete microbiomes and microbial cores, underlining the role of species-specific factors in shaping microbial assemblages. However, and as predicted, these interspecific differences disappeared when species cohabitated the same nests, implying either social transmission of symbionts among nestlings, or transmission of microbes via feeding adults and/or from the shared physical environment (c.f., Diez-Méndez *et al.* 2023). In hetero-specific nests, we observed a consistent change of the magpie microbiota to resemble that of their hetero-specific nest-mates that most likely reflect horizontal transmission of microbes between cuckoos and magpies via their altered social environment. Lastly, we observed a negative impact of social environment on certain candidate pathogenic bacteria in magpies, suggesting a potential positive feedback of the presence of cuckoos on magpie skin microbiomes.

The interspecific differences in uropygial gland skin microbiomes in mono-specific nests cannot only be accounted by vertical transmission of microbes from the biological or foster parents. This is particularly true for cuckoos, as vertical transmission of microbes is restricted to the pre-egg laying period (Soler *et al.* 1999; Soler & Soler 1999). These interspecific differences may thus result from intrinsic factors, e.g., species-specific chemical composition of the uropygial gland secretion (Jacob & Ziswiler 1982; Javůrková *et al.* 2019; Martínez-Renau *et al.* 2022). Birds preen their feathers and skin with uropygial secretion, including during the nestling stage (Soler *et al.* 2022), thus the antimicrobial properties of these secretions (Martín-Platero *et al.* 2006; Martín-Vivaldi *et al.* 2010) may prevent specific bacteria from establishing (Soler *et al.* 2012; Martín-Vivaldi *et al.* 2014; Soler *et al.* 2016), while secretions themselves may stimulate the growth of other microbial taxa (Javůrková *et al.* 2019). Consequently, it is likely that the particularities of the uropygial secretion of magpies and cuckoos promote a species-specific selective environment that favours certain microbes to grow on the skin, a possibility worth to be explored in the future by testing for the promoting or inhibitory effects of the uropygial secretion on the bacterial strains.

In gut microbial communities of cuckoos and magpies, species specificities in cloacal microbiomes are retained in hetero-specific nests (Ruiz-Rodríguez *et al.* 2018; Lee *et al.* 2020). In contrast, we found that skin microbiomes converged in the host-parasite species pair. This indicates that the magnitude of the effect of social and shared physical environment

varies depending on whether the host-associated microbiomes are external or internal (Hussa & Goodrich-Blair 2013; Valles-Colomer *et al.* 2023). Despite similarities in the microbial composition (e.g., saliva microbiomes) and diet between cuckoo and magpie nestlings in the same nests (Lee *et al.* 2020), species-specific cloacal microbiomes indicate internally-maintained digestive tract microbiomes that are resistant to perturbations from the shared environment (Ruiz-Rodríguez *et al.* 2018), while skin microbiomes are more susceptible to horizontal transfer of microbes.

The evidence for social transmission of microbes stems mainly from the potential transfer of cuckoo-specific microbes to magpies. This unidirectional transfer of microbes from cuckoos to magpies might indicate that the resistance of skin microbiomes to social environment also varies interspecifically. Given the brood-parasite lifestyle, cuckoos may depend more on *in ovo* vertical transmission of microbes than magpie nestlings (c.f., Trevelline *et al.* 2018), while having more resistant skin microbiomes to ensure transgenerational transfers of symbionts (c.f., Diez-Méndez *et al.* 2023). This skewed opportunity for vertical transmission of microbes in cuckoos may alter host-symbiont associations in this species with potential ramifications for losses or replacement of microbial symbionts across generations. However, the effects of sharing a nest with hetero-specifics might be only temporal as shown by a cross-foster experiment on captive zebra finch (*Taeniopygia guttata*) and Bengalese finch (*Lonchura striata domestica*) nestlings (Maraci *et al.* 2022). In these cases, effects of foster families on cloacal microbiomes early in the nestling period disappeared in later stages, where interspecific differences among nest mates were detected (Maraci *et al.* 2022). In the case of the uropygial gland, its secretion might not be fully developed during the nestling stage (Jacob & Ziswiler 1982) and, thus, the associated microbiome is likely shaped during the second part of the nesting phase (Martínez-García *et al.* 2016), explaining why we detected the expected effect of social environment at the late developmental stage of magpie nestlings. However, to fully grasp the breadth of how early life social environment influences long-term associations and generational transfers of skin symbionts, we need to explore the fate of skin microbes over time during an individual's life.

The social transfer of potentially pathogenic bacteria contradicted the patterns for non-pathogenic bacteria, where the relative abundances of potentially pathogenic genera *Pseudomonas*, *Escherichia – Shigella*, *Enterococcus* and *Staphylococcus* were significantly higher in magpies in mono-specific than hetero-specific nests. The lower prevalence of these genera in hetero-specific nests could be mediated by the parallel increase in the richness and diversity of the skin microbiota of nestlings. This might be because increased microbial

diversity provides increased resistance to pathogen colonization (Lozupone *et al.* 2012; Kriss *et al.* 2018), but also stimulate the host immune system (Weiss, Maltz & Aksoy 2012). Alternatively, the properties of the uropygial secretion, or symbiotic defensive bacteria within the gland of cuckoos, may counter potential pathogens. Even if this might have a positive effect of reduced pathogens on host magpies, it would be unlikely to counter the negative fitness effects of brood parasitism (Soler *et al.* 2017). Nevertheless, because we did not test for pathogenicity of strains, these results are only tentative and, to fully understand the implication of social environment on pathogenic taxa, future research is needed to explore the specific bacterial strains with detrimental effects on birds, and how they are distributed among con- and hetero-specific nests. Furthermore, to disentangle the mechanisms underlining the observed negative effect of social environment on potentially pathogenic bacteria, we need to isolate these bacterial taxa and conduct co-culture assays with cuckoo and magpie uropygial gland secretions.

Using a natural host-brood parasite system, we were able to separate the effects of genetic relatedness and shared environment on the skin microbiota from those of interacting individuals, elucidating a role of social environment determining the skin microbiomes of wild birds. Taken together, our study implies that skin microbiomes are amenable to horizontal transfer of microbes from the social and the nest environment, but that the magnitude and identities of bacterial genera transferred depend on host ecology. Early-life exposure to hetero-specific microbes thus alters the wild bird skin microbiomes, which should influence both short and long-term stability of beneficial and antagonistic symbiotic interactions.

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Supplementary material

Additional table 1. Taxonomy of strains considered potentially pathogenic. We considered as potentially pathogenic strains every bacteria inside a genus described as pathogenic in one of the three sources. Information was extracted from PHI-base (Urban *et al.* 2020), FAPROTAX (Louca, Parfrey & Doebeli 2016) and a review published by Benskin *et al.* (2009).

Domain	Phylum	Order	Class	Family	Genus	Species	Source
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*_sensu_stricto_1		PHI database
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*		Benskin et al. 2009
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*_sensu_stricto_1	uncultured_bacterium	PHI database
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*_sensu_stricto_1	uncultured_organism	PHI database
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*_sensu_stricto_1	*Clostridium_celatum*	PHI database
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*_sensu_stricto_7	uncultured_Clostridium	PHI database
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*_sensu_stricto_9		PHI database
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Escherichia-Shigella*		PHI database
Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae			Benskin et al. 2009
Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae			Benskin et al. 2009
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*Pseudomonas_caeni*	Benskin et al. 2009
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*		Benskin et al. 2009
Bacteria	Firmicutes	Bacilli	Staphylococcales	Staphylococcaceae	*Staphylococcus*		Benskin et al. 2009
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*_sensu_stricto_1	*Clostridium_perfringens*	PHI database
Bacteria	Proteobacteria	Gammaproteobacteria	Coxiellales	Coxiellaceae	*Coxiella*		FAPROTAX
Bacteria	Verrucomicrobiota	Chlamydiae	Chlamydiales	cvE6	cvE6	uncultured_Chlamydia	FAPROTAX
Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	*Haemophilus*		FAPROTAX
Bacteria	Firmicutes	Bacilli	Lactobacillales	Listeriaceae	*Listeria*		Benskin et al. 2009
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Yersiniaceae	*Serratia*	*Serratia_myotis*	Benskin et al. 2009
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Yersiniaceae	*Serratia*		Benskin et al. 2009
Bacteria	Firmicutes	Bacilli	Staphylococcales	Staphylococcaceae	*Staphylococcus*	*Staphylococcus_simulans*	Benskin et al. 2009
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*		Benskin et al. 2009
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*	*Streptococcus_alactolyticus*	Benskin et al. 2009
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*	*Streptococcus_equi*	Benskin et al. 2009
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Yersiniaceae	*Yersinia*		Benskin et al. 2009
Bacteria	Chlamydiota	Chlamydiae	Chlamydiales	Parachlamydiaceae			FAPROTAX

Additional table 2. Results from mixed model ANOVAs exploring the effects of species ID in either, mono- or hetero-specific nests, as well as the effect on social environment on the alpha diversity indexes (Chao1, Shannon index and Faith's phylogenetic diversity (PD)) on magpies bacterial communities. The analyses are controlled for the number of nestlings in the nest. We analysed the effects of species ID and social environment on the whole bacterial community and on the subsets of core microbiome and potentially pathogenic ASVs isolated from the uropygial gland skin of magpies and great spotted cuckoos. Results with associated p-value lower than 0.05 are shown in bold.

		All ASVs			**Potentially pathogenic ASVs**			**Core microbiome**		
		F	*df*	p	F	*df*	p	F	df	p
Chao1	*Species effect (monospecific nests)*									
	Brood size	1.75	1, 12.8	0.209	<0.01	1, 12.5	0.992	0.28	1, 12.8	0.604
	Species ID	**4.90**	**1, 12.1**	**0.047**	3.74	1, 12.1	0.077	0.31	1, 12.1	0.591
	Nest (Species ID)	2.59	12, 13	0.051	**4.63**	**12, 13**	**0.005**	2.78	12, 13	0.040
	Species effect (hetero-specific nests)									
	Brood size	0.40	1, 6.9	0.548	3.41	1, 69	0.108	<0.01	1, 6.5	0.952
	Species ID	0.65	1, 9	0.441	1.53	1, 9.8	0.245	0.01	1, 7.6	0.917
	Nest	**3.72**	**7, 7.7**	**0.045**	**6.59**	**7, 7.6**	**0.009**	1.93	7, 7.2	0.199
	Species ID*Nest	**4.71**	**8, 8**	**0.021**	**3.63**	**8, 8**	**0.043**	1.72	8, 8	0.231
	Effect social environment magpies									
	Brood size	2.33	1, 19.2	0.143	0.02	1, 19.1	0.900	1.09	1, 19.5	0.309
	Social environment	**23.23**	**1, 18.8**	**<0.001**	2.18	1, 18.9	0.156	**18.34**	**1, 18.3**	**<0.001**
	Nest (Social environment)	**5.20**	**19, 18**	**<0.001**	**8.65**	**19, 18**	**<0.001**	1.81	19, 18	0.130
Shannon	*Species effect (monospecific nests)*									
	Brood size	0.17	1, 12.4	0.687	0.09	1, 12.7	0.770	0.49	1, 13.4	0.495
	Species ID	0.27	1, 12.1	0.612	2.11	1, 12.1	0.172	0.02	1, 12.2	0.880
	Nest (Species ID)	**6.03**	**12, 13**	**0.001**	**3.06**	**12, 13**	**0.028**	1.51	12, 13	0.235
	Species effect (hetero-specific nests)									
	Brood size	1.87	1, 6.8	0.215	0.15	1, 6.8	0.710	0.07	1, 6	0.798
	Species ID	0.86	1, 8.9	0.379	0.80	1, 8.5	0.397	0.99	1, 7.2	0.350
	Nest	**4.01**	**7, 7.6**	**0.038**	2.27	7, 7.8	0.139	0.94	7, 7.2	0.531
	Species ID*Nest	3.32	8, 8	0.055	**5.96**	**8, 8**	**0.010**	1.71	8, 8	0.233
	Effect social environment magpies									
	Brood size	**5.61**	**1, 19.15**	**0.029**	0.68	1, 19.2	0.420	3.78	1, 19.8	0.066
	Social environment	**13.40**	**1, 18.8**	**0.002**	0.44	1, 18.8	0.515	2.97	1, 18	0.102
	Nest (Social environment)	**5.45**	**19, 18**	**<0.001**	**5.10**	**19, 18**	**<0.001**	1.10	19, 18	0.421
PD	*Species effect (monospecific nests)*									
	Brood size	0.42	1, 12.5	0.529	0.10	1, 13.3	0.758	2.06	1, 12.4	0.173
	Species ID	1.43	1.12.1	0.255	0.01	1, 12.2	0.922	**11.38**	**1, 12.1**	**0.005**
	Nest (Species ID)	**4.64**	**12, 13**	**0.005**	1.57	12, 13	0.217	**4.78**	**12, 13**	**0.004**
	Species effect (hetero-specific nests)									
	Brood size	0.37	1, 6.8	0.562	3.62	1, 6	0.106	0.19	1, 6.6	0.676
	Species ID	0.42	1, 8.6	0.534	0.04	1, 7.3	0.854	4.56	1, 6.9	0.071
	Nest	**4.59**	**7, 7.3**	**0.029**	0.85	7, 7.3	0.582	4.88	7, 6.2	0.033
	Species ID*Nest	1.89	8, 8	0.193	1.95	8, 8	0.183	0.73	8, 8	0.665
	Effect social environment magpies									
	Brood size	2.83	1, 19.2	0.109	0.19	1, 19.3	0.670	1.68	1, 19.4	0.210
	Social environment	**28.51**	**1, 18.8**	**<0.001**	2.05	1, 18.6	0.168	**41.34**	**1, 18.5**	**<0.001**
	Nest (Social environment)	**4.67**	**19, 18**	**<0.001**	**2.74**	**19, 18**	**0.019**	2.07	19, 18	0.064

To download Additional Tables 3-6: http://www.eeza.csic.es/documentos/users/smart/anexos.zip

Additional table 3.xlsx: Rarefied ASV table of the whole bacterial community with six taxonomic levels. Samples are identified regarding species and whether they share or not share the nest with hetero-specifics. GenBank accession number specified above sample number.

Additional table 4.xlsx: Taxonomy of detected bacterial genera on the skin of the uropygial gland of magpie and great spotted cuckoo nestlings in monospecific (Sharing) and hetero-specific (Not sharing) nests. We also show mean relative abundance of different bacterial taxa per group.

Additional table 5.xlsx: Wilcoxon Rank Hum Test results on differential abundance of bacterial genera between Eurasian magpies and great spotted cuckoos from mono- (M) or hetero-specific (H) nests. Group column refers to the group with higher abundance of the specific strain.

Additional table 6.xlsx: Subset of the ASV table with six taxonomic levels including ASVs classified as core-microbiome in Eurasian magpies or great spotted cuckoos. Samples are identified regarding species and whether they share or not share the nest with hetero-specifics.

CAPÍTULO II

Microbial infection risk predicts antimicrobial potential of avian symbionts

Abstract

Symbiotic bacteria on animal hosts can prevent pathogenic bacterial infections by several mechanisms. Among them, symbiotic bacteria can indirectly enhance host's immune responses or, directly, produce antimicrobial substances against pathogens. Due to differences in life-style, different host species are under different risks of microbial infections. Consequently, if symbiotic bacteria are somewhat selected by genetically determined host characteristics, we would expect the antimicrobial properties of bacterial symbionts to vary among host species and to be distributed according to risk of infection. Here we have tested this hypothesis, by measuring the antimicrobial ability of the bacterial strains isolated from the uropygial-gland skin of 19 bird species differing in nesting habits, and, therefore, in risk of microbial infection. In accordance with our predictions, intensity and range of antimicrobial effects against the indicator strains assayed varied among bird species, with hole- and open-nesters showing the highest and the lowest values, respectively. Since it is broadly accepted that hole-nesters have higher risks of microbial infection than open nesters, our results suggest that the risk of infection is a strong driver of natural selection to enhance immunocompetence of animals through selecting for antibiotic-producing symbionts. Future research should focus on characterizing symbiotic bacterial communities and detecting coevolutionary processes with particular antibiotic-producing bacteria within-host species.

Keywords*: antimicrobial activity, antibiotic-producing bacteria, birds, natural selection, symbiotic bacteria, uropygial gland, uropygial secretion*

Introduction

Current view of symbiotic microbiomes acknowledges the beneficial effect of microorganisms on their hosts, in opposition to the classical view of microorganisms as pathogens. Thus, symbiotic bacteria are essential for understanding the evolution and functioning of their animal hosts (McFall-Ngai 2002; Archie & Tung 2015; Sherwin *et al.* 2019). Some bacteria, for instance, allow animals to achieve better digestion and use of nutrients (Backhed *et al.* 2004), enhance the immune system (Umesaki *et al.* 1995), promote chemical communication (Ezenwa & Williams 2014; Carthey, Gillings & Blumstein 2018; Maraci, Engel & Caspers 2018), or even trigger direct defenses against parasite and/or predator enemies (McFall-Ngai *et al.* 2013; Suzuki 2017; Mazorra-Alonso, Tomás & Soler 2021). Indeed, animals using bacterial symbionts or their metabolites for self-protection against infections is widely spread within the animal kingdom (Currie *et al.* 1999; Brucker *et al.* 2008; Soler *et al.* 2008; Brownlie & Johnson 2009; Soler *et al.* 2010). Birds, for instance, tend to use antimicrobial-producing bacteria growing in nest lining materials (i.e., feathers; Peralta-Sánchez *et al.* 2010; Peralta-Sánchez *et al.* 2014; Ruiz-Castellano *et al.* 2016; Ruiz-Castellano *et al.* 2019) or in their uropygial gland (Soler *et al.* 2008), to prevent pathogenic infection of embryos (Martín-Vivaldi *et al.* 2014), nestlings (Soler *et al.* 2017) or breeding adults (Ruiz-Rodríguez *et al.* 2009).

Birds use their uropygial gland secretion to preen their feathers, which also confers protection against pathogenic infections during reproduction (Soler *et al.* 2012). The secretion is mainly composed of monoester and diester waxes of aliphatic alcohols and fatty acids with antimicrobial activity (Jacob & Ziswiler 1982). This could partially explain the detected negative associations between size of the uropygial gland of different bird species and their eggshell bacterial loads and hatching success (Soler *et al.* 2012). Interestingly, in recent years, evidence showing that the uropygial gland of birds hosts symbiotic bacteria is accumulating in the literature. Following the pioneering works in woodhoopoes (*Phoeniculus purpureus*) (Law-Brown & Meyers 2003) and European hoopoes (*Upupa epops*) (Soler *et al.* 2008), symbiotic bacteria have been detected in the uropygial secretion of turkeys (*Meleagris gallopavo*) (Braun *et al.* 2016), great spotted woodpeckers (*Dendrocopos major*) (Braun *et al.* 2018b), American barn owls (*Tyto furcata*) (Braun *et al.* 2018a), Egyptian geese (*Alopochen aegyptiacus*) (Braun *et al.* 2018c), dark-eyed juncos (*Junco hyemalis*) (Whittaker & Theis 2016), great tits (*Parus major*) (Bodawatta *et al.* 2020) and house sparrows (*Passer domesticus*) (Videvall *et al.* 2021). Moreover, in hoopoes some of the bacteria isolated from the gland secretion produce bacteriocins active against a wide range of bacterial strains,

including potential pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus* and the feather degrading *Bacillus licheniformis* (Martín-Platero *et al.* 2006; Martín-Vivaldi *et al.* 2010; Ruiz-Rodríguez *et al.* 2013). Thus, it is possible that the previously described antimicrobial properties of the uropygial secretions of birds (Shawkey, Pillai & Hill 2003; Reneerkens *et al.* 2008) were, at least partially, mediated by their antibiotic-producing bacterial symbionts.

In birds, antibiotic-producing bacteria, have also been found in avian body feathers, the bill, brood patch and eggshells (Cook *et al.* 2005; Shawkey *et al.* 2005; Martín-Vivaldi *et al.* 2014; Peralta-Sánchez *et al.* 2014; Soler *et al.* 2014; Martínez-García *et al.* 2015; Soler *et al.* 2016; Javůrková *et al.* 2019). All these locations are spread with uropygial secretion during preening and, thus, it is possible that some of these antibiotic-producing bacteria come from those inhabiting the uropygial gland of birds (Martínez-García *et al.* 2015; Soler *et al.* 2016). Another non-exclusive possibility is that some of these bacteria do not come directly from the bacterial communities in the secretion, but rather the secretion acts as a nutritional substrate facilitating or driving the colonization and growth of antibiotic-producing bacteria on the avian integumentary structures. In other words, it is possible that, thanks to the uropygial secretion, birds cultivate antibiotic-producing bacteria not only in their gland, but also on their feathers, beak and skin. Those symbiotic bacteria would help animals to prevent infections and, thus, would complement or add to the defensive responses of the immune system (Soler *et al.* 2010). If this is the case, since antibiotic-producing bacteria development would depend on the bird's physiology (e.g., by producing a large amount of uropygial secretion of special chemical characteristics), birds might be able to select the antimicrobial characteristics of their bacterial symbionts. In this scenario, as it occurs with the immune system, antimicrobial capacity of those bacteria should adjust to the expected level of risk of infection. Here, we explore some predictions to test this hypothesis in a comparative interspecific framework.

The general predictions of the hypothetical selection of antimicrobial capacities of bacterial symbionts according to risk of infection for hosts are: i) antimicrobial capacity of isolated symbiotic bacteria should differ among species; and ii) species-specific values of the antimicrobial potential should correlate with the strength of selection pressure, i.e. with the risk of infection experienced by each studied species. Life-style and life history strategies may condition risk of infection of animal populations. For instance, breeding in cavities entails high risks of ectoparasitism and pathogenic infections (Møller 1997; Peralta-Sánchez *et al.* 2018). Secondary-cavity nesters are in some way obligated to re-use holes due to the

scarcity of natural ones. This life-style facilitates parasite transmission and multiple infections by more virulent parasites (Wiebe, Koenig & Martin 2007; Møller *et al.* 2009), and, on the other hand, selects for more efficient immune systems (Møller & Erritzøe 1996). Thus, if antimicrobial capacity of symbiotic bacteria is selected according to risk of infection of their hosts, we expect that those isolates from hole-nester species should be more efficient antagonizing the proliferation of other microorganims than isolates from non-hole nester species.

Immune responses also vary with age, either because of ageing of different components of the immune system (Cichon, Sendecka & Gustafsson 2003; Müller, Fülöp & Pawelec 2013), or because juveniles have not yet fully developed (Palacios *et al.* 2009; Stambaugh *et al.* 2011). However, the effect of age depends on the immune parameters considered (Vermeulen *et al.* 2017). Nestlings typically show lower innate immune response (Ricklefs 1992; Killpack & Karasov 2012), but higher cell mediated immunity (Tella, Scheuerlein & Ricklefs 2002) than adults. As suggested above, birds could cultivate antibiotic-producing bacteria on their feathers, beak, and skin. The uropygial gland is not fully developed before fledging (Jacob & Ziswiler 1982) and characteristics and quantity of secretion typically vary among nestlings of different ages and between nestlings and adults (Jacob & Ziswiler 1982; Soler *et al.* 2022). Thus, due to age differences in immunity and in uropygial secretion properties, it can be predicted that the bacterial community associated with the uropygial secretion, and, thus, its antimicrobial capacity, varies with age too.

In this study, we characterized the antimicrobial capacity of bacterial isolates from the uropygial gland skin of nestlings and/or adults of 19 bird species against several referential bacterial strains. We then explore the intra- and interspecific variations in intensity and range of antimicrobial capacity and tested the hypothesis that antimicrobial capabilities of microbial symbionts is adjusted to the risk of infections of their avian hosts with the expectations that they should vary according to species identity, age and nesting habits.

Material and methods

Study area and species

Fieldwork was carried out during the breeding seasons of 2018 and 2019, in southern Spain; in the Hoya de Guadix (37°15′N; 3°01′W), a semiarid high-altitude plateau, and in the Charca de Suárez, a wetland near the coast in Motril (36° 43' 18.707"N, 3° 32' 30.836"W). Most nestlings and adults of hole-nester species were sampled during breeding in cork-made nest boxes (internal dimensions: 180 mm x 210 mm and 350 mm high, 240 mm from the bottom

to the hole entrance) located in the Guadix study area, where we also sampled nestlings of open-nester species few days before leaving the nests. We used mist nets to capture adults or fledglings few days after abandoning the nest, mainly in the Charca de Suárez, but also in Guadix.

Fieldwork

Since March 15th, we visited nest boxes every 7-8 days, and intensively looked for nests of non-hole nesters in the surroundings until egg detection, which allowed us to calculate the hatching date and, thus, planning the sampling date of adults and nestlings. Hole-nester adults were captured within the nest boxes during brooding, and nestlings were sampled during the last quarter of the nestling period. Briefly, we sampled the bacterial community of the uropygial gland skin by rubbing the surface area of the UG, including the opening, with a sterile cotton swab slightly wetted in sterile Phosphate Buffer Saline (PBS, 0.2 M). The surface of the sampled uropygial gland skin was estimated by multiplying the length and the width of the gland. The swab was kept in a sterile microfuge vial containing 1 mL of sterile PBS and stored at 4ºC until further processing. We also measured tarsus length with a digital caliper (accuracy 0.01 mm), wing length with a ruler (accuracy 1 mm), body mass with a digital scale (accuracy 0.01 g), and gland dimensions (length, width and height) with a digital caliper (accuracy 0.01 mm) following Martín-Vivaldi et al. (Martín-Vivaldi *et al.* 2009). Finally, we ringed all individuals with numbered metal rings (Ministerio de Agricultura, Spain) to avoid resampling.

Laboratory procedure

Isolation of bacteria from the uropygial gland skin

We processed the uropygial skin samples in the laboratory the same day of collection. After vortexing the microfuge tubes containing the swabs, we plated decimal dilutions up to 10^{-4} on Tryptic Soy Agar (TSA, Scharlau, Barcelona, Spain). Petri dishes were incubated aerobically for 24 h at 37 ºC. Bacterial counts were estimated by standardization of the number of colonies per cm^2 of sampled uropygial gland skin.

Antimicrobial activity of colonies isolated from uropygial gland skin

We isolated five morphologically different colonies from each plate. To assure that a pure culture was achieved, each of these colonies were serially cultivated by the streak-plate method onto TSA plates for three times, incubating them for 24 h at 37 ºC. We then assayed their production of antimicrobial substances by the double-layer technique (Gratia &

Fredericq 1946) against 9 indicator bacterial strains. To this end, each isolate was replicated by spotting onto 9 TSA petri dishes (30 isolates per plate), and incubated for 24 h at 37 ºC before performing the antagonistic tests used to estimate antimicrobial capacity of sampled individuals. After producer bacteria were grown, plates were covered with 7 mL of soft agar (BHI added 0.8% agar, Scharlau Chemie S.A., Barcelona) previously heated until liquefied and tempered to 50ºC. Once liquid, the soft agar was inoculated with 100 µL of an overnight culture of the indicator strain (see below) at 37 ºC. Finally, covered plates were incubated for 24 h at 37 ºC. The antimicrobial activity of each isolated colony was measured as the width of the inhibition halo around the spot of the colony, measured with a ruler to the nearest 0.5 mm. No control strains were used to standardize the halo width, then raw data was employed for statistical analyses (for more details see Ruiz-Castellano et al. (2019) and Ruiz-Rodriguez et al. (2012)).

The antimicrobial assays were performed against nine typified strains covering a wide range of bacterial taxa, which include potential pathogenic bacteria for birds (Pinowski *et al.* 1994; Hubalek 2004; Benskin *et al.* 2009). These strains come from the Spanish Type Culture Collection (CECT) and from our laboratory. We used *Bacillus licheniformis* D13, *Enterococcus faecalis* S47, *Escherichia coli* CECT774, *Listeria inocua* CECT340, *Micrococcus luteus* 241, *Mycobacterium sp*., *Pseudomonas putida, Salmonella choleraesuis* CECT443, and *Staphylococcus aureus* CECT240.

Statistical analysis

We sampled 326 individuals from 19 species, including non-hole (N = 29 adults, 12 nests, 16 nestlings, Table 1) and hole nesters (N = 84 adults, 105 nests, 200 nestlings, Table 1). For eight of those species we collected information from both adults and nestlings close to fledging, for six species we only sampled nestlings, and for five species we only sampled adults.

For each bacterial isolate, antimicrobial capacity was estimated as the average of the width of antagonistic halos (hereafter intensity of antimicrobial activity) when tested against each of the nine indicator bacteria. Moreover, we also estimated the diversity (Shannon index) of antimicrobial activity, which inform on the range of antimicrobial activity of each bacterial isolate. Then, for each individual, we averaged the antimicrobial activity and antimicrobial range values of the five isolates from its uropygial gland skin. Bacterial density of the uropygial gland skin, as well as intensity and range of the antimicrobial activity of bacteria isolated from individuals (i.e. nestlings) of the same nest, were consistent ($R^2 > 70\%$)

and, then, we used nest mean values, which would appropriately account for the non-independence data of siblings. Bacterial density were log10 transformed to approach a normal distribution, while intensity and range of antimicrobial activity did not differ significantly from the Gaussian distribution.

Table 1. Sample sizes of adults, nests and nestlings sampled for each species of hole nester and non-hole nester species.

		Sample size		
	Species	**Nests**	**Nestlings**	**Adults**
Hole nesters	Athene noctua	8	14	6
	Columba oenas	6	12	
	Coracias garrulus	8	15	3
	Corvus monedula	6	12	
	Otus scops	8	16	9
	Parus major	7	12	3
	Passer domesticus	8	16	11
	Petronia petronia			5
	Picus viridis	4	8	
	Pyrrhocorax pyrrhocorax	6	11	
	Sturnus unicolor	9	17	9
	Upupa epops	35	67	38
Non-hole nesters	Acrocephalus scripaceus			7
	Cettia cetti			4
	Chloris chloris			3
	Columba palumbus	3	5	
	Corvus corone	3	5	
	Muscicapa striata			4
	Serinus serinus	6	6	11

To explore the effects of age (nestling *versus* adult) and species identity on the intensity and the range of the antimicrobial activity of bacteria isolated from their uropygial gland (hereafter antimicrobial variables), and on bacterial density, we first used General Linear Models (GLMs). These models included age and species identity as fixed factors, while the interaction between these two factors was explored in separate models. Because antimicrobial variables consistently differed for adults and nestlings, (even after controlling the effect of species identity (see Results)), the effect of nesting habits and species identity (nested within nesting habits to control for the non-balance species-data) were explored in separate GLM models for adults and nestlings. This approach allows increasing the number of considered species from 8 species with information for adult and nestlings, to 14 and 13 species, respectively.

Furthermore, we also explored the effects of nesting behavior and species identity on the antimicrobial profile of bacteria isolated from the uropygial gland skin of birds. To do this, we first calculated average individual/nest values of intensity of antimicrobial activity against each of the indicator bacterium used, and estimated distance matrices among sampled individuals/nests based on the Bray-Curtis similarity measure. Then, we explored the effects of fixed factors (age, nesting habits and species identity) on the distance matrix by means of PERMANOVAs as implemented in Primer7 v.7.0.17 (PRIMER-e). Similarly to the approach described for GLMs, we first checked the effect of age on the antimicrobial profile of the eight species with information for both nestlings and adults. Since we found a strong effect of age even after controlling for the effect of species identity (see Results), subsequent models directed to explore the effects of nesting habits were computed independently for adults and nestlings. These PERMANOVAs included nest type and species identity nested within nest type as independent fixed factors. Principal Coordinates Analyses (PCoA) were used to visualize the relative position of species centroids (± 90% CI of ellipses) in the multidimensional space.

Finally, as nesting habits have a strong phylogenetic component, we tried to control the analyses for phylogeny by means of Bayesian phylogenetic mixed models (MCMCglmm). First, we downloaded 100 trees for our set of species from http://birdtree.org/ (source of trees was Ericson all species; (Jetz *et al.* 2012)) and estimated the predicted effects for each of the trees using the MCMCglmm package (Hadfield 2010) in R (R-Core-Team 2020) environment that also included the packages "ape" (Paradis, Claude & Strimmer 2004), "MASS" (Paradis, Claude & Strimmer 2004) and 'mvtnorm' (Venables & Ripley 2002). To run the model we used the uninformative prior [list(G=list(G1=list(V=1,nu=0.002)),R=list(V=1,nu=0.002)], and adjusted the number of iterations, to 100000, the burn-in period to 10000 and the thinning interval to 10. That model was run for each of the 100 trees and calculated average values and the minimum and maximum values of lower and upper 95% credibility intervals of estimates. We also used

Geweke's convergence diagnostic for Markov chains (Geweke 1992), which is based on a standard z-score of means of the first (10%) and the last part (50%) of a Markov chain. These z-scores never exceeded the critical value of 1.96. The random effect of phylogeny is reported as heritability (h2) (Hadfield 2010),which is a measure of phylogenetic signal analogous to Pagel's lambda that ranges from zero (non-phylogenetic signal) to one (high phylogenetic signal).

Results

Age effects

Bacterial isolates from the uropygial gland skin of adults demonstrated higher intensity and range of antimicrobial activity than those from nestlings, although the effect depended on species identity (Table 2, Fig. 1). Moreover, density of bacteria on the uropygial gland skin was higher in nestlings than in adults, although it again depended on species identity (Table 2, Fig. 1).

Table 2. Results from General Linear Models (GLM) and PERMANOVAs with antimicrobial activity (average values of the width of antagonistic halos when tested against each of the nine indicator bacteria), antimicrobial range (Shannon index of the antimicrobial activity), bacterial density and the antimicrobial profile as dependent variables. Species identity and age (nestling vs adult) were used as the independent factors. The interaction between species identity and age was explored in separate models that also included main effects. Values in bold are statistically significant.

	General Linear Models (GLM)									**PERMANOVA**		
	Antimicrobial activity			**Antimicrobial range**			**Bacterial density**			**Antimicrobial profile**		
	F	df	p	F	df	p	F	df	p	Pseudo-F	df	p
Species	4.14	8, 173	**<0.001**	9.17	8, 173	**<0.001**	6.89	8, 164	**<0.001**	3.7	8, 165	**<0.001**
Age	35.19	1, 173	**<0.001**	12.87	1, 173	**<0.001**	52.97	8, 164	**<0.001**	9.56	1, 165	**<0.001**
Species*Age	1.86	8, 165	0.07	3.7	8, 165	**<0.001**	2.89	8, 156	**0.005**	1.61	8, 165	**0.003**

Nesting habits effects on adults and nestlings

Although total bacterial density of the uropygial gland skin of adults did not differ significantly among species, intensity and range of antimicrobial activity of bacterial isolates did (Table 3). As expected, adults of species with bacterial symbionts of higher intensity and range of antimicrobial activity (the hoopoe (*Upupa epops*), the great tit (*Parus major*), the little owl and the Eurasian scops owl (*Otus scops*)) were hole-nesters, while those with bacterial symbionts with lower values (reed warblers (*Achrocephalus scirpaceus*) and green finches (*Chloris chloris*)) were non-hole nesters (Fig. 1).

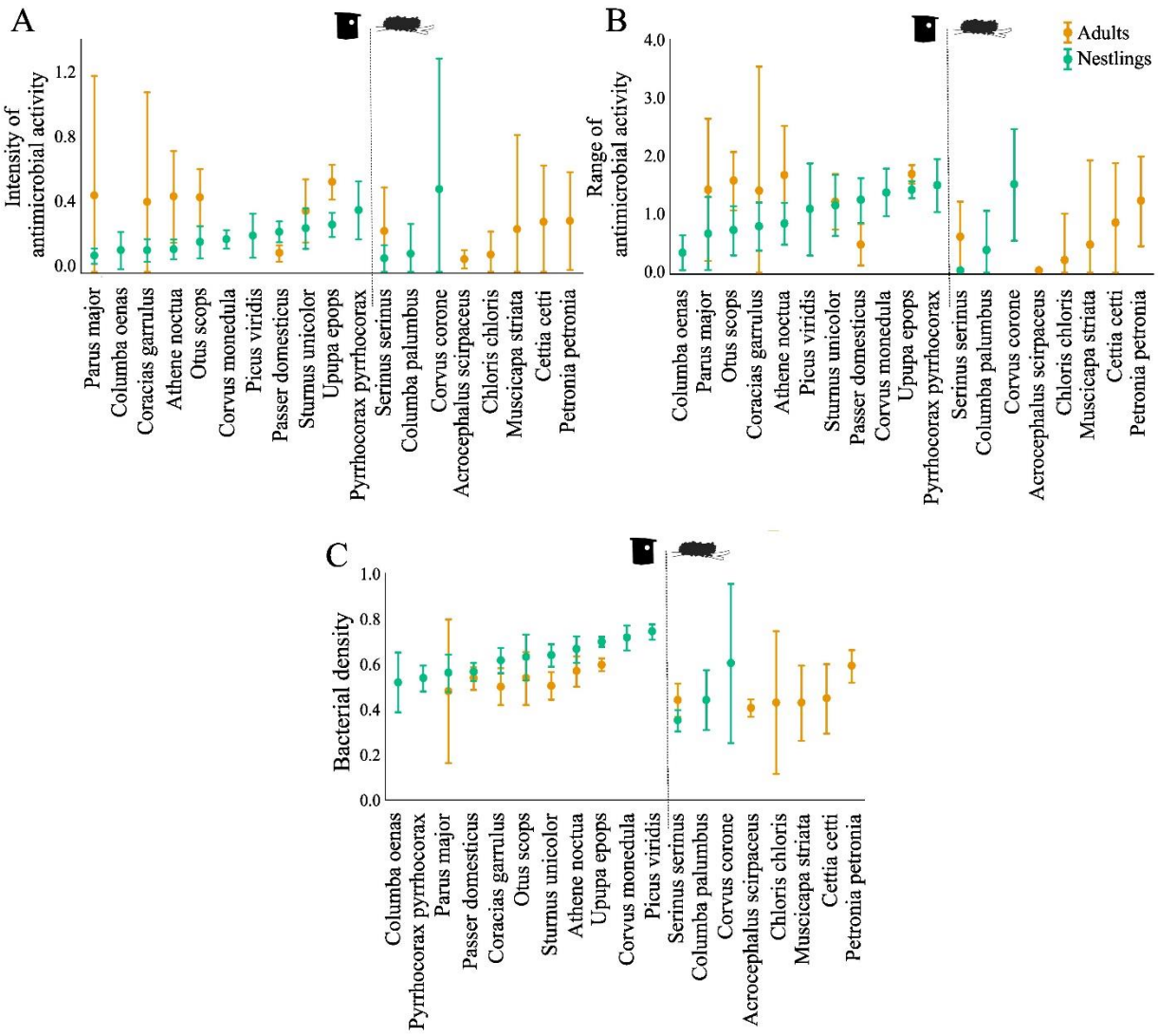

Figure 1. Antimicrobial capacity: **(A)** antimicrobial activity (average values of the width of antagonistic halos (mm) when tested against each of the nine indicator bacteria) and **(B)** antimicrobial range (Shannon index of the antimicrobial activity), of the bacterial communities associated to the uropygial gland skin of different bird species. **(C)** Bacterial density on the gland surface of the same species. Results for adults and nestlings are shown in different colours. Values are means ± 95% CI. CI are not symmetric because negative values are not possible.

Thus, on average, antimicrobial capacity of symbiotic bacteria isolated from adults of hole-nester species were more intense and diverse than those isolated from non-hole nesters (Fig. 2, Table 3). Similarly, when considering compositional variance of the antimicrobial profile, they significantly differed among species, and between hole-nester and non-hole-nester (Table 3, Fig. 3). Interestingly, bacterial density on the skin of adults depended on nesting habits, but not on species identity within hole and non-hole nester groups (Table 3). The bacterial density on uropygial gland skin of hole-nester species was higher than that on non-hole nesters (Fig. 2), suggesting that hole-nester species experience higher risk of bacterial infection. Phylogenetic corrected analyses confirmed all those results, but

differences in intensity of antimicrobial activity of bacteria isolated from hole- and non-hole-nester species was only close to statistical significance (Table 4). Interestingly, the three models showed significant phylogenetic components suggesting that antimicrobial capacity of bacteria from close-related species is similar to each other.

Similar to the results for adults, we found significant interspecific differences in bacterial density, and in the intensity, range and profile of antimicrobial capacity of bacteria isolated from the skin of the uropygial gland of nestlings (Table 3, Fig. 2, Fig. 3). In this case, the species with bacterial symbionts of higher intensity and range of antimicrobial activity (the carrion crow (*Corvus corone*)) was a non-hole nester, although it was followed by two hole-nester species; the red-billed chough (*Pyrrhocorax pyrrhocorax*) and the hoopoe. Moreover, two out of three species with bacterial symbionts with lower values of intensity of antimicrobial activity and of range of antimicrobial capacity were non-hole nesters (Fig. 1). Thus, although differences among hole and non-hole nesting species followed similar pattern in nestlings and adults, the intensity of antimicrobial activity of bacterial symbionts isolated from the gland skin of nestlings of hole- and non-hole-nester species did not differ significantly (Table 3, Fig. 2).

Table 3. Results of nested General Linear Models (GLM) and PERMANOVAs exploring the effects of nest type (hole vs non-hole) and species identity nested in nest type on the antimicrobial activity (average values of the width of antagonistic halos when tested against each of the nine indicator bacteria), antimicrobial range (Shannon index of the antimicrobial activity), bacterial density and the antimicrobial profile in separated models for each of the dependent variable, and for adults and nestlings. * F statistic is Pseudo-F in PERMANOVA analysis for antimicrobial profile. Values in bold are statistically significant.

	Adults			**Nestlings**		
	F*	df	p	F*	df	p
Antimicrobial activity						
Nest type	8.02	1, 97	**0.006**	0.26	1, 104	0.609
Species (Nest type)	2.45	11, 97	**0.01**	4.34	12, 104	**<0.001**
Antimicrobial range						
Nest type	36.74	1, 97	**<0.001**	6.69	1, 104	**0.011**
Species (Nest type)	4.32	11, 97	**<0.001**	6.47	12, 104	**<0.001**
Total bacterial density						
Nest type	23.14	1, 93	**<0.001**	33.98	1, 104	**<0.001**
Species (Nest type)	1.51	11, 93	0.142	5.21	12, 104	**<0.001**
Antimicrobial profile						
Nest type	8.54	1, 97	**<0.001**	5.74	1, 104	**<0.001**
Species (Nest type)	2.18	11, 97	**<0.001**	1.7	12, 104	**<0.001**

In any case, similarly to what we found in adults, estimated antimicrobial range (Fig. 2) and antimicrobial profiles (Fig. 3) of nestlings of hole- and non-hole nester species differed significantly (Table 3). Moreover, antimicrobial range, and bacterial density, were of higher values in hole-nester species (Table 3, Fig. 2). Phylogenetic corrected analyses only confirmed detected differences in bacterial density, but all three models showed significant phylogenetic components (Table 4).

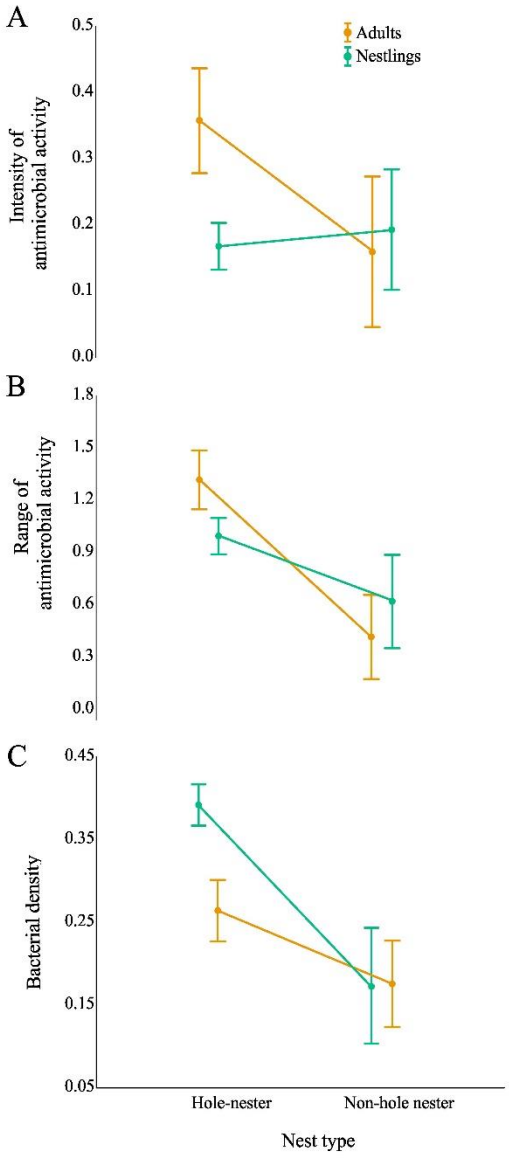

Figure 2. Effect of nesting habits of birds (hole *vs* non-hole) on the antimicrobial capacity and bacterial density of the community living on their uropygial gland skin. Graphs show the estimated antimicrobial capacity: **(A)** intensity of antimicrobial activity (average values of the width of antagonistic halos (mm) when tested against each of the nine indicator bacteria) and **(B)** antimicrobial range (Shannon index of the antimicrobial activity), as well as **(C)** density of bacteria isolated from the birds' gland skin after controlling for species identity nested in nest type. Results for adults and nestlings are shown in different colours. Values are least square means ±95% CI.

Table 4. Results of MCMCglmm models with nest type as a fixed factor, the bird phylogeny as a random factor and the antimicrobial activity (average values of the width of antagonistic halos when tested against each of the nine indicator bacteria), antimicrobial range (Shannon index of the antimicrobial activity) and bacterial density as dependent variables. The average of the 100 trees was calculated for each factor, as well as the 95% CI calculated as the lower and upper values for 95% Credibility Intervals of each estimate. Values in bold are statistically significant.

		Lower 95% CI	Upper 95% CI	ESS Lower 95% CI	ESS Upper 95% CI	Autocorr Lower 95% CI	Autocorr Upper 95% CI	(z-score) Lower 95% CI	(z-score) Upper 95% CI	pMCMC (-95%CI)	pMCMC (+95%CI)
Adults	**Antagonistic activity**										
	Nest type	-0.374	0.018	8960.682	9120.342	-0.002	0.002	-0.337	0.049	0.072	0.074
	heritability	0.012	0.354								
	Antagonistic range										
	Nest type	-1.358	-0.288	9012.492	9124.574	-0.004	0	-0.308	0.054	**0.006**	**0.006**
	heritability	0.07	0.53								
	Bacterial density										
	Nest type	-0.164	-0.002	8966.204	9061.441	-0.001	0.003	-0.254	0.2	**0.045**	**0.047**
	heritability	0.012	0.28								
Nestlings	**Antagonistic activity**										
	Nest type	-0.128	0.155	8839.062	9037.088	0	0.005	-0.109	0.25	0.876	0.886
	heritability	0.034	0.514								
	Antagonistic range										
	Nest type	-0.805	0.205	8948.682	9050.966	-0.002	0.002	-0.119	0.275	0.222	0.227
	heritability	0.178	0.68								
	Bacterial density										
	Nest type	-0.314	-0.065	8932.945	9032.842	-0.001	0.003	-0.319	0.097	**0.006**	**0.006**
	heritability	0.17	0.705								

Discussion

In the present study, we examined the antimicrobial capacities of the bacterial community harbored on the skin of the uropygial gland of nestlings and adults of hole- and non-hole-nester bird species. Our results reveal that, although the effects of age and nesting habits depended on the species identity, antimicrobial capacity of bacteria isolated from adults were more intense and diverse than those of bacteria isolated from nestlings. The uropygial skin of nestlings, however, carried bacteria at a higher density than that of adults. Moreover, intensity and range of antimicrobial capacities of bacterial isolates were higher in hole-nester species, which also harbored bacteria at a higher density. These effects of nesting habits were mainly detected for adult birds. Finally, variables describing either antimicrobial capacities or bacterial density of different bird species have a moderate but significant phylogenetic component. Because adult birds might have a more stable bacterial community than nestlings, and hole-nesters are under higher risk of bacterial infection than non-hole nesters are, those results might suggest that natural selection favors the establishment and growth of competent antibiotic-producing bacteria on the skin of birds. Below, we discuss that possibility and the potential role of uropygial secretion selecting antibiotic-producing bacteria that would contribute to the bird's antimicrobial defenses.

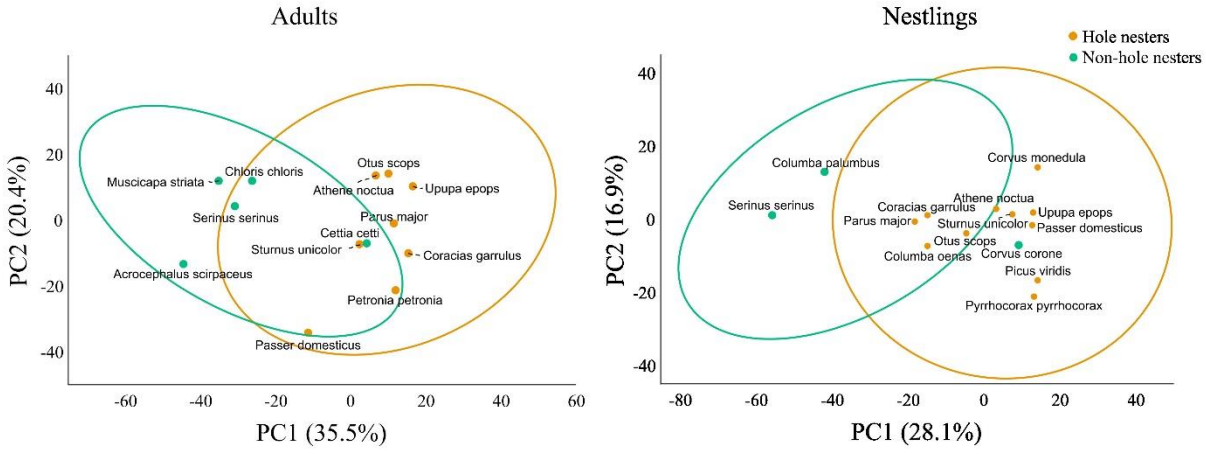

Figure 3. Principal Coordinates Analisis (PCoA) ordination plot using bray-curtis distances of the antimicrobial profile of bacterial communities isolated from the uropygial gland skin. Points are centroids per species and ellipses indicate 90% CI.

We detected consistent among species differences in characteristics of the bacterial communities, which occurred even after controlling for the effect of age or nesting habits. These differences could be explained by different species exploiting different resources or habitats where the pool of bacteria able to colonize the skin of birds might also differ

(Thompson *et al.* 2017). Bacterial communities of holes and open habitats that birds use for breeding differ (Godard *et al.* 2007; Peralta-Sánchez *et al.* 2012) and, thus, skin bacterial communities of hole and non-hole nesters should accordingly differ. Although our results fit that prediction in terms of bacterial density, it is important to highlight that bacteria from hole-nester species consistently demonstrated higher intensity and range of antimicrobial activity, which could also be explained because of a higher competition among bacterial strains in environments with higher bacterial densities, such as the gland skin of hole-nester species, thus favoring the establishment of antibiotic-producing bacteria.

Another non-exclusive explanation is that different species might differ in mechanisms preventing bacterial colonization of skin and some other integuments of birds (Soler *et al.* 2011; Peralta-Sánchez *et al.* 2018; Javůrková *et al.* 2019; Azcárate-García *et al.* 2020) that would result in species-specific skin bacterial communities with particular antimicrobial properties. Appropriate holes for bird breeding are scarce in nature (Newton 1994) and, thus, they are likely reused from season to season (Aitken, Wiebe & Martin 2002; Wiebe, Koenig & Martin 2007). Interestingly, nest reuse increases the risk of horizontal transmission of ectoparasites and pathogens (Møller & Erritzøe 1996; Tomás *et al.* 2007) and density of bacteria in nestlings' skin (González-Braojos *et al.* 2012). In accordance, we found higher bacterial density in the uropygial gland skin of hole-nester than in that of non-hole-nester species. Importantly, bacterial density of avian nests predicts the probability of hatching failure (Peralta-Sánchez *et al.* 2018), and, thus, selection pressures favoring the evolution of antibacterial defenses (i.e. innate humoral immunity (Soler *et al.* 2011)). In this scenario, natural selection should be stronger for hole-nester species and, accordingly the size of the bursa of Fabricius and the spleen of these species is larger than that of non-hole-nester species (Møller & Erritzøe 1996). Here, fitting with this pattern, we found that the antimicrobial capacity of bacteria isolated from hole-nester species were higher than that of non-hole nesters isolates. However, to conclude in favor of the hypothesis that our findings are the consequence of natural selection, a mechanism resulting from bird phenotypes must first be demonstrated as the cause of the detected differences in antimicrobial properties of bacteria isolated from hole-nester and non-hole-nester species.

A third non-exclusive possibility explaining interspecific differences in antimicrobial properties of bacteria isolated from the skin of the uropygial gland of birds is that characteristics of the uropygial secretion, which differ among species (Jacob & Ziswiler 1982), are associated with interspecific variation of bacterial communities (Soler *et al.* 2012). Uropygial secretion might thus be the avian trait where natural selection works favoring

antimicrobial potential of the microbial symbionts. Birds preen their feathers, bills and skin with uropygial secretion and, thus, the detected interspecific effects might be due to interspecific differences in antimicrobial properties of secretion (Moreno-Rueda 2017). Moreover, the preen secretion of different species also varies in chemical composition (Jacob & Ziswiler 1982) that might differentially enhance the growth of particular bacteria explaining detected interspecific variation in bacterial communities. Interestingly, chemical composition of the uropygial secretion of birds typically changes with phenology. The uropygial secretion of hoopoe females, for instance, changes during reproduction and as does its antimicrobial capabilities (Soler *et al.* 2008), which is mediated by the microbial symbionts (Ruiz-Rodríguez *et al.* 2009). Thus, although we know that microbial symbionts affect the chemical composition of secretion (Martín-Vivaldi *et al.* 2010), it is plausible that the remarkable changes in chemical composition of uropygial secretions command colonization and growth of their antibiotic-producing bacteria. Similarly, it is also possible that, because birds use the uropygial secretion for preening, interspecific variation in chemical composition of the secretion was the cause of the detected interspecific variation in antimicrobial capability of microbial symbionts. It has also been suggested that uropygial secretion modulated the microbiota of body feathers (Jacob *et al.* 2018), which might suggest that the uropygial secretion is also responsible for the detected antimicrobial characteristics of the bacteria that we isolated from the skin of the uropygial gland of different species.

Apart from its antimicrobial properties, the uropygial secretion of birds might function in scenarios of chemical communication including that related to inadvertent social information (Danchin *et al.* 2004; Caro, Balthazart & Bonadonna 2015; Mazorra-Alonso, Tomás & Soler 2021). Accordingly, variations in chemical composition have been described in association with individual characteristics including age, sex and phenology. (Reneerkens, Piersma & Damste 2002; Leclaire *et al.* 2011a; Leclaire *et al.* 2011b; Amo *et al.* 2012a; Díez-Fernández *et al.* 2021). Here, we have detected that the antimicrobial capacity of bacterial isolates differed depending on age, which might also be interpreted as a consequence of the age related variation of the chemical composition of the uropygial secretion between adults and nestlings. Age differences, however, would be hardly explained as a consequence of selection pressures due to risk of infections, mainly because bacteria grew at a larger density in nestlings than in adults, but also because antimicrobial activity of bacteria isolated from nestlings skin of hole-nester species did not differ from that of non-holer species. However, in accordance with the hypothetical role of natural selection driving antimicrobial capacity of nestling symbionts, the antimicrobial profile of nestlings of holer- and of non-holer species

differed significantly, with antimicrobial range of nestlings of the former species being larger than that of the latter group of species. Similarly to the age effects detected in antimicrobial capacity of bacterial symbionts, the immune responses of nestling birds are also typically weaker than that of adults (see Introduction), which is mainly explained as an ontogenetic effect of developing immune system (Apanius 1998). Thus, it is possible that the detected age effect was also due to the ontogenetic effects of developing uropygial secretion responsible of known differences in characteristics of the uropygial secretion of birds (Amo *et al.* 2012a; Amo *et al.* 2012b; Soler *et al.* 2022); a possibility worth exploring in the future.

Summarizing, we detected parallelism in the antimicrobial capacities of microbial symbionts and the strength of selection due to parasitic infections associated with nesting habits. Because detected differences could be hardly explained by random effects, we suggest that natural selection should favor mechanisms (i.e., characteristics of uropygial secretion) allowing cultivation of antibiotic-producing bacteria on the uropygial glands. These results therefore suggest a new line of animal immunity mediated by natural selection acting on traits determining antimicrobial capacity of their bacterial symbionts. Future research should focus on characterizing symbiotic bacterial communities and detecting coevolutionary processes with particular antibiotic-producing bacteria within-host species.

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CAPÍTULO III

Pathogen risks of nesting wild birds drives their skin and uropygial microbiome

Abstract

Animals associate with a diverse array of antibiotic producing bacteria that protect them against pathogens. In some instances, these defensive bacteria are cultivated in specialised body locations, but they or their antimicrobial products are used in some others parts as skin, eggs, or crops. Thus, finding associations (i.e., bacterial similarities) between the bacterial communities of locations where mutualistic bacteria are growing and where they are acting, as well as how this association varies according to risk of infection, is of crucial interest to understand that kind of symbiotic associations. Symbionts growing in exocrine glands are usually spread to other body locations and, then, are good candidates to study the similarities between different bacterial communities. In the case of birds, some species have antibiotic-producing bacteria with antimicrobial properties in their uropygial gland, yet these kind of bacteria are more often detected in the skin of species that suffer higher probabilities of pathogenic infections (i.e., cavity nesters). Here, we explore whether the microbiota of the secretion of 26 species of birds were correlated with the microbiota of the skin of the uropygial gland, and if the expected association depended on the risk of infection (i.e., nesting habits). Our results support the expected similarities between bacterial communities of the skin and secretion, and that those bacterial communities were tighter related in cavity than in non-cavity nester species. Our findings therefore suggest that the risk of infection experienced by the different species determine the level of community overlap among bacterial communities. Since birds under higher risk of pathogenic infections have bigger uropygial glands, we discuss the possibility that behavioural mechanisms (i.e., preening) were responsible of the flow of antibiotic-producing bacteria from the body location where they are being grown through other body parts where they are acting.

Keywords: *birds, microbiota, nesting habits, symbiotic bacteria, uropygial gland, uropygial secretion*

Introduction

Bacteria dominate the world in which animals live, reproduce and evolve (McFall-Ngai *et al.* 2013), and where these microorganisms have parasitic to mutualistic interactions with animals. Although most bacteria have neutral effects on animal hosts, some play key roles in ecology and evolution of their hosts (Fraune & Bosch 2010). Animal microbiota, for instance, facilitate essential functions of their hosts related to nutrition (Backhed *et al.* 2004; Krajmalnik-Brown *et al.* 2012), development (Bates *et al.* 2006; Heijtz *et al.* 2011; Schwarzer *et al.* 2016), activation of the immune system (Umesaki *et al.* 1995; Riley & Wertz 2002; Weiss, Maltz & Aksoy 2012; Mazorra-Alonso, Tomás & Soler 2021), chemical communication (Archie & Theis 2011; Ezenwa & Williams 2014; Wada-Katsumata *et al.* 2015; Maraci, Engel & Caspers 2018; Grieves *et al*. 2019; Mazorra-Alonso, Tomás & Soler 2021), or direct defence against pathogens (Harris *et al.* 2009; Martín-Vivaldi *et al.* 2014; Hoyt *et al.* 2015; Martínez-Renau *et al.* 2022). Bacterial communities in symbiosis with animals are not isolated from each other or from those in the animal surroundings (Martinez-Garcia *et al.* 2016; Soler *et al.* 2016; van Veelen, Salles & Tieleman 2017). Moreover, the beneficial effects of these symbionts might rely on these bacteria reaching the body location in which they act, and therefore on the associations between bacterial communities from different body locations. In some cases, animals might even facilitate some bacterial species or strains to expand and disperse from one to another location where they are incorporated into particular microbial communities (Martinez-Garcia *et al.* 2016; Soler *et al.* 2016; van Veelen, Salles & Tieleman 2017). This is especially true for bacterial communities associated with exocrine glands that, due to animal grooming behaviour, will colonise other locations. The bacterial communities of the exocrine glands can include beneficial strains that, for instance, could prevent hosts from pathogenic infections, as it is the case of fungus-growing ants and Eurasian hoopoes (*Upupa epops*). The fungus-growing ants have special body locations (crpyts) where exocrine glands tip out and where they cultivate the antibiotic-producing bacteria that are used in fungal gardens to keep them free of microbial pathogens (Currie *et al.* 2006). The hoopoes use the antibiotic-producing bacteria cultivated in their uropygial gland to smear their feathers (Ruiz-Rodríguez *et al.* 2009) or eggshells, thus preventing pathogenic infections (Martín-Vivaldi *et al.* 2014). Detecting associations between bacterial communities of locations where animals cultivate antibiotic-producing bacteria and those locations where these bacteria act protecting their hosts is important to understand mutualistic interactions between bacteria and their animal hosts, and would allow

to predict co-evolutionary patterns between bacteria and the body locations where animals cultivate those antibiotic-producing microorganisms (Currie *et al.* 2006).

Animal integuments (i.e., skin, hair or feathers) constitute the first defensive barrier against pathogens and they are often smeared with secretions produced by animal exocrine glands (Mills & Prum 1984; Kaltenpoth *et al.* 2005; Soler *et al.* 2016; Bush & Clayton 2018) that contain bacteria (Theis *et al.* 2013; Leclaire, Nielsen & Drea 2014; Whittaker & Theis 2016; Braun *et al.* 2018; Braun *et al.* 2019; Bodawatta *et al.* 2020; Rojas *et al.* 2020). In the case of birds, the uropygial secretion has a species-specific chemical composition (Jacob & Ziswiler 1982) that includes antimicrobial components. Interestingly, in some species, antimicrobials in the secretion are, at least partially, produced by the bacterial community harboured inside the gland (Soler *et al.* 2008), which opens the possibility that birds cultivate particular antibiotic-producing bacteria in their uropygial gland. Birds spread this secretion onto the skin, feathers and other body parts while preening and, thus, the antibiotic-producing bacteria can reach different body regions beyond the uropygial gland. In accordance, Soler *et al.* (2016) reported a nested pattern of bacterial communities from the uropygial gland of hoopoes to their bill, brood patch and eggshells, suggesting that the antibiotic-producing bacteria found on the eggshells derive from the bacterial community inhabiting the uropygial gland secretion (see also Martín-Vivaldi *et al.* 2014). This pattern may be generalized to other bird species since antibiotic-producing bacteria has also been detected in the skin of the uropygial gland of several bird species (Martínez-Renau *et al.* 2022), particularly in those experiencing higher risk of pathogenic infection. Since the gland skin is in close contact with the uropygial secretion, it was hypothesised that antibiotic-producing bacteria detected in the skin might derived from those cultivated inside the gland (Martínez-Renau *et al.* 2022).

Here, by characterizing the bacterial communities of the uropygial gland and skin, as well as the similarities among them, we explore the possibility that antibiotic-producing bacteria detected on the skin derived from those cultivated inside the gland in adults and nestlings of 26 bird species. This hypothesis predicts that characteristics (i.e., diversity and/or composition) of the bacterial communities of the skin and the secretion should be related to each other. Thus, exploring how this kind of association varies according to species-specific ecological factors related to risk of infection can provide insight into possible adaptive functions of animal behaviours that enhance bacterial flow among body locations. Ecological factors and lifestyle of animals should explain particularities of the animal phenotypes including traits that determine characteristics of mutualistic symbionts (Currie *et al.* 2006; Li *et al.* 2018). Among these traits, some will determine environment for bacterial symbionts

cultivation, while some others will enhance the association among microbial communities or locations (source and sink dynamics). Therefore, we expect that selection pressures due to risk of pathogenic infection should not only affect characteristics of the bacterial communities (i.e. their antimicrobial potential) of the skin and secretion (see Martínez-Renau *et al.* 2022), but also the level of association among them. Thus, if birds cultivate antimicrobial-producing bacteria within their gland, we predict that the association between the microbiota of the gland and that of the skin should be stronger in species experiencing higher risk of pathogenic infection. To explore this possibility and following previous works (Tella, Scheuerlein & Ricklefs 2002; Pap *et al.* 2015; Martínez-Renau *et al.* 2022), we considered the nesting habits as a proxy of risk of pathogenic infection. This is because second-cavity nesters tend to reuse breeding sites due to scarcity of natural cavities, and hence, are more susceptible to horizontal transmission of pathogens than non-cavity breeders (Møller & Erritzøe 1996).

Bird species that phylogenetically are closely related are more likely exploiting similar resources, and experiencing similar environmental conditions and selection pressures due to parasitism (Harvey & Pagel 1991). Then, they are more likely to interact with similar bacterial communities (Martínez-Renau *et al.* 2022). Consequently, if host phylogeny and parasitism selection pressure accounts for variability of their microbial symbionts, it should be reflected by patterns of phylosymbiosis (Lim & Bordenstein 2020) that should be clearer in species under stronger selection pressures (i.e., cavity nesters). Here we also tested this prediction and, because phylosymbiosis is more likely detected in communities isolated from the external environment (Kohl, Dearing & Bordenstein 2018; Ross *et al.* 2018; Lim & Bordenstein 2020), we predicted that those patterns should be more clearly detected for bacterial communities of the uropygial secretion than for those of the gland skin.

Methods

Study area and species

Fieldwork was carried out during the breeding seasons of 2018, 2019 and 2021 in southern Spain; at the Hoya de Guadix (37°15′N; 3°01′W), a semiarid high-altitude plateau, and in the Charca de Suárez, a wetland by the coast in Motril (36° 43' 18.707"N, 3° 32' 30.836"W). Most nestlings and adults of cavity-nester species were sampled while nesting in cork-made nest boxes (internal dimensions: 180 mm x 210 mm and 350 mm high, 240 mm from the bottom to the hole entrance) during the breeding period at the Guadix study area. In the same area, when possible, we also sampled nestlings of open-nester species few days before

leaving the nests. By mist netting in both study areas, we captured adults and fledglings few days after abandoning the nest.

Fieldwork

Since mid-March, we visited the installed nest boxes in the area every 3 days and looked intensively for nests of non-cavity nester species until egg detection, which allowed us to infer the laying date and, hence, to plan the sampling date of adults and nestlings. We sampled nestlings during their last quarter of the nesting period. Cavity-nester adults were captured from the nest box during brooding, while non-cavity nester adults and fledglings were captured using mist nets. We sampled the microbiota of the uropygial gland skin and of the uropygial secretion from each captured bird species (Table 1). The uropygial gland skin was sampled by rubbing the surface skin of the uropygial gland, including the opening, with a sterile cotton swab (APTACA, ref. 2160, Canelli, Italy) wetted in sterile Phosphate Buffer Saline (1X PBS, 0.2 M). The swab was kept in a sterile microfuge vial with 1 mL of sterile PBS, and stored at -18°C until DNA extraction. The uropygial secretion was extracted using a sterile micro-capillary (32mm, 10ul), placed on the gland opening, while slightly pressing the gland until emptying it. Once emptied, the micro-capillary was kept in a sterile microfuge vial and stored at -18°C until DNA extraction. We also measured tarsus length with a digital calliper (accuracy 0.01 mm), wing length with a ruler (accuracy 1 mm), body mass with a digital scale (accuracy 0.01 g), and gland dimensions (length, width and height) with a digital calliper (accuracy 0.01 mm) following Martín-Vivaldi *et al.* (2009). We calculated the gland volume as the width*height*length and log10 transformed to adjust the values to a normal distribution. Finally, we ringed all individuals with numbered metal rings (Ministerio de Agricultura, Spain) to avoid resampling.

DNA extraction and high-throughput sequencing

DNA from the uropygial gland skin bacterial communities was extracted using the FavorPrep^{TM} Blood Genomic DNA Extraction Mini Kit (Favorgen Biotech Crop., Taipei, Taiwan), with the following protocol: first, we sonicated the sample for 2 minutes at 120 Hz trying to release the bacterial cells from the swab. We then removed the swab and centrifuged the PBS with the bacteria at 13,000 rpm for 5 min. Afterwards, we discarded the supernatant, and added to the pellet 180 µl of TES (25 mM Tris-HCl, pH 8, 10 mM EDTA and 10% sucrose), 10 mg/ml of lysozyme and 4 µl of RNasa (10 mg/mL). Subsequent steps were performed following the FavorPrep^{TM} instructions.

Table 1. Sample sizes of adults and nestlings of uropygial gland skin and secretion with taxonomic information.

Order	Family	Species	Cavity nesters	Age class	Gland skin	Gland secretion
Bucerotiformes	Upupidae	*Upupa epops*	Yes	Adults	41	
				Nestlings	67	
Columbiformes	Columbidae	*Columba oenas*	Yes	Adults		
				Nestlings	12	21
		Columba livia	No	Adults		
				Nestlings		6
		Columba palumbus	No	Adults		
				Nestlings	6	9
Coraciiformes	Coraciidae	*Coracias garrulus*	Yes	Adults	4	7
				Nestlings	17	17
Passeriformes	Corvidae	*Corvus monedula*	Yes	Adults		
				Nestlings	12	22
		Pyrrhocorax pyrrhocorax	Yes	Adults		
				Nestlings	11	23
		Corvus corone	No	Adults		
				Nestlings		4
	Paridae	*Parus major*	Yes	Adults	3	10
				Nestlings	12	13
	Passeridae	*Passer domesticus*	Yes	Adults	11	10
				Nestlings	15	33
		Passer montanus	Yes	Adults		4
				Nestlings		
		Petronia petronia	Yes	Adults	5	10
				Nestlings		25
	Sturnidae	*Sturnus unicolor*	Yes	Adults	11	
				Nestlings	17	
	Acrocephalidae	*Acrocephalus scirpaceus*	No	Adults	6	13
				Nestlings		
	Fringillidae	*Carduelis carduelis*	No	Adults	4	
				Nestlings		
		Chloris chloris	No	Adults	4	9
				Nestlings		
		Serinus serinus	No	Adults	11	13
				Nestlings	8	10
	Cettiidae	*Cettia cetti*	No	Adults	4	8
				Nestlings		
	Muscicapidae	*Luscinia megarhynchos*	No	Adults		5
				Nestlings		
		Muscicapa striata	No	Adults	5	11
				Nestlings		
	Sylviidae	*Sylvia atricapilla*	No	Adults		6
				Nestlings		
		Curruca melanocephala	No	Adults		11
				Nestlings		
Piciformes	Picidae	*Picus viridis*	Yes	Adults		
				Nestlings	9	
Strigiformes	Strigidae	*Athene noctua*	Yes	Adults	8	9
				Nestlings	13	18
		Otus scops	Yes	Adults	8	11
				Nestlings	15	11
		Asio otus	No	Adults		
				Nestlings		3

DNA extractions from the bacterial communities within uropygial secretions were performed following the protocol in Boom *et al.* (2000) with the subsequent modifications to optimize lysis and DNA extraction. The sample was resuspended in 30 µl of lysis solution and incubated for 10 min at 75 ˚C. Afterwards, a volume of 30 µl of neutralization solution

was added. From this extraction 5 µl were used to perform PCR reaction. To verify the presence of bacterial DNA in both uropygial gland skin and uropygial secretions we conducted PCRs using primers B969F (ACGCGHNRAACCTTACC) and BA1406R (ACGGGCRGTGWGTRCAA) (Comeau *et al.* 2011). The PCR products were visualized on a 1% agarose gel with electrophoresis. The same primers were also used to construct Illumina libraries targeting the V6-V8 region of the bacterial 16S rRNA gene. Library construction and sequencing in the MiSeq platform was carried out at the Institute of Parasitology and Biomedicine "López-Neyra" facilities (IPBLN, Granada, Spain).

MiSeq sequence analyses

Raw reads from uropygial gland skin and uropygial secretion obtained from Illumina MiSeq were processed together with QIIME2 v2020.6 (Bolyen *et al.* 2019) using the following pipeline: first, we used the DADA2 (Callahan *et al.* 2016) plugin implemented in QIIME2 for primer trimming, sequence quality filtering, chimera removal and clustering into ASVs (Amplicon Sequence Variants). Then, the representative sequence of each ASV was taxonomically assigned using Silva 138 database (Quast *et al.* 2012). Mitochondrial, chloroplastidial, archeal and sequences not identified at the phylum level, and non-bacterial sequences were removed from the ASV table. To avoid potential contaminant sequences, possible contaminant ASVs were identified with Decontam package in R (Davis *et al.* 2018; R-Core-Team 2020), comparing against laboratory and field controls, using the prevalence method and a threshold of 0.4. Identified contaminant ASVs were removed from the ASV table. A sequences alignment and a rooted bacterial phylogeny was generated using the function “align-to-tree-mafft-fasttree” in QIIME2. The ASV table was rarefied to the minimum sampling depth (1,651) using the function “rarefy_even_depth” on “phyloseq” package (McMurdie & Holmes 2013) within R v4.0.2 (R-Core-Team 2020).

Statistical analyses

Gland size and nesting habits

To explore the effect of the nesting habits on the gland size of the bird species, we controlled the gland volume for the interspecific variability, by using residuals of gland size after correcting for body mass that were estimated for adults and nestlings of each considered species separately. Those residuals were used as the dependent variable in subsequent analyses. The final model included the nest type and the species ID nested within nest type as fixed factors, and in nestling models we also included the nest identity nested within

species (in turn nested within nest type) as the random factor. We computed these analyses using TIBCO Statistica™ software (version 14).

Alpha and beta diversity

The Chao1 richness and the Shannon's diversity of microbiomes were calculated using "microbiome" package (Lahti & Shetty 2017), and Faith's phylogenetic diversity (PD) was computed using "picante" package (Kembel *et al.* 2010) within R v4.0.2 (R-Core-Team 2020). We first tested the effect of age (adults *versus* nestlings) and species identity (ID) by means of General Linear Models (GLM) using only species with samples for both age classes. The used models included age class and species ID as fixed factors and nest identity nested within the interaction between species and age as the random factor, while the interaction between the fixed factors was explored in separate models that also included the main effects. Because of the possibility that the effect of age differed for cavity and open nester species, we repeated these analyses for the cavity nester species. The effect of nest type was explored independently for adults and nestlings, which allowed increasing the number of species considered in each analyses. In these cases, the GLMs included nest type (cavity nester vs non-cavity nester species) and species ID nested within nest type as fixed factors, whereas in nestling models we also included the nest identity nested within species (in turn nested within nest type) as the random factor. GLM analyses were computed using TIBCO Statistica™ software (version 14).

Beta diversity distance matrices were calculated using Bray-Curtis, Jaccard, weighted UniFrac and unweighted UniFrac distances by means of "phyloseq" package (McMurdie & Holmes 2013) in R environment (v4.0.2) (R-Core-Team 2020). The generated distance matrices were used in Permutational ANOVAs (PERMANOVAs) computed by PRIMER7 v.7.0.17 (PRIMER-e). To test for the effect of age class and species ID, we included both variables as fixed factors, and nest identity nested within the interaction between age class and species ID as random factor. The effect of the interaction between age class and species ID was explored in separate models. Similarly, as in alpha diversity, the effect of nest type was explored in independent PERMANOVAs for nestlings and adults, which allowed increasing the species number used in each model. The statistical model for adults included nest type and species ID nested within nest type as fixed factors, while the model for nestlings also included the nest identity nested within species (which in turn was nested within nest type) as random factor to account for the non-independence data of nestlings from the same nest. Microbial community differences (only for Bray-Curtis distances) were visualized in

Non-Metric Multidimensional Scaling (NMDS) plots in "phyloseq" package (McMurdie & Holmes 2013).

Since nesting habits have a strong phylogenetic component, we controlled the alpha diversity analyses for the bird's phylogeny by means of Bayesian phylogenetic mixed models (MCMCglmm). Firstly, we generated the host species phylogeny using three mitochondrial genes (Cytochrome b [cyt b], NADH dehydrogenase subunit 2 [ND2] and cytochrome oxidase subunit I [COI]) sourced from GenBank and built individual gene alignments using MAFFT (Katoh *et al.* 2002) as implemented in SEAVIEW (Gouy, Guindon & Gascuel 2010). We then analysed individual gene partitions in BEAST v1.8.4 (Drummond *et al.* 2012) as an initial way to check the quality of the downloaded sequence data. Subsequently, we generated a concatenated dataset (2,905 base pairs) partitioned by genes in BEAST v1.8.4 (Drummond *et al.* 2012) using the GTR + I + G nucleotide substitution model as determined by jmodeltest (Guindon & Gascuel 2003; Darriba *et al.* 2012). We used a relaxed uncorrelated lognormal distribution for the molecular clock model, and assumed a birth-death speciation process as a tree prior. The Markov chain Monte Carlo (MCMC) algorithm was run several times for 100 million iterations with trees sampled every 10,000th generation. Convergence of individual runs was assessed using Tracer 1.6 (Rambaut *et al.* 2014) ensuring all ESS > 200 and graphically estimating an appropriate burn-in). TreeAnnotator 1.8.2 (Rambaut & Drummond 2015) was used to summarize a single maximum clade credibility (MCC) tree using mean node heights. We constrained the relationships of our tree by locking avian orders according to Jarvis *et al.* (2014) and Prum *et al.* (2015) and by locking passerine bird families according to Oliveros *et al.* (2019). To obtain absolute dates, we applied 1) a rate of 0.0145 substitutions per site per lineage (2.9%) per Myr to our ND2 data, 2) a rate of 0.007 substitutions per site per lineage (1.4%) per Myr to our cyt-b data, and 3) a rate of 0.008 substitutions per site per lineage (1.6%) per Myr to our COI data following Lerner *et al.* (2011).

The resulting phylogenetic tree of avian hosts was used to estimate the predicted effects of nesting habits using the MCMCglmm package (Hadfield 2010) in R environment. To run the model we used the uninformative prior [list(G = list(G1 = list(V = 1,nu = 0.002)),R = list(V = 1,nu = 0.002)], and adjusted the number of iterations to 2,000,000, the burn-in period to 100,000 and the thinning interval to 2,000. We also used Geweke's convergence diagnostic for Markov chains (Geweke 1992), which is based on a standard z-score of means of the first (10%) and the last part (50%) of a Markov chain. These absolute z-scores never exceeded the critical value of 1.96. The random effect of phylogeny is reported as heritability

(h2) (Hadfield 2010), which is a measure of phylogenetic signal analogous to Pagel's lambda that ranges from zero (non-phylogenetic signal) to one (high phylogenetic signal).

In order to control the effects of the nesting habits on the beta diversity for birds' phylogeny, we performed mantel tests including any of the distance matrices for the bacterial communities of the uropygial gland skin or secretion as dependent variables. As independent matrices, we used a binary matrix with information on the nest type, where the "0" and "1" values indicated that pairs of samples had the same or different nesting habits respectively, and a matrix with phylogenetic distances between bird species (see above). For nestling's analyses, we also included a binary matrix with "0" and "1" values, which indicates if particular pair of nestlings belonged to the same or different nest.

Comparison between gland skin and gland secretion bacterial communities

To compare the bacterial community of the two body locations, we used a subset of the individuals for which we had samples from the uropygial gland skin and secretion (n = 80). Under the assumption that uropygial gland skin microbiome partially comes from that of the uropygial gland secretions, we investigated the association between these two microbiomes using partial mantel test with 10,000 permutations. For these models, we used the distance matrix of the uropygial gland skin microbiome as the dependent variable, and the uropygial secretion distance matrix as the independent variable. We also added a binary matrix where the "0" and the "1" values respectively indicated that the cells corresponded to the interaction between the same or different species ID. The mantel test was performed with the whole set of samples and for cavity and non-cavity nesters separately, which allowed exploring if the correlation between both bacterial communities was consistent among different ecological scenarios. We used several methods to estimate and build the distance matrix (Bray-Curtis, Jaccard, Weigthed UniFrac and Unweighted UniFrac) and repeated the analyses with the ASV table, and after collapsing information at genus level. We also controlled these analyses for the bird's phylogeny by replacing the species matrix for the phylogenetic distances between bird species.

In order to explore whether the bacterial community found in the uropygial gland skin comes, at least partially, from the bacterial community inhabiting the secretion (inside the uropygial gland), we conducted source tracking analysis using SourceTracker 1.0. We categorized the secretion bacterial community as the "source", and the skin bacterial community as the "sink". After training the data set with default parameters, for each species, we estimate the proportion of bacteria in the uropygial skin that might originate from the

uropygial secretion. The species with samples for nestlings and adults were analysed separately for each age class. Furthermore, using statistical models similar to those specified above for the alpha diversity, we conducted statistical analyses to explore whether the proportions of the bacterial community of the uropygial gland skin coming from secretion differed among age class, species, and nest type.

Phylosymbiosis analyses

The methods used for testing the phylosymbiosis require the number of bird species in the phylogenetic tree to match the number of bird species in the microbial distance matrix. Thus, for adults and nestlings separately, we calculated the average abundance for each ASV per species. Hierarchical clustering was performed to build the microbial dendogram from the distance matrices created with Bray-Curtis, Jaccard, weighted UniFrac and unwegithed UniFrac, while the phylogenetic distances between host species were measured with the "adephylo" package (Jombart & Dray 2010).

We used Mantel tests to explore the correlations between the bird phylogenetic distances and those of their microbial community by means of Pearson's correlations and using 10,000 random permutations. Mantel tests were performed with the "vegan" package (Oksanen *et al.* 2022). Furthermore, we used a cluster-matching method (Robinson-Foulds metric) to explore the topological dissimilarities between bird phylogeny and bacterial dendogram. We used the function "RF.dist" from the package "phangorn" (Schliep 2011) to calculate the normalized distances, and compared them with a random null model with 1,000 random trees to assess significance. Robinson-Foulds metrics ranges from 0 (complete congruency between topologies) to 1 (no matching nodes).

Results

Gland size and nesting habits as proxies of parasitism selection pressure

The uropygial gland was bigger in adults of cavity nester species than in non-cavity nesters after controlling for body mass, species ID, and nest ID (nesting habits: $F_{(1, 14)} = 24.5$, $p < 0.001$; LS means ± SE cavity nesters = 0.05 ± 0.03; LS means ± non-cavity nesters = -0.19 ± 0.04). For nestlings, we found a similar non-significant trend (nesting habits: $F_{(1, 233.5)} = 0.85$, $p = 0.359$; LS means ± SE cavity nesters = -0.04 ± 0.03; LS means ± non-cavity nesters = -0.16 ± 0.05).

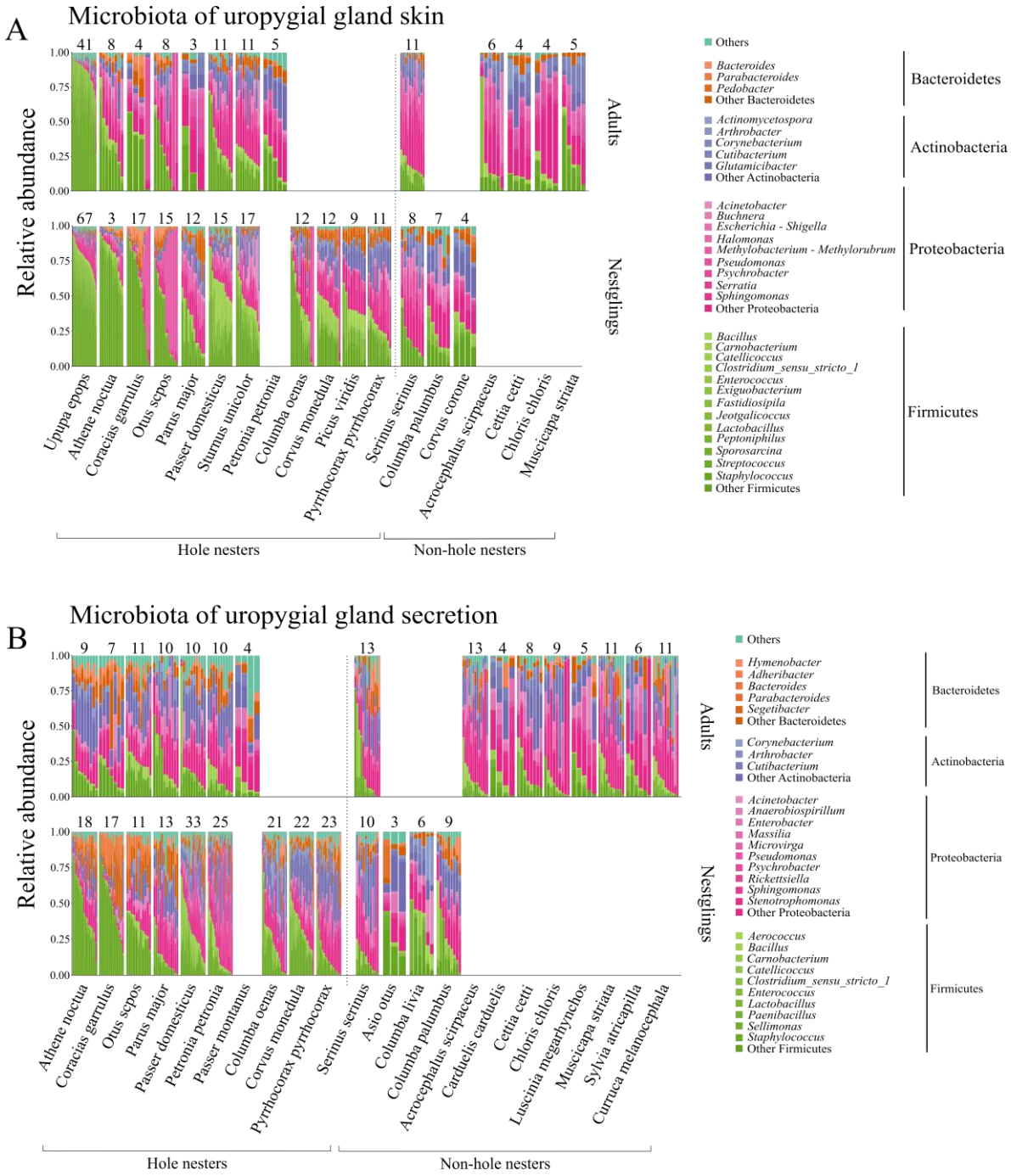

Figure 1. Stacked barplot showing the relative abundance of the most frequently detected genera for each individual. The figure provides information on the species identity, age class and nest type, as well as the sampling size per species (number above bars) for both kinds of bacterial communities (uropygial gland skin and secretion).

Composition of the uropygial gland and secretion microbiota

We successfully sequenced the microbiota of 691 samples: 339 from the gland skin (121 from adults and 218 from nestlings), and 352 from uropygial gland secretion (148 from adults and 204 from nestlings) (Table 1). Both data sets included cavity (gland skin: 12 species; gland secretion: 10 species) and non-cavity nester species (gland skin: 7 species; gland

secretion: 12 species) (Table 1). From the gland skin microbiota, we obtained 10,077,955 sequences classified into 23,101 ASVs. Each sample had an average of 29,728.5 (SD = 20,876.3) sequences. Rarefaction was set to the minimum sampling depth of 1,651 sequences, leading to 15,981 ASVs. The data set was dominated by Firmicutes (51.7%), Proteobacteria (29.9%), Actinobacteriota (10.5%) and Bacteroidota (4.9%) (Fig. 1). From the uropygial secretion, we obtained 8,223,291 sequences before rarefaction, classified into 33,658 ASVs. Each sample had an average of 23,361.6 (SD = 16,269) sequences. After rarefaction, we retained 27,083 ASVs. The data set of uropygial secretion was dominated by Proteobacteria (34%), Firmicutes (24.17%), Actinobacteriota (22.5%) and Bacteroidota (13.2%) (Fig. 1).

Table 2. Results of General Linear Models (GLMs) exploring the effects of species identity (ID), age class and nest type on alpha diversity variables (Chao1, Shannon index and PD). Species identity and age class effects were explored together in models including both variables, while the interaction was calculated in separate models that also included the main effects. The effect of nest type was explored in a nested GLM models that included nest type and species ID nested within nest type. Values in bold are those with associate alpha value lower than 0.05.

		Chao1			**Shannon index**			**PD**		
		F	*df*	p	F	*df*	p	F	*df*	p
Effect of age class										
	Gland skin									
	Species	**11.63**	**7, 195.4**	**<0.001**	**5.45**	**7, 198.5**	**<0.001**	**4.35**	**7, 252**	**<0.001**
	Age	**41.36**	**1, 196.3**	**<0.001**	**12.33**	**1, 199.7**	**<0.001**	0.01	1, 252	0.927
	Species*Age	1.42	7, 193.5	0.200	**3.38**	**7, 199.4**	**0.002**	**3.31**	**7, 192.4**	**0.002**
	Nest(Species*Age)	**2.61**	**182, 70**	**<0.001**	**2.09**	**182, 70**	**<0.001**	**2.97**	**182, 70**	**<0.001**
	Gland secretion									
	Species	**5.72**	**6, 126.8**	**<0.001**	5.14	**6, 131.9**	**<0.001**	**5.27**	**6, 126.1**	**<0.001**
	Age	**4.28**	**1, 129.1**	**0.040**	0.00	1, 135	1.000	0.02	1, 128.3	0.892
	Species*Age	1.67	6, 118.8	0.135	**2.23**	**6, 123.9**	**0.045**	**3.04**	**6, 119.4**	**0.008**
	Nest(Species*Age)	**3.45**	**113, 76**	**<0.001**	**2.50**	**113, 76**	**<0.001**	**3.65**	**113, 76**	**<0.001**
Effect of nest type										
Adults	*Gland skin*									
	Nest type	**13.38**	**1, 108**	**<0.001**	**9.75**	**1, 108**	**0.002**	**16.64**	**1, 108**	**<0.001**
	Species (nest type)	1.79	11, 108	0.065	**8.36**	**11, 108**	**<0.001**	**3.25**	**11, 108**	**<0.001**
	Gland secretion									
	Nest type	**55.17**	**1, 125**	**<0.001**	**54.80**	**1, 125**	**<0.001**	**88.92**	**1, 125**	**<0.001**
	Species (nest type)	**4.14**	**14, 125**	**<0.001**	**2.74**	**14, 125**	**0.001**	**3.62**	**14, 125**	**<0.001**
Nestlings	*Gland skin*									
	Nest type	3.06	1, 144.7	0.083	2.00	1, 143.9	0.160	0.35	1, 151.2	0.558
	Species (nest type)	**30.44**	**12, 113.6**	**<0.001**	**5.53**	**12, 113.5**	**<0.001**	**12.74**	**12, 114.5**	**<0.001**
	Nest (Species(Nest type))	**2.43**	**109, 95**	**<0.001**	**2.48**	**109, 95**	**<0.001**	**2.02**	**109, 95**	**<0.001**
	Gland secretion									
	Nest type	**9.86**	**1, 131.5**	**0.002**	**4.84**	**1, 148**	**0.029**	**4.11**	**1, 140.4**	**0.045**
	Species (nest type)	**2.51**	**11, 93.6**	**0.008**	**2.32**	**11, 97.8**	**0.014**	**1.99**	**11, 95.8**	**0.037**
	Nest (Species(Nest type))	**2.53**	**83, 115**	**<0.001**	**1.82**	**93, 115**	**0.002**	**2.10**	**83, 115**	**<0.001**

Microbial diversity, composition and the influence of age class and nesting habits

Alpha diversity

Overall, the microbial communities of the gland skin and gland secretion of nestlings were richer (Chao1 richness estimate) than those of adults (Table 2, Additional Fig. 1). Similarly, Shannon's diversity index was significantly higher in nestlings than adults for the uropygial gland skin; but were similar for the gland-secretion community (Table 2, Additional Fig. 1). PD values did not differ between adults and nestlings in neither the skin nor the secretion bacterial communities (Table 2, Additional Fig. 1). The results were similar when only considering cavity-nester species (Additional Table. 1). Moreover, the richness and diversity of the bacterial community also varied interspecifically (Table 2), and the interaction between age class and species identity reached statistical significance, for the Shannon and PD models, which suggest that the significant effect of age differed depending on the species (Additional Fig. 1).

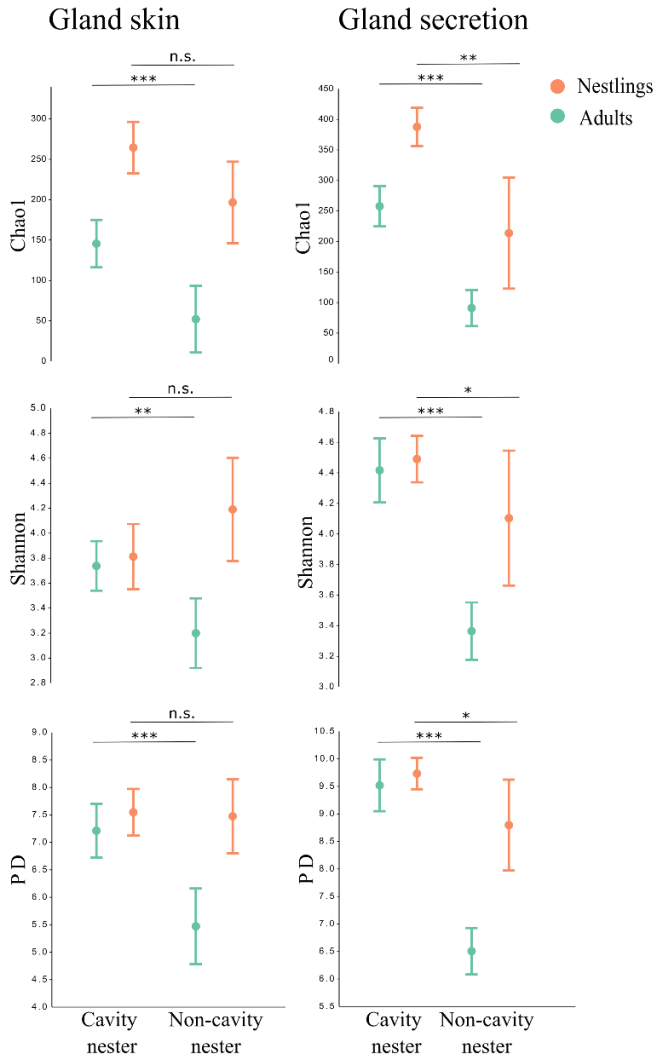

Figure 2. Least square means of alpha diversity indexes (Chao1, Shannon index and PD) for cavity and non-cavity nester species, in adults and nestlings, after controlling for species identity. Significant comparisons are labelled with * (<0.001), ** (<0.01) or *** (<0.05).

When considering only adult samples, cavity nesters harboured richer and more diverse bacterial communities in both the gland skin and secretions (Table 2, Fig. 2). The secretion bacterial communities of cavity-nester nestlings were also richer and more diverse than that of nestlings from non-cavity nester species, but nesting habits did not associate with alpha diversity indexes of the bacterial community of nestlings' skin (Table 2, Fig. 2). The statistical inferences for adults did not change after controlling for the bird's phylogeny, although the alpha diversity differences between cavity and non-cavity nester nestlings mainly disappeared (Additional Table 2).

Beta diversity

Regardless of the distance matrix used, the composition of the bacterial community of gland skin and gland secretion differed among species and age classes, (Table 3). Moreover, the interaction between age class and species ID was statistically significant in all cases. In addition, nest type was a good predictor of the bacterial community composition of gland skin and gland secretion for adults, since it differed significantly among cavity and non-cavity nester species when using any of the considered distance matrices (Table 3, Fig. 3A). Similarly, nest type influenced the bacterial composition of nestling's gland skin, irrespective of the distance matrix used. In the case of nestling's gland secretion, the bacterial community composition varied with nest type when considering Bray-Curtis or Jaccard. However, these associations in nestlings did not reach statistical significance when considering weighted or unweighted UniFrac (Table 3). Phylogenetically controlled models provided similar results although some (Bray-Curtis, Jaccard and unweighted UniFrac for the gland skin of adults, unweighted UniFrac for the gland secretion of adults and weighted UniFrac for the gland skin of nestlings) of the effects described above did not reach statistical significance (Additional Table 3). Moreover, pairwise comparisons of both bacterial communities (uropygial gland skin and secretion) in dispersion tests (average distance to spatial median) showed that adults and nestlings of cavity-nester species had higher distances to spatial median than non-cavity-nester adults (Fig. 3B, Additional Table 4).

Table 3. Results of PERMANOVA analyses exploring the effects of species identity (ID), age class and nest type on beta diversity (distance matrices constructed with Bray-Curtis, Jaccard, weighted UniFrac and unweighted UniFrac). Species ID and age class effects were explored together in models including both variables and the nest identity nested within the interaction of species ID and age class. The interaction between species ID and age was calculated in separate models that also included the main effects. The effect of nest type was explored in nested models that included nest type, species ID nested within nest type and nest identity nested within species ID (in turn nested within nest type). Values in bold are those with associate alpha value lower than 0.05.

		Bray-Curtis			**Jaccard**			**Unweighted UniFrac**			**Weighted UniFrac**		
		Pseudo-F	*df*	p	Pseudo-F	*df*	p	Pseudo-F	*df*	p	Pseudo-F	*df*	p
Effect of age class													
	Gland skin												
	Species	**8.71**	**7, 175**	**<0.001**	**5.31**	**7, 175**	**<0.001**	**7.71**	**7, 175**	**<0.001**	**36.05**	**7, 175**	**<0.001**
	Age	**6.08**	**1, 191.1**	**<0.001**	**4.05**	**1, 192.8**	**<0.001**	**8.63**	**1, 198.5**	**<0.001**	**8.08**	**1, 205.9**	**<0.001**
	Species*Age	**1.51**	**6, 126.5**	**<0.001**	**1.78**	**7, 195.9**	**<0.001**	**2.87**	**7, 200.3**	**<0.001**	**6.15**	**7, 199.9**	**<0.001**
	Nest (Species *Age)	**2.03**	**175, 77**	**<0.001**	**1.81**	**175, 77**	**<0.001**	**1.35**	**175, 77**	**<0.001**	1.00	175, 77	0.508
	Gland secretion												
	Species	**2.53**	**6, 107.0**	**<0.001**	**1.88**	**6, 107.0**	**<0.001**	**4.06**	**6, 107.0**	**<0.001**	**3.81**	**6, 107.0**	**<0.001**
	Age	**2.07**	**1, 127.3**	**<0.001**	**1.58**	**1, 131.0**	**<0.001**	**3.28**	**1, 130.3**	**<0.001**	**3.71**	**1, 127.0**	**0.011**
	Species*Age	**1.52**	**6, 127**	**<0.001**	**1.30**	**6, 131.1**	**<0.001**	**2.05**	**6, 128.7**	**<0.001**	**2.38**	**6, 123.9**	**0.004**
	Nest (Species *Age)	**1.74**	**107, 82**	**<0.001**	**1.46**	**107, 82**	**<0.001**	**1.51**	**107, 82**	**<0.001**	**1.80**	**107, 82**	**<0.001**
Effect of nest type													
Adults	*Gland skin*												
	Nest type	**4.02**	**1, 108**	**<0.001**	**2.64**	**1, 108**	**<0.001**	**7.53**	**1, 108**	**<0.001**	**10.77**	**1, 108**	**<0.001**
	Species (nest type)	**3.40**	**11, 108**	**<0.001**	**2.34**	**11, 108**	**<0.001**	**2.84**	**11, 108**	**<0.001**	**16.68**	**11, 108**	**<0.001**
	Gland secretion												
	Nest type	**2.65**	**1, 125**	**<0.001**	**1.88**	**1, 125**	**<0.001**	**16.35**	**1, 125**	**<0.001**	**10.06**	**1, 125**	**<0.001**
	Species (nest type)	**1.31**	**14, 125**	**<0.001**	**1.18**	**14, 125**	**<0.001**	**1.93**	**14, 125**	**<0.001**	**1.56**	**14, 125**	**0.015**
Nestlings	*Gland skin*												
	Nest type	**2.59**	**1, 135.2**	**<0.001**	**1.83**	**1, 143.5**	**<0.001**	**2.92**	**1, 155.8**	**0.001**	**11.67**	**1, 141.1**	**<0.001**
	Species (nest type)	**4.75**	**12, 112.4**	**<0.001**	**3.15**	**12, 113.5**	**<0.001**	**6.96**	**12, 115.3**	**<0.001**	**9.46**	**12, 113.2**	**<0.001**
	Nest (Species (Nest type))	**3.29**	**109, 95**	**<0.001**	**2.45**	**109, 95**	**<0.001**	**1.75**	**109, 95**	**<0.001**	**2.65**	**109, 95**	**<0.001**
	Gland secretion												
	Nest type	**1.99**	**1, 148.1**	**<0.001**	**1.53**	**1, 158.9**	**<0.001**	1.73	1, 146.6	0.133	2.50	1, 159.46	0.090
	Species (nest type)	**2.36**	**11, 97.7**	**<0.001**	**1.78**	**11, 100.8**	**<0.001**	**3.80**	**11, 97.3**	**<0.001**	**3.10**	**11, 101**	**<0.001**
	Nest (Species (Nest type))	**1.83**	**83, 115**	**<0.001**	**1.51**	**83, 115**	**<0.001**	**1.88**	**83, 115**	**<0.001**	**1.49**	**83, 115**	**<0.001**

Association between gland skin and gland secretion

Mantel tests revealed that the two bacterial communities were significantly correlated for both adults and nestlings at the ASV-level for all tested distance matrices except for unweighted UniFrac distances (Table 4). Interestingly, when considering only nestlings from cavity-nester species, the correlation between both bacterial communities was highly significant at the ASV and genus level, whereas the correlation between both bacterial communities in adults of cavity nester species was only significant when considering weighted or unweighted UniFrac. In contrast, we did not find an association between both bacterial communities in adults or nestlings from non-cavity nester species (Table 4, Fig. 4A). These results did not change after controlling for bird's phylogeny (Additional Table 5). Moreover, source-tracking analyses revealed that the proportions of the gland skin bacterial community originated in the gland secretion differed among species (Table 5, Additional Fig. 2), and the nest type was only a significant predictor of the proportions for nestlings. While nestlings of cavity nesters had a higher proportion of bacteria coming from the uropygial gland secretion (Table 5, Fig. 4B), the nestlings of non-cavity nesters and adults of all considered species presented lower proportions of bacteria originated in the secretion (Table 5, Fig. 4B)

Table 4. Results from Mantel tests exploring correlations between uropygial gland skin and uropygial gland secretion microbiomes using the whole set of samples, and for cavity and non-cavity nesters separately, both at ASV and Genus level. Bray-Curtis, Jaccard, weighted UniFrac and unweighted UniFrac were used to build the distance matrices. Number of permutations was set to 10,000. Values in bold are those with associate alpha value lower than 0.05.

		Bray-Curtis		**Jaccard**		**Unweighted UniFrac**		**Weighted UniFrac**	
		R	p	R	p	R	p	R	p
Adults	All samples								
	ASV-level	**0.16**	**0.038**	**0.18**	**0.020**	-0.01	0.541	**0.25**	**0.013**
	Genus-level	0.02	0.378	0.02	0.386	-0.02	0.569	**0.19**	**0.049**
	Cavity-nesters								
	ASV-level	-0.09	0.780	-0.04	0.624	**0.31**	**0.019**	**0.36**	**0.029**
	Genus-level	0.09	0.238	0.08	0.256	**0.42**	**0.002**	**0.42**	**0.021**
	Non- cavity nesters								
	ASV-level	0.19	0.078	0.20	0.065	-0.02	0.565	0.18	0.119
	Genus-level	0.08	0.268	0.07	0.286	-0.08	0.784	-0.01	0.485
Nestlings	All samples								
	ASV-level	**0.33**	**<0.001**	**0.35**	**<0.001**	0.04	0.299	**0.23**	**0.003**
	Genus-level	**0.35**	**<0.001**	**0.30**	**<0.001**	0.05	0.263	0.11	0.106
	Cavity-nesters								
	ASV-level	**0.45**	**<0.001**	**0.43**	**<0.001**	0.02	0.381	**0.19**	**0.040**
	Genus-level	**0.35**	**<0.001**	**0.29**	**<0.001**	0.07	0.190	0.03	0.357
	Non- cavity nesters								
	ASV-level	-0.17	0.884	-0.18	0.897	0.04	0.376	-0.07	0.602
	Genus-level	-0.12	0.725	-0.14	0.771	-0.01	0.422	-0.16	0.758

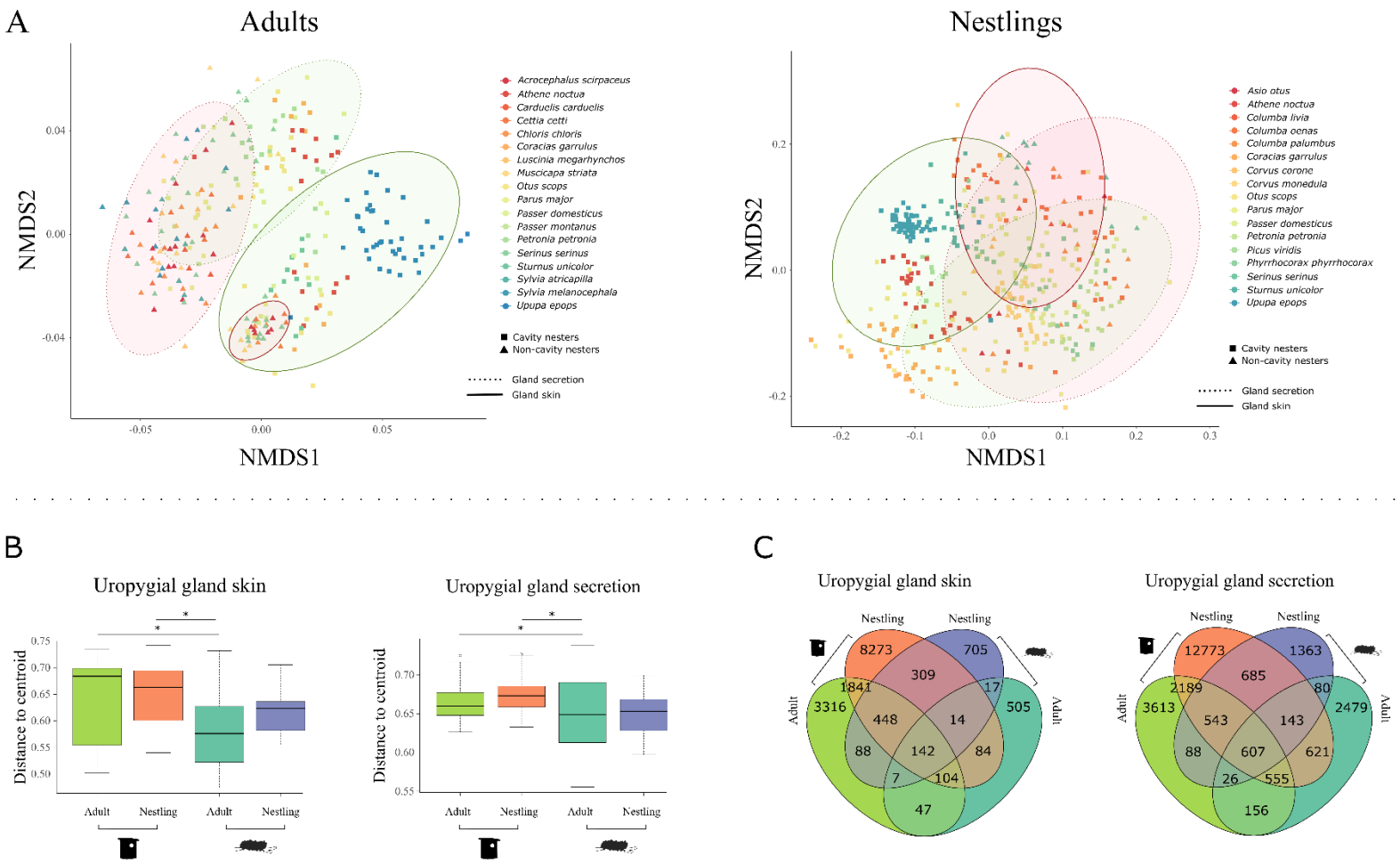

Figure 3. (A) NMDS ordination plots of adults and nestlings showing the diversity of bacterial communities of uropygial gland skin and secretion for the different sampled species including cavity (squares) and non-cavity nesters (triangles). Green (cavity nesters) and red (non-cavity nester ellipses correspond to 95% CI for each group. (B) Box plots showing group dispersions with Bray-Curtis distance matrix (betadisper analyses) of adults and nestlings from cavity and non-cavity nester species. (C) Venn diagram showing the number of shared ASVs among groups of adults and nestlings from cavity and non-cavity nesters.

Table 5. Results of General Linear Models using source tracking output. The proportion of the gland skin bacterial community originated in the gland secretion was used as dependent variable. We explored the effects of age class, species identity (ID) and nest type in two different models. The first model included the species ID and age class as fixed factors, while the interaction between them was analysed in separate models including also the main effects. The effect of nest type was explored separately for adults and nestlings, including the nest type and the species ID nested within nest type as independent variables. Values in bold are those with associate alpha value lower than 0.05.

		F	*df*	p
Effect of age class				
	Species	**5.68**	**3, 36**	**0.003**
	Age	2.35	1, 35.7	0.134
	Species*Age	**12.22**	**3, 31.1**	**<0.001**
	Nest (Species*Age)	**8.89**	**36, 3**	**0.048**
Effect of nest type				
Adults	Nest type	1.473	1, 34	0.233
	Species (nest type)	**2.47**	**7, 34**	**0.037**
Nestlings	Nest type	**32.45**	**1, 21.6**	**<0.001**
	Species (nest type)	**5.50**	**5, 18.3**	**0.003**
	Nest(Species(Nest type))	**2.54**	**21, 7**	**0.105**

Phylosymbiosis analyses

The Robinson-Foulds test was not significant for any comparison, lacking of congruence between the topologies of host phylogeny and tested microbial community dendograms. We observed multiple significant mantel correlations between uropygial gland skin or uropygial gland secretion and the bird's phylogeny for adults and nestlings only when considering Bray-Curtis or Jaccard distance matrices (Table 6). Moreover, when splitting the samples into cavity or non-cavity nesters, the phylogeny of the bacterial communities only associated with that of their host birds for nestlings of cavity nester species (Table 6, Fig. 4C).

Figure 4. (A) Linear regression plots showing the correlation among individual distances of the uropygial gland skin bacterial community and the bacterial community from the uropygial gland secretion of adults and nestlings on cavity and non-cavity nesters using Bray-Curtis and weighted UniFrac distance matrices. (B) Results of the source tracking analyses showing the proportions of the uropygial gland skin microbiota that is originated in the microbiota of the uropygial gland secretion or in an unknown source. (C) Linear regression plots showing the correlation of the bacterial community distances and the host phylogenetic distances.

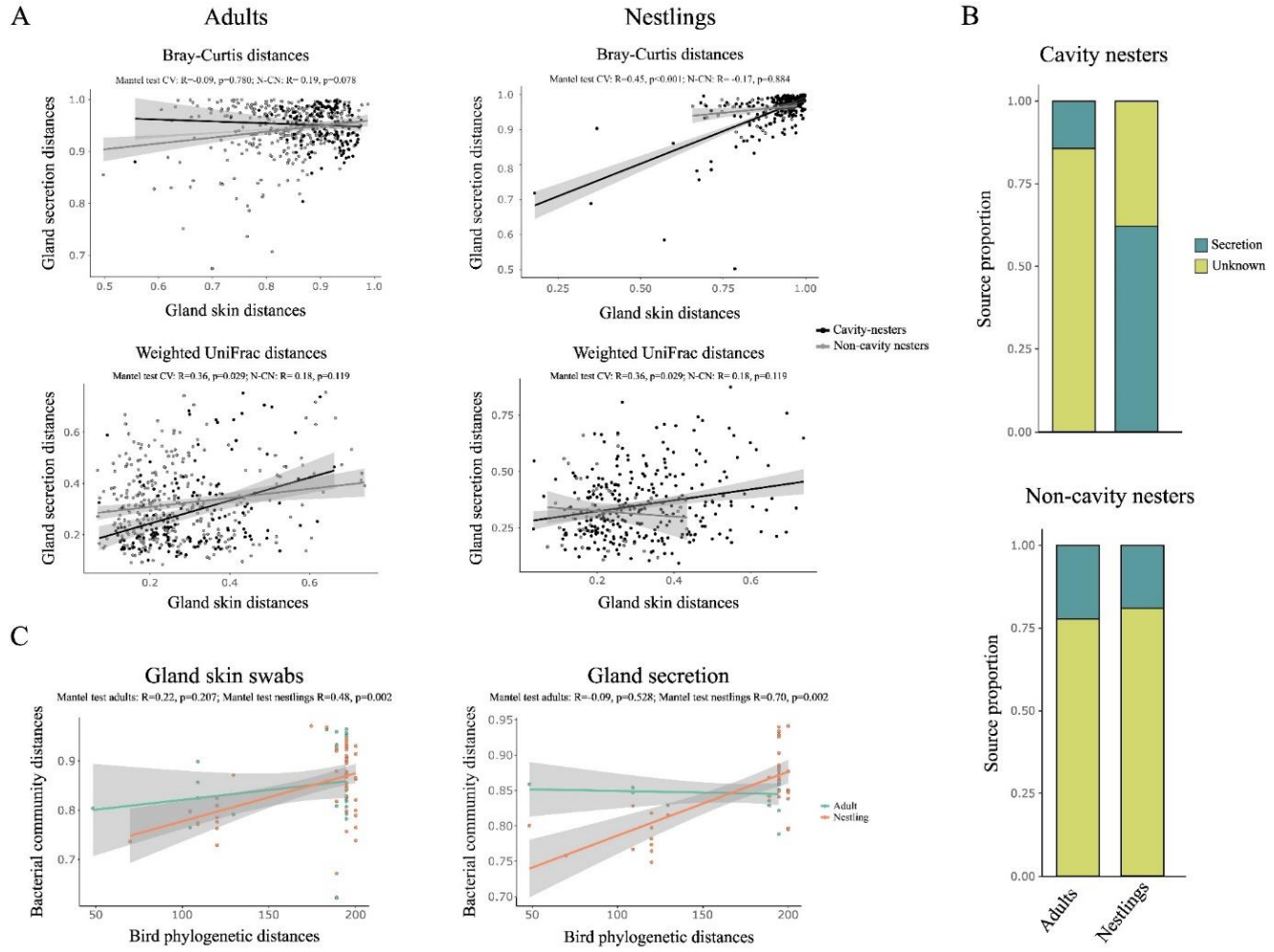

Table 6. Results of the phylosymbiosis analyses using either the whole data set, or data of cavity and non-cavity nester species separately. We compared the bacterial communities (uropygial gland skin and secretion) against the phylogeny of birds (host phylogeny). We used the Mantel test with 10,000 permutations to compare the distance matrices and the Robinson-Foulds test to compare the topology of the dendrograms. The distance matrices for the bacterial community were calculated using Bray-Curtis, Jaccard, unweighted and weighted UniFrac. Significance did not change after FDR correction. Values in bold are those with associate alpha value lower than 0.05.

			Uropygial gland skin					**Uropygial gland secretion**				
			Mantel test		Robinson-Foulds distances			Mantel test		Robinson-Foulds distances		
			R	**P**	**RF (Obs)**	**Null mean**	**p**	**R**	**P**	**RF (Obs)**	**Null mean**	**p**
Whole set of samples	Adults	bray	**0.46**	**0.014**	0.91	0.95	0.907	**0.48**	**0.006**	1.00	0.97	0.733
		jaccard	**0.46**	**0.015**	0.91	0.95	0.905	**0.48**	**0.007**	1.00	0.97	0.735
		unifrac	-0.05	0.613	0.91	0.97	0.963	0.08	0.241	1.00	0.99	0.868
		wunifrac	0.34	0.125	1.00	0.97	0.733	0.13	0.159	1.00	0.97	0.644
	Nestlings	bray	**0.48**	**<0.001**	1.00	0.98	0.757	**0.47**	**0.003**	1.00	0.94	0.578
		jaccard	**0.48**	**0.001**	1.00	0.98	0.761	**0.48**	**0.004**	1.00	0.94	0.577
		unifrac	0.09	0.231	1.00	0.98	0.752	0.15	0.135	0.90	0.97	0.966
		wunifrac	0.19	0.096	1.00	0.98	0.780	0.17	0.123	1.00	0.99	0.879
Cavity nesters	Adults	bray	0.22	0.207	1.00	0.95	0.756	-0.09	0.528	1.00	0.93	0.759
		jaccard	0.20	0.215	1.00	0.95	0.758	-0.09	0.533	1.00	0.92	0.750
		unifrac	-0.08	0.585	1.00	0.96	0.800	-0.33	0.889	1.00	0.92	0.756
		wunifrac	0.30	0.105	1.00	0.95	0.735	-0.16	0.706	1.00	0.93	0.765
	Nestlings	bray	**0.48**	**0.002**	0.89	0.94	0.887	**0.70**	**0.003**	0.71	0.91	0.970
		jaccard	**0.48**	**0.002**	0.89	0.94	0.888	**0.71**	**0.002**	0.71	0.91	0.968
		unifrac	**0.60**	**<0.001**	0.78	0.91	0.956	0.25	0.068	0.71	0.91	0.968
		wunifrac	0.20	0.133	1.00	0.98	0.865	0.24	0.116	0.86	0.94	0.919
Non-Cavity nesters	Adults	bray	0.68	0.100	0.67	0.87	0.930	-0.11	0.735	1.00	0.98	0.863
		jaccard	0.70	0.100	0.67	0.87	0.934	-0.11	0.739	1.00	0.98	0.860
		unifrac	0.46	0.100	0.67	0.88	0.933	-0.04	0.580	1.00	0.97	0.847
		wunifrac	0.18	0.367	1.00	0.88	0.705	-0.25	0.942	1.00	0.96	0.766
	Nestlings	bray	0.72	0.333	0.00	0.67	1.000	-0.33	0.667	1.00	0.67	0.667
		jaccard	0.73	0.333	0.00	0.67	1.000	-0.34	0.667	1.00	0.68	0.676
		unifrac	-0.32	0.667	1.00	0.67	0.668	-0.80	1.000	1.00	0.66	0.665
		wunifrac	-0.89	1.000	1.00	0.67	0.669	-0.78	1.000	1.00	0.67	0.667

Discussion

In the present study, we aimed to explore the association between the bacterial communities of the uropygial gland skin and its secretion, and the influence of parasitism selection pressure on this association. We recently detected species-specific differences in antimicrobial potential of bacterial communities of the uropygial gland skin that associated to risk of pathogenic infection (Martínez-Renau *et al.* 2022). Bacteria on the gland skin were hypothesized to partially derive from the bacteria cultivated within the uropygial gland, and we here explore this prediction. Our findings of the remarkable overlap between skin and uropygial microbiomes provide support for this prediction. Our results also revealed interspecific differences in both bacterial communities, emphasising the role of species-specific factors in shaping the microbial assemblages. Moreover, both types of bacterial communities as well as the association between them varied according to the nesting habits, which we used here as a proxy of the selection pressures due to pathogenic infection. All these results suggest that the risk of infection by pathogenic bacterial strains partly determines the assemblage and association of the bacterial communities of gland skin and secretion. Here, we discuss the importance of preening as a possible mechanism explaining the detected associations as well as the importance of species-specific intrinsic factors, ontogenetic and evolutionary processes driving detected interspecific differences.

The bacterial communities of the uropygial gland skin and of the secretion varied between species. Interspecific differences occurs in most of the animal symbiotic microbial communities (McKenzie *et al.* 2012; Chiarello *et al.* 2018; Ronque *et al.* 2020; Bodawatta *et al.* 2021), and are commonly explained by species-specific differences in habitat characteristics (Hernández *et al.* 2022; Matheen, Gillings & Dudaniec 2022). Associations between symbiotic bacterial communities from different body locations are expected because within individuals (i) the substrate or location of different bacterial communities are chemically similar (Byrd, Belkaid & Segre 2018; DeCandia *et al.* 2021), (ii) the bacterial communities are physically close to each other (Yasuda *et al.* 2015), and (iii) grooming behaviours allow direct contacts and exchanges between bacterial communities (Martín-Vivaldi *et al.* 2014). The bacterial communities of the uropygial gland skin and secretion are particularly close to each other and, when the birds collect the secretion for preening, they repetitively grasp their glands and the secretion is spread through the gland skin. Thus, the secretion might not only act as a source of bacterial strains but also provide a chemical substrate where bacteria from the gland would be able to grow. These two characteristics of

birds would then explain the detected resemblance of the studied bacterial communities in both adults and nestlings.

In a previous study using the same pool of species, we found interspecific differences on the antimicrobial capabilities of bacterial isolates from the uropygial gland skin (Martínez-Renau *et al.* 2022). Different species are exposed to different environments that would select for morphological and physiological characteristics of the gland and, then, of its bacterial communities (Li *et al.* 2018). Nesting habits explained the antimicrobial capabilities of the bacterial communities from the uropygial skin (Martínez-Renau *et al.* 2022), suggesting that the selection pressures due to pathogenic infections are likely explaining those differences. Similarly, here we also observed that the richness and composition as well as the inter-individual variability of the bacterial communities of the uropygial gland skin of adults and nestlings differed among species with different nesting habits, which might indicate that the risk of pathogenic infections is influencing the bacterial community assemblage. Previous works have detected evidence supporting links between bacterial communities and selection pressures due to parasitism or pathogenic infections (Scarborough, Ferrari & Godfray 2005; Rebollar *et al.* 2016), and more specifically to nesting habits (Godard *et al.* 2007; Peralta-Sánchez *et al.* 2012). We found similar results in the bacterial community of the secretion of adults and nestlings, suggesting that these selection pressures are also modulating the studied bacterial communities.

Because bacterial communities of the skin and of the secretion are established on the same individual experiencing particular selection pressures, the risk of pathogenic infections should also influence the strength of the association between them (see above). We found that this expected association is stronger and more consistent in nestlings. At the time of sampling, adults visit the nests mainly to feed their nestlings and, thus, different bacterial strains from the environments other than the nests can colonise their uropygial gland skin. In contrast, only bacteria from the uropygial secretion or nest environment will reach the nestling skin, which might explain that the associations between the microbiota of the skin and secretion were clearer in nestlings than in adults. Another possible explanation would be that nestlings preen more frequently than adults do, and, thus, the contact between microbial communities occurs more often. The uropygial gland of adults is fully developed and its associated bacterial community has higher antimicrobial properties than that of nestlings (Martínez-Renau *et al.* 2022). Therefore, they might need to preen less frequently than nestlings to maintain their feathers in a good condition. Moreover, due to nest reusing (Møller & Erritzøe 1996) and longer nesting periods (Martin & Li 1992), nestlings that develop in

cavities, experience higher risk of pathogenic infection than those developing in open nests, and thus it would be expected higher rates of preening with antimicrobial secretion in the former species. The rate of preening and the uropygial gland size are highly correlated with the amount of secretion produced (Pap *et al.* 2010). Our results did not detect statistical significant differences in gland sizes of nestlings from cavity and non-cavity nesters, but these differences are highly significant in adults, which evidence that hole-nesters preen more frequently than non-cavity nester species. Thus, in accordance with the previous argument, we found that the bacterial communities of uropygial gland skin and secretion were more closely related to each other in nestlings of cavity nester than in non-cavity nester species. Interestingly, the results of source-tracking analyses supported the hypothesis that, at least for hole-nesting species, the direction of the association is likely to be from the secretion towards the skin of nestlings, which suggest that particular bacteria are responsible of the detected associations.

The nesting habits (Wallace 1889), the gland size (Jacob & Ziswiler 1982) and the secretion composition (Jacob & Ziswiler 1982) have a phylogenetic component, and then the detected effects of cavity nesting on (i) the bacterial communities of the uropygial gland skin and secretion, (ii) the association among them and (iii) the gland size, could also be the result of phylogenetic relatedness among considered species. However, we only found a weak association between bird phylogeny and bacterial composition, suggesting that co-evolutionary processes between birds and their symbionts would unlikely explain the detected correlations between parasitism selection pressures and host traits determining characteristics of the studied bacterial communities. Therefore, the adaptive explanation of the risk of pathogenic infections shaping bacterial communities that include antimicrobial-producing bacteria would be the most likely. Although the number of species that we considered was relatively high, looking for these patterns in a wider range of species is necessary to reach a stronger conclusion.

Summarising, our results strongly suggest that the ecological factors are responsible, not only of the species-specific differences on the symbiotic bacterial communities that include antibiotic-producing bacteria, but also of the connectance or relation between them. Since the ecological factor that explained the differences and associations of the bacterial communities was the nesting habits, our results point towards the selection pressures due to risk of pathogenic infections as an important driver of the microbiota of the uropygial gland skin and secretion of birds. Future research should focus on searching specific antibiotic-producing bacteria on the uropygial gland skin and in the secretion.

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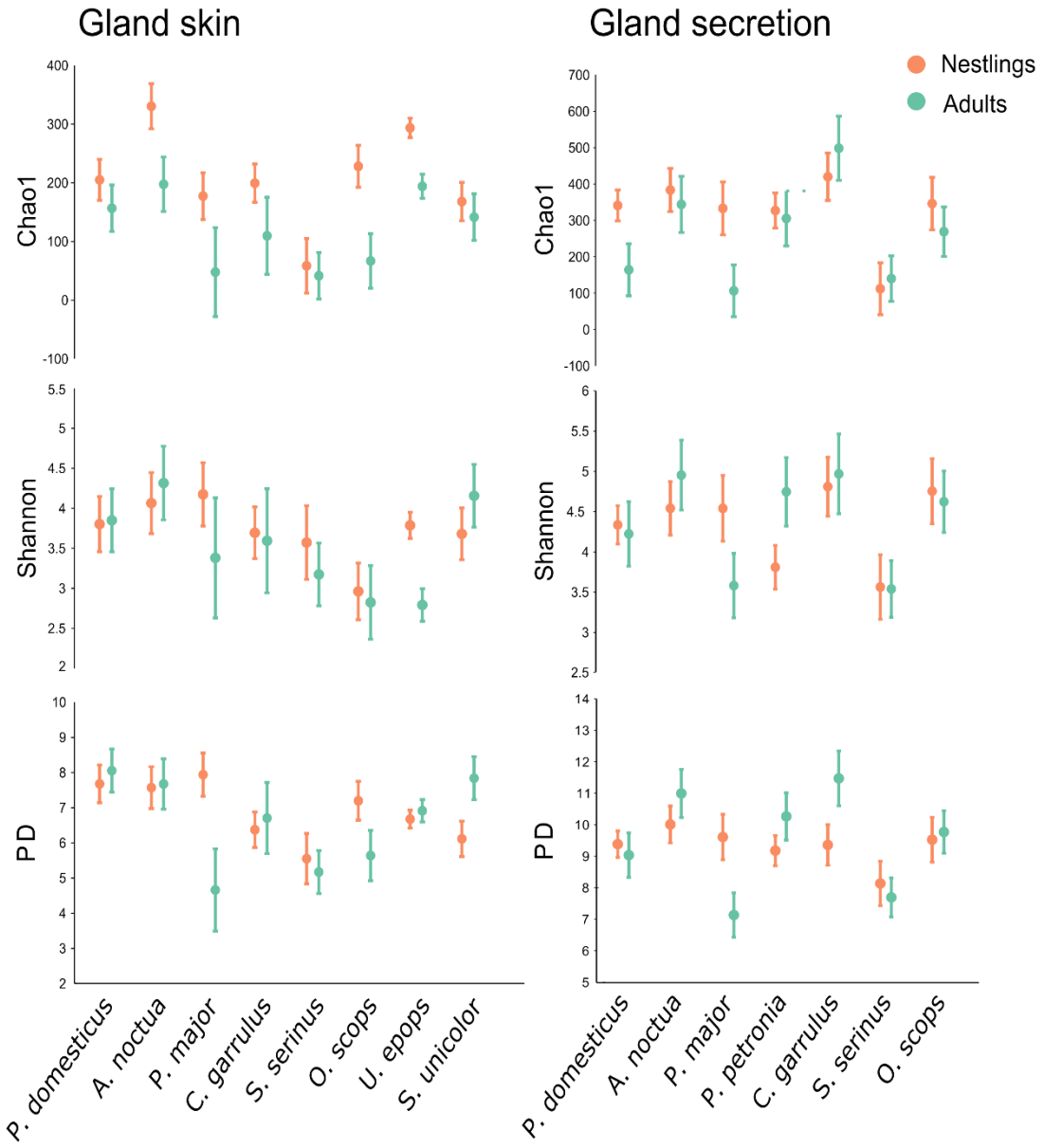
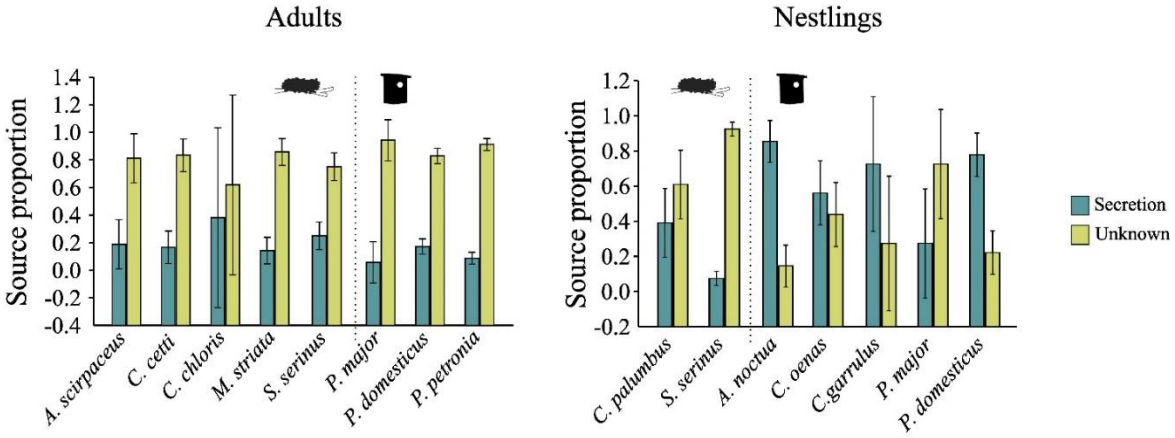
Supplementary material



Additional Figure 1. Least square means of alpha diversity indexes (Chao1, Shannon index and PD) for adults and nestlings after controlling for species identity.



Additional Figure 2. Results of the source tracking analyses showing the proportions of the uropygial gland skin microbiota that is originated in the microbiota of the uropygial gland secretion versus an unknown source for each species.

Additional Table 1. Results of General Linear Models (GLMs) exploring the effects of species identity (ID) and age class on alpha diversity variables (Chao1, Shannon index and PD) of cavity-nester species. Species identity and age class effects were explored together in models including both variables, while the interaction was calculated in separate models that also included the main effects. Values in bold are those with associate alpha value lower than 0.05.

	Chao1			**Shannon index**			**PD**		
	F	*df*	p	F	*df*	p	F	*df*	p
Effect of age class									
Gland skin									
Species	**6.92**	**6, 175**	**<0.001**	**5.77**	**6, 177.6**	**<0.001**	**2.17**	**6, 173.8**	**<0.001**
Age	**41.71**	**1, 176.3**	**<0.001**	**10.65**	**1, 179.2**	**0.001**	0.07	1, 175	0.788
Species*Age	1.34	6, 173.8	0.345	**3.65**	**6, 179.3**	**0.002**	**3.57**	**6, 173.4**	**0.002**
Nest(Species*Age)	**2.84**	**164, 70**	**<0.001**	**2.29**	164, 70	**<0.001**	**3.20**	164, 70	**<0.001**
Gland secretion									
Species	**3.19**	**5, 103**	**0.010**	**2.56**	**5, 109.4**	**0.031**	**3.53**	**5, 103.8**	**0.005**
Age	**5.70**	**1, 103.2**	**0.018**	0.002	1, 109.7	0.969	0.20	1, 104	0.654
Species*Age	1.45	5, 96.4	0.124	**2.66**	**5, 103.3**	**0.026**	**3.58**	**5, 98.9**	**0.005**
Nest(Species*Age)	**4.10**	**91, 76**	**<0.001**	**2.64**	**91, 76**	**<0.001**	**3.84**	**91, 76**	**<0.001**

Additional Table 2. Results of MCMCglmm models with nest type as a fixed factor, the bird phylogeny as a random factor, and the alpha diversity variables (Chao1, Shannon index and PD) as dependent variables. Values in bold are those with associate alpha value lower than 0.05.

			Lower 95% CI	Upper 95% CI	pMCMC	Auto-correlation	Heritability Lower 95% CI	Heritability Upper 95% CI	z-score
Gland skin	Adults	Chao1	**-164.07**	**-58.79**	**0.002**	-0.026	3.00E-08	0.18715	0.734
		Shannon	-1.56	0.05	0.093	-0.024	8.62E-06	8.24E-05	-1.228
		PD	**-3.48**	**-0.64**	**0.011**	-0.087	4.16E-08	0.00026	1.463
	Nestlings	Chao1	-220.86	136.42	0.785	-0.005	0.52272	0.88074	1.475
		Shannon	-0.22	1.15	0.225	0.057	3.48E-08	1.45E-05	-0.307
		PD	-1.09	2.65	0.478	0.004	1.16E-05	0.00016	0.173
Gland secretion	Adults	Chao1	**-270.79**	**-74.51**	**0.001**	-0.025	0.08782	0.51564	0.185
		Shannon	**-1.57**	**-0.59**	**0.006**	0.004	0.00105	0.37008	-1.048
		PD	**-4.42**	**-1.75**	**0.001**	0.060	0.05744	0.48349	-1.663
	Nestlings	Chao1	**-247.18**	**-24.77**	**0.027**	-0.001	6.79E-09	0.23434	-1.864
		Shannon	-0.74	0.36	0.387	-0.018	0.00073	0.41321	1.120
		PD	-1.51	0.46	0.307	-0.020	7.63E-05	0.20679	-0.615

Additional Table 3. Results of mantel test analyses exploring the effects of nest type on beta diversity (distance matrices constructed with Bray-Curtis, Jaccard, weighted UniFrac and unweighted UniFrac) of the bacterial communities of secretion and skin of adult and nestling birds after controlling for the bird phylogeny. Nest ID was also included in the models of nestlings to control for the non-independence of the data. Values in bold are those with associate alpha value lower than 0.05.

		Bray-Curtis		Jaccard		Unweighted UniFrac		Weighted UniFrac	
		R	p	R	p	R	p	R	p
Adults	*Gland skin*								
	Nest type	0.000	0.416	0.000	0.509	0.016	0.092	**0.052**	**0.005**
	Phylogeny	**<0.001**	**<0.001**	<0.001	<0.001	**<0.001**	**<0.003**	-0.0002	0.204
	Gland secretion								
	Nest type	**0.087**	**<0.001**	**0.038**	**<0.001**	0.030	0.068	**0.051**	**0.020**
	Phylogeny	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.020**	<0.0002	0.204
Nestlings	*Gland skin*								
	Nest type	**0.153**	**<0.001**	**0.103**	**<0.001**	**1.020**	**0.001**	0.040	0.241
	Phylogeny	**<0.001**	**<0.001**	**<0.001**	**<0.001**	9.918	0.106	**<0.001**	**<0.001**
	Nest ID	**0.055**	**<0.001**	**0.048**	**<0.001**	-6.291	0.655	-0.047	0.152
	Gland secretion								
	Nest type	**0.054**	**<0.001**	**0.033**	**<0.001**	**0.018**	**0.019**	0.000	0.999
	Phylogeny	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.003**
	Nest ID	**0.009**	**<0.001**	**0.006**	**<0.001**	**-0.007**	**0.230**	**0.030**	**0.007**

Additional Table 4. Post-hoc comparisons between groups (adults and nestlings of cavity (CN) and non-cavity (NCN) nesters) from multivariate homogeneity of group dispersions analyses (*betadisper*). Values in bold are statistically significant. Values in bold are those with associate alpha value lower than 0.05.

	Comparisons	Lower 95% CI	Upper 95 % CI	p adj
Gland skin	**Adult_NCN - Adult_CN**	**-0.097**	**-0.031**	**0.000**
	Nestling_CN - Adult_CN	-0.011	0.029	0.666
	Nestling_NCN - Adult_CN	-0.062	0.019	0.523
	Nestling_CN - Adult_NCN	**0.042**	**0.104**	**<0.001**
	Nestling_NCN - Adult_NCN	-0.004	0.090	0.092
	Nestling_NCN - Nestling_CN	-0.069	0.009	0.185
Gland secretion	**Adult_NCN - Adult_CN**	**-0.026**	**0.000**	**0.045**
	Nestling_CN -Adult_CN	-0.004	0.019	0.317
	Nestling_NCN - Adult_CN	-0.037	0.003	0.114
	Nestling_CN - Adult_NCN	**0.011**	**0.031**	**0.000**
	Nestling_NCN - Adult_NCN	-0.023	0.015	0.951
	Nestling_NCN - Nestling_CN	**-0.042**	**-0.007**	**0.002**

Additional Table 5. Results from Mantel tests exploring correlations between uropygial gland skin and uropygial gland secretion microbiomes using the whole set of samples, and for cavity and non-cavity nesters, separatly, at ASV and Genus levels after controlling for the bird phylogeny. Bray-Curtis, Jaccard, weighted UniFrac and unweighted UniFrac were used to build the distance matrices. Values in bold are statistically significant. Values in bold are those with associate alpha value lower than 0.05.

			Gland skin microbiota							
			Bray-Curtis		**Jaccard**		**Unweighted UniFrac**		**Weighted UniFrac**	
			R	p	R	p	R	p	R	p
Adults	All samples	ASV-level								
		Secretion microbiota	0.054	0.711	**0.299**	**0.046**	-0.005	0.949	**0.217**	**0.018**
		Phylogeny	<0.001	0.575	**<0.001**	**0.011**	<0.001	0.157	<0.001	0.517
		Genus-level								
		Secretion microbiota	0.006	0.931	0.003	0.961	-0.010	0.852	0.188	0.065
		Phylogeny	<0.001	0.457	<0.001	0.436	<0.001	0.913	<0.001	0.352
	Cavity-nesters	ASV-level								
		Secretion microbiota	-0.137	0.389	-0.100	0.514	**0.301**	**0.023**	**0.303**	**0.029**
		Phylogeny	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.206**	**0.000**	**0.788**
		Genus-level								
		Secretion microbiota	0.044	0.734	0.039	0.782	**0.505**	**0.001**	**0.308**	**0.022**
		Phylogeny	<0.001	<0.001	<0.001	<0.001	<0.001	0.400	<0.001	0.470
	Non- cavity nesters	ASV-level								
		Secretion microbiota	0.295	0.167	0.347	0.127	-0.018	0.826	0.167	0.205
		Phylogeny	**<0.001**	**0.008**	**<0.001**	**0.009**	<0.001	0.681	**<0.001**	**0.013**
		Genus-level								
		Secretion microbiota	0.092	0.580	0.094	0.617	-0.075	0.422	-0.014	0.930
		Phylogeny	**<0.001**	**0.003**	**<0.001**	**0.005**	<0.001	0.699	**<0.001**	**0.020**
Nestlings	All samples	ASV-level								
		Secretion microbiota	**0.703**	**<0.001**	**0.818**	**<0.001**	0.0034	0.394	**0.240**	**0.004**
		Phylogeny	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	**<0.001**
		Genus-level								
		Secretion microbiota	**0.299**	**<0.001**	**0.236**	**<0.001**	0.0641	0.443	0.096	0.154
		Phylogeny	<0.001	0.133	**<0.001**	**0.043**	<0.001	0.088	<0.001	0.666
	Cavity-nesters	ASV-level								
		Secretion microbiota	**0.831**	**<0.001**	**0.945**	**<0.001**	-0.023	0.768	0.166	0.055
		Phylogeny	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
		Genus-level								
		Secretion microbiota	**0.393**	**<0.001**	**0.400**	**<0.001**	0.316	0.936	0.038	0.759
		Phylogeny	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	Non- cavity nesters	ASV-level								
		Secretion microbiota	-0.311	0.239	-0.387	0.214	0.044	0.803	-0.048	0.693
		Phylogeny	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.004**	<0.001	0.502
		Genus-level								
		Secretion microbiota	-0.111	0.547	-0.162	0.449	-0.001	0.995	-0.096	0.481
		Phylogeny	**<0.001**	**0.002**	**<0.001**	**0.001**	**<0.001**	**0.015**	<0.001	0.348

CAPÍTULO IV

Colouration of spotless starling nestlings shows genetic and environmentally determined characteristics while begging for food

Abstract

Similar to other signals, colouration of traits expressed by nestlings while they are begging for food are assumed to reflect their phenotypic conditions, and, therefore, they should be environmentally determined. However, these colourations are also species specifics and, thus, a genetic component explaining the evolution of nestling colouration should exist; a possibility rarely explored in the literature. Cross-fostering experiments allowed us to quantify genetic and environmental components of colouration of mouth, flanges and skin in a spotless starling *Sturnus unicolor* population. In addition, by supplementing some nestlings within each brood with non-pigmented antioxidants (Vitamin E or Vitamin complex), we explored the effects of food supplementation on colouration of begging related traits and plasma carotenoid concentration. We detected significant genetic components of colourations of skin and mouth, but not of flanges or plasma carotenoid concentration. Moreover, experimental supplementation with Vitamin E affected UV colouration of mouth, flanges and skin, while vitamin-complex supplementation exclusively affected flange colouration. Furthermore, colouration of flanges, but not that of other traits, predicted plasma carotenoid concentration suggesting a direct link between these two traits. All these results suggest that colouration of begging related traits is a multifaceted signal informing parents about genetic characteristics, and of short-term needs of nestlings, including anti-oxidative capacity, that parents could use to adjust feeding efforts, and deciding which chick to feed. Future research should focus on the parents' perspective, taking into account their feeding effort and feeding preferences in relation to genetically and/or environmentally determined colouration of begging related traits.

Key words*: antioxidants, begging signals, carotenoid-based colouration, cross-fostering experiment, environmental component of signals, genetic component of signals, plasma carotenoid concentration*

Introduction

Animal communication is paramount to understanding inter- and intra-specific interactions at different social levels, as well as, to facilitate the resolution of conflicts of interest among counterparts. Individuals for instance might use signals to inform receivers on their physiological characteristics, phenotypic or genotypic capabilities, or even behavioural intentions. Receivers will use the obtained information to modulate or optimize their responses, which will also benefit the individual that emits the signal (Maynard-Smith & Harper 2003). Phenotype of a signalling trait might vary very little when informing on genetic characteristics, but be plastic or environmentally driven when its phenotype is directly or indirectly determined by environmental conditions (Roulin & Ducrest 2013). Detecting additive genetic variance or the environment-genetic interaction of a signalling character is necessary for explaining the evolution of signalling traits. However, mainly because signalling traits are considered to reflect environmentally driven conditions of the signaller, genetic and environmental components explaining signalling traits have rarely been estimated in the literature (Roulin & Ducrest 2013).

Parent-offspring interactions, in which young individuals communicate their needs to their parents, and parents adaptively respond to offspring signalling, has been used as a model system for exploring different functional characteristics and hypotheses explaining the evolution of animal communication. Offspring are selected to demand more resources than parents are able to provide, but signals of need should reliably reflect physiological requirements (Godfray 1995). Most of these studies have been performed in birds (Mock & Parker 1997; Mock, Dugas & Strickler 2011), and, although the main interest is to understand the evolution of parent-offspring communication, heritability of involved signals has not been quantified so far except in a couple of studies (Casagrande *et al.* 2009; Dugas 2012).

Resource solicitation to parents by young individuals implies a series of complex and conspicuous behaviours (i.e., begging behaviour) that are costly to produce in terms of, for instance, energy (Kilner 2001; Moreno-Rueda & Redondo 2011; Soler *et al.* 2014), inclusive fitness (Trivers 1974), or predatory or parasitism risk (Redondo & Castro 1992a; Haskell 1994; Tomás & Soler 2016). Thus, begging related signals are supposed to inform parents about their physiological needs (Godfray 1991; Kilner & Johnstone 1997; Johnstone & Godfray 2002) or phenotypic quality (Grafen 1990; De Ayala *et al.* 2007; Jacob & Heeb 2013) honestly. Moreover, both offspring needs and phenotypic quality (i.e., probability of successful fledging) are mainly explained by food received from parents. Thus, begging related signals should mainly be environmentally determined. Similarly, parents respond to

offspring needs would also depend on food availability and, therefore, also be environmentally influenced (Bize *et al.* 2006; Mock, Dugas & Strickler 2011; Boncoraglio *et al.* 2013; Ruiz-Castellano *et al.* 2016). Consequently, mainly because begging related traits are by definition signals of needs, most should be environmentally determined with very little genetic influence if any (Casagrande *et al.* 2009; Dugas 2012).

The begging display includes several kinds of signals including acoustic (begging calls) (Kilner, Noble & Davies 1999), postural (head movements and gaping (Redondo & Castro 1992b) and position of the nestlings when adults arrive to the nest (McRae, Weatherhead & Montgomerie 1993)), or flamboyant coloured mouth and flanges contrasting with body skin and nest material (Avilés *et al.* 2008; Soler & Avilés 2010). Relative genetic and environmental influences on begging related traits might vary for different traits and species. The two previous studies estimating genetic and environmental components of begging related traits dealt with the conspicuous colours of mouth, flanges and skin of nestlings, and both studies concluded that the heritability of these traits is nil or very low (Casagrande *et al.* 2009; Dugas 2012). However, mouth and flange colouration consistently vary among species, depending for instance on environmental light conditions (Avilés *et al.* 2008), parental visual performance (Avilés & Soler 2009), or level of sibling competition for parental attention (Soler & Avilés 2010). These interspecific differences adjusted to environmental conditions provide evidence that these structures may have a significant genetic component that might vary among species. Therefore, the existence of genetic components explaining coloured mouth, flanges and skin of nestlings is an open question, and its estimation is necessary for understanding consistent interspecific differences and, thus, function and evolution of begging related characteristics.

Colouration of begging related traits reflects the state of need of a nestling (Kilner 1997) and physiological conditions including immune response (Soler *et al.* 2007; Pirrello *et al.* 2017), which has a low but significant genetic component (Brinkhof *et al.* 1999; Tella *et al.* 2000; Soler, Moreno & Potti 2003; Erin, Daniel & Ethan 2009; Drobniak *et al.* 2010). Mouth and flange colouration of nestlings likely reflect concentration of pigmentary (i.e., carotenoids) (Saino *et al.* 2000a; De Ayala *et al.* 2007; Thorogood *et al.* 2008; Dugas & Rosenthal 2010; Dugas, McGraw & Strickler 2018) and non-pigmentary but potent antioxidants such as vitamin E, vitamin A, vitamin C or antioxidant enzymes in the blood (Hartley & Kennedy 2004). The colourless antioxidants would prevent the oxidation of carotenoids in the signalling traits (Bertrand, Faivre & Sorci 2006; Pike *et al.* 2007), which suggest the existence of an intricate relationship between pigmentary and non-pigmentary

antioxidants in the diet, in the blood, and in coloured signals. For instance, diets rich in colourless antioxidants may break the predicted positive associations between carotenoids in the blood and in signals (i.e., colouration), or even turn them negative if higher concentration of colourless antioxidants in the blood provokes a more elevate mobilization of carotenoids to signals.

In the present study, we explored the above-described scenario in a population of spotless starlings *Sturnus unicolor*. Briefly, (i) we experimentally tested the effects of antioxidant (i.e., Vitamin E, hereafter VitE and Nutratom, an antioxidant complex) supplemented diet on the colouration of begging related traits of developing nestlings. Moreover, (ii) we also quantified carotenoid concentration in the blood of experimental birds, and explored (a) the effect of experimental supplementation, and (b) the association with colouration of begging related traits. In addition, since it can be predicted that traits influenced by food supplementation are mainly those with low or nil genetic components, (iii) we estimated the genetic and the environmental components, not only of colouration of nestling traits, but also of carotenoid concentration in the blood. Finding significant associations between carotenoid concentration in blood and nestling colouration will confirm that the colouration of nestlings reflects their antioxidant capacity, while an experimental effect of supplemented non-pigmentary antioxidants on carotenoid concentration and on colouration of nestlings will not only demonstrate an association between pigmented and non-pigmented antioxidants, but also the environmental component of begging related traits. Furthermore, since interspecific differences in colourations of begging related traits should be the result of evolutionary processes, we expect to detect significant genetic components of such characters. Here, we estimate the relative contributions of genetic and environmental components by means of a cross-fostering experimental approach (see Merilä (1996)), which allows partitioning the phenotypic variance (V_P) in environmental (V_E; i.e., nest of rearing), and genetic variances (V_G; nests of origin); the latter also including possible maternal effects (V_M, that we tried to control statistically by removing variance of nestling colouration that were explained by eggshell pigmentation), and dominance variance (Falconer 1989).

By joining results from the food supplementation experiments with those of cross-fostering experiments, we were able to discuss the importance of both genetic and environmental components of the colour expression of begging related traits in scenarios of multiple signalling. Spotless starling nestlings show a flashy yellow colouration in the mouth and flanges, and their featherless skin has a reddish colouration to the human eye, but mainly

reflecting the UV part of the light spectrum. Moreover, colourations of begging related traits of spotless starling nestlings are related to their phenotypic condition, including immune response (Soler *et al.* 2007; Pirrello *et al.* 2017). That makes this species a model system for studying these type of signals, and, thus, parent-offspring communication in general, and the above described scenarios in particular.

Methods

Study area and species

Fieldwork was carried out during the breeding seasons 2016 and 2019 in a spotless starling (hereafter starlings) population located in southern Spain, at the old railway station of La Calahorra (37°15' N, 3°01'W), situated in the high altitude plateau of the semiarid Hoya de Guadix, where starlings breed in 94 cork-made nest boxes (internal dimensions: 180 mm x 210 mm and 350 mm high, 240 mm from the bottom to the hole entrance) attached to tree trunks or walls.

The starling is a medium-sized, hole-nesting altricial passerine. In our study population, starlings start laying their eggs in mid-April, the clutch size is commonly of 4-5 eggs and they lay one egg per day. The incubation period lasts 11 days and they begin the incubation before laying the last egg, making the last nestling hatch later (Azcárate-García *et al.* 2020). Incubation is mainly performed by females, while both parents feed nestlings during development (Veiga & Polo 2016).

Fieldwork

We visited nest boxes every three days since late March until egg detection, which allows to inference of the date of laying of the first eggs (hereafter, laying date). We then visited nests every other day until detecting clutch completion, when we measured colouration of eggshells (see below). A total of 12 days after laying, we visited nest boxes again and then daily until hatching when, in 2019, we randomly decided the experiment (VitE or cross-fostering) to be performed in each nest. In 2016, only second clutches were used to perform the antioxidant supplementation experiment.

Cross-fostering experiment

One day after hatching, we exchanged two nestlings between two nests of the same hatching date and similar (± 1 egg) clutch size. On day 10, we took biometric measurements of nestlings, ringed them, and measured the colouration of begging related traits. 4 days later,

we collected blood samples to extract the plasma (for more details on measurements and blood collection, see Additional Methods). A total of 34 nests and 107 nestlings were included in the cross-fostering experiment.

Antioxidant supplementation experiments

The experiment started the day after hatching and consisted in oral administration of an age-dependent dose of VitE (α-Tocopherol) diluted in corn oil (for details of dose estimates, see Additional Methods) to half of the nestlings (alternating nestlings ranked depending on their body mass), and doses of only oil to the rest of the brood. We fed them every other day, and ten days after hatching, we took biometric measurements, ringed them, and measured the colouration of begging related traits. On day 14, we also collected blood samples (see details in Additional Methods). In 2016, we performed a similar food supplementation experiment, but in that case we used the antioxidant complex Nutratom (FARDI, S.A.) (for details see Additional Methods). A total of 239 nestlings from 70 starling nests were included in the 2019 experiment, while 45 nestlings from 14 nests were included in the antioxidant experiment performed in 2016.

Colour measurements and estimation of colour variables

We used a spectrophotometer (Ocean Optics S2000) to measure colouration as spectral reflectance between wavelengths of 300 to 700 nm. Before performing the colour analyses, all negative values of reflectance were corrected to zero and, to reduce noise, a triangular correction was applied as implemented in AVICOL v.6 (Gomez 2006). We measured eggshells at three haphazardly selected areas along the long axis, and mouth, flanges and breast skin of nestlings as described in the Additional Methods. We estimated brightness, UV hue and chroma (300-400 nm), Yellow-Red hue and chroma (580-680nm), and carotenoid chroma (reflectance value at 700 nm minus that at the 450 nm wavelengths (Cuthill 2006; Isaksson *et al.* 2008; Charmantier *et al.* 2017)) for eggshells and nestlings. Spectrum of each measured trait is shown in Figure 1. For details on considered colour variables and spectra characteristics of each measured trait, see Additional Methods.

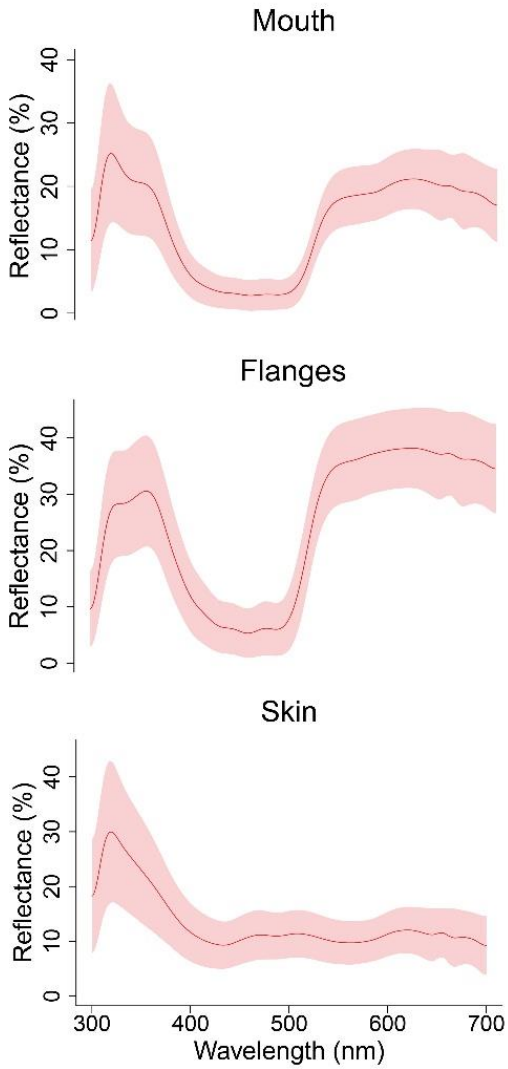


Figure 1. Reflectance spectra of starling nestling mouth, flange and skin. Only non-moved nestlings from cross-fostering experiment (n=107) were used. Curves show means and shaded areas show ± Standard Deviation.

Estimating blood plasma carotenoid concentration

We measured carotenoid concentration in blood plasma by means of a spectrophotometric assay. Briefly, following Bertrand, Faivre and Sorci (2006), we added 135 µl of ethanol to 15 µl of plasma, vortexed the mix and centrifuged at 4ºC and 1,500 x *g* RCF for 10 min, and measured the absorbance of the supernatant at 450 nm in a spectrophotometer (Sunrise-basic Tecan, 16039400). Calibration curves of absorbance at 450 nm and carotenoid concentration (from 0 to 200 µg x mL^{-1}) were obtained by using lutein (CAYM10010811-1, VWR) (R^2 = 0.999), which allowed us to extrapolated absorbance values to those of lutein concentrations, which we use as a proxy of carotenoid concentration in blood plasma.

Statistical analysis

We implemented a multiple regression model to calculate the residuals of body mass after controlling for the effects of tarsus and wing length. These residuals tend to reflect body condition index (Peig & Green 2009) and are associated with probability of local recruitment of nestling birds (see Moreno *et al.* (2005), Cichon and Dubiec (2005) and references therein). These estimates were, therefore, used as independent variable in a linear regression model to study its association with colouration of begging related traits and plasma carotenoid concentration. In order to assess the effect of body mass on nestling colouration or plasma carotenoid concentration, only those nestlings that were not antioxidant supplemented were included in the analyses (i.e. nestlings under control treatment or from cross-fostering experiment).

Repeatability of all colour measurement was relatively high ($R > 0.70$) (for detailed results of each measured trait, see Additional Methods) and, then, we used mean values in subsequent analyses.

We used mixed model ANOVAs to explore the association between carotenoid concentration and nestling colouration, with plasma carotenoid concentration, study year, breeding attempt and nest identity as additional independent factors (see Additional Methods for details). The effects of experimental food supplementation on nestling colouration and plasma carotenoid concentration were explored in similar models that also include the experimental treatment as fixed effects (see Additional Methods for details).

In order to explore the genetic and environmental components of colouration of begging related traits and the concentration of plasma carotenoids of cross-fostered nestlings, we used hierarchized nested ANOVAs with identity of nest of rearing as the variable explaining the environmental component, and nest-of-origin identity nested within nest of rearing as the factor dealing with the genetic component (see Additional Methods for details). Experimental effects on different body parts and colour variables were explored in separate statistical models. All these statistical analyses were run in STATISTICA v.12 (Statsoft 2015).

Results

Body condition, carotenoid concentration, and colouration of nestlings

Nestlings with brighter flanges (mean brightness, $r = 0.170$, $F_{(1,220)} = 7.14$, $P = 0.008$), were those with better body condition (Fig. 2). Similarly, mouth brightness ($r = 0.111$, $F_{1,220} =$

2.77, P = 0.097), skin UV hue (r = 0.117, $F_{(1,215)}$ = 3.02, P = 0.083) and plasma carotenoid concentration (r = 0.141, $F_{(1,185)}$ = 3.73, P = 0.054) were, to some extent, positively related with body condition of nestlings too (Additional Table 1).

Table 1. Results from Mixed model ANOVAs exploring the association between plasma carotenoid concentration and colouration of begging-related traits (mouth, flanges and skin) of spotless starling nestlings after controlling for the effect of study year, breeding attempt (nested within study year) and the random effect of nest identity (nested within breeding attempt and study year). Colour variables used include the achromatic component (Brightness), as well as hue and chroma of the total (Total) or particular wavelength intervals (UV, Yellow-red and Carotenoids). We only have information on mouth colouration from the 2019 breeding season. Only nestlings from cross-fostering experiment and control-non-feeding nestlings were used to test these associations. Statistical effects of the experimental treatment with associated alpha-values lower than 0.05 are highlighted in bold font. Associations between plasma carotenoid concentration and colour variables that reached statistical significance in interaction with breeding attempt (flange total hue ($F_{2,101}$ = 3.41, P = 0.037) or with study year (flange total hue ($F_{2,102}$ = 6.78, P = 0.011) and flange yellow-red chroma ($F_{2,102}$ = 4.50, P = 0.036)) were highlighted in italic bold fonts. Results from complete models are shown in Additional Table 2, while here we only show effects associated to plasma carotenoid concentration.

	Plasma Carotenoid Concentration and Begging Related Traits											
	Mouth				**Flanges**				**Skin**			
Dependent Factors	F	df	P	β	F	df	P	β	F	df	P	β
Brightness	0.001	1, 95	0.972	0.00	**6.263**	**1, 103**	**0.014**	**0.25**	0.359	1, 101	0.550	-0.08
Hue												
Total	1.012	1, 95	0.317	-0.10	***0.147***	***1, 103***	***0.703***	***-0.05***	1.455	1, 101	0.231	0.00
UV	0.014	1, 95	0.904	0.15	0.790	1, 103	0.376	-0.11	0.470	1, 101	0.494	0.07
Yellow-Red	0.111	1, 95	0.739	-0.04	**4.710**	**1, 103**	**0.032**	**-0.21**	1.286	1, 101	0.259	0.14
Chroma												
UV	0.241	1, 95	0.625	-0.04	1.299	1, 103	0.257	0.13	0.032	1, 101	0.858	-0.02
Yellow-Red	1.165	1, 95	0.283	0.13	***0.482***	***1, 103***	***0.489***	***-0.09***	0.410	1, 101	0.523	-0.09
Carotenoid	0.027	1, 95	0.870	-0.02	0.028	1, 103	0.867	-0.02				

Concentration of plasma carotenoids was correlated with brightness and yellow-red hue of flanges (Table 1, Fig. 2). Nestlings with brighter and more yellow flanges were those with higher carotenoid concentration in the blood. Furthermore, neither the interaction between carotenoid concentration and breeding attempt, nor that between carotenoid concentration and study year explained a significant proportion of variance in brightness (Additional Table 2) and yellow-red hue of flanges (Additional Table 2). However, the expected association between carotenoid concentration and total hue of flanges depended on breeding attempt and study year (see interactions in Additional Table 2), while the association between carotenoid concentration and yellow-red chroma of flanges depended on the study year (see interactions in Additional Table 2).

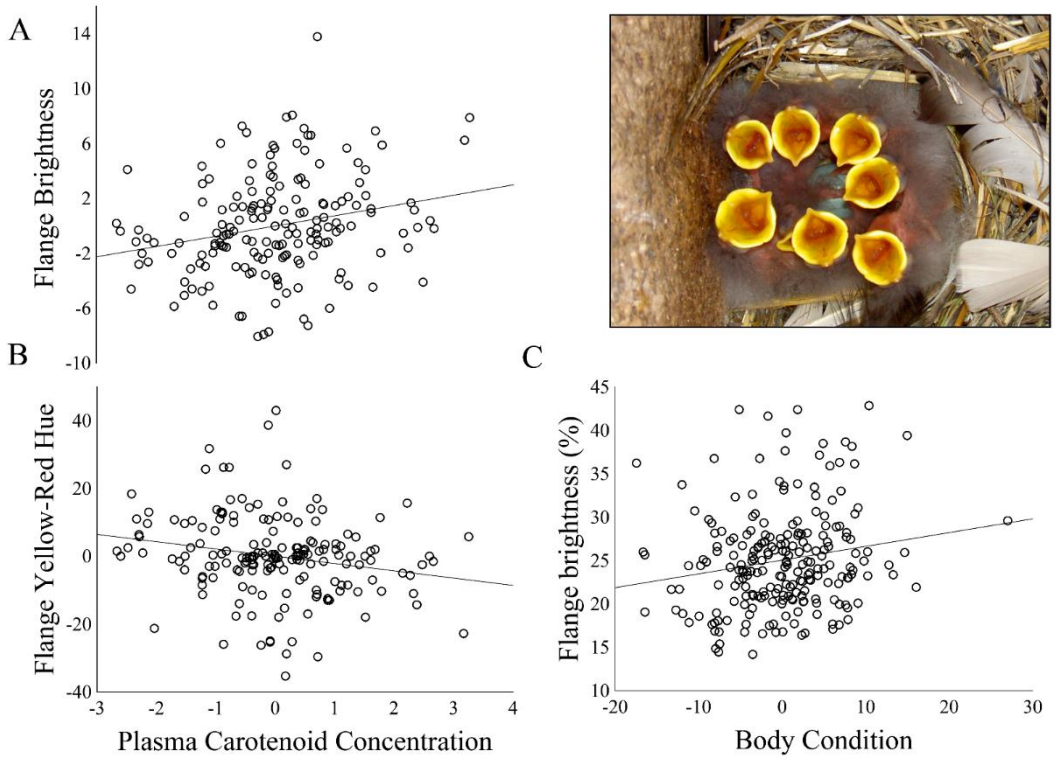


Figure 2. Association between residual plasma carotenoid concentration and (A) residual flange brightness or (B) residual flange yellow-red hue. The residuals were controlled for experimental year, breeding attempt and nest identity. (C) Association between body condition index and flange brightness. Lines are regression lines and the image shows nestlings begging for food.

Environmental and genetic components of nestling colouration and plasma carotenoid concentration

Except for mouth carotenoid chroma, and flange total hue, most of the colour variables of begging related traits of nestlings, as well as plasma concentration of carotenoids showed a strong environmental component (Table 2). In contrast, genetic components were only apparent for chromatic coloration of skin and hue of mouth of nestlings (Table 2). We did not find any statistical support for a genetic component determining flange colouration or plasma carotenoid concentration.

Food supplementation experiments, colouration and plasma carotenoid concentration

Mouth, flanges and skin of VitE supplemented nestlings showed higher values of UV-hue than those of control nestlings (Table 3). In addition, mouth and flanges of VitE supplemented nestlings were, respectively, slightly more carotenoid coloured and brighter than those of nestlings under control treatment (Table 3). Except for flange UV-hue, breeding

attempt did not have a large effect on colouration of begging related traits of starling nestlings (Table 3).

Experimental food supplementation with antioxidants complex also affected colouration of nestlings. Similar to the VitE experiment, flanges of experimental nestlings were brighter than those of control nestlings (Table 3). In addition, UV-chroma and yellow-red chroma of flanges of experimental nestlings were respectively higher and lower than those of control nestlings (Table 3). Importantly, the detected effects of both experiments were consistent among nests as suggested by the statistically non-significant interaction between experimental treatment and nest identity (Additional Table 4 and Additional Table 5).

Table 2. Results from hierarchized ANOVA exploring the effect of nest of rearing and nest of origin (nested within nest of rearing) on colouration of begging-related characters (mouth, flanges and skin) and carotenoid concentration in plasma of spotless starling nestlings after controlling for maternal effects. Colour variables used include the achromatic component (Brightness), as well as hue and chroma of the total (Total) or particular wavelength intervals (Yellow-red and UV). Statistical effects of experimental treatment associated with two-tailed alpha-values lower than 0.1 are in bold font, and degrees of freedom associated to comparisons (F) of carotenoid concentration are marked with *. Results from the analysis before controlling for maternal effects are shown in the Additional Table 3.

		Independent Factors					
		Nest of rearing			Nest of origin (nest of rearing)		
Dependent factors		$F_{28, 41}$ $F_{25, 33*}$	P	Estimated variance (%)	$F_{28, 41}$ $F_{24, 33*}$	P	Estimated variance (%)
Mouth							
Brightness		**3.754**	**< 0.001**	35.5	1.472	0.127	14.7
Hue	Total	**2.184**	**0.011**	44.9	**2.305**	**0.007**	0.0
	UV	**1.961**	**0.024**	13.2	1.357	0.183	15.8
	Yellow-Red	**4.492**	**< 0.001**	50.6	1.072	0.411	2.1
Chroma	UV	**8.364**	**< 0.001**	68.9	1.054	0.431	1.0
	Yellow-Red	**3.568**	**< 0.001**	45.6	0.906	0.603	0.0
	Carotenoid	1.212	0.282	18.2	0.516	0.966	0.0
Flanges							
Brightness		**2.408**	**0.005**	32.9	0.850	0.671	0.0
Hue	Total	1.632	0.075	21.9	0.747	0.789	0.0
	UV	**4.186**	**< 0.001**	47.1	1.125	0.360	3.8
	Yellow-Red	**4.716**	**< 0.001**	53.5	1.016	0.474	0.4
Chroma	UV	**2.399**	**0.005**	19.6	1.414	0.154	16.5
	Yellow-Red	**2.132**	**0.013**	20.2	1.214	0.281	9.4
	Carotenoid	**2.167**	**0.011**	22.9	1.136	0.349	6,0
Skin							
Brightness		**2.489**	**0.004**	22.5	1.351	0.186	14,0
Hue	Total	**2.356**	**0.006**	16.1	1.529	0.106	20.9
	UV	**14.524**	**< 0.001**	56.8	**3.562**	**< 0.001**	26.6
	Yellow-Red	**8.588**	**< 0.001**	51.9	**2.309**	**0.007**	21.7
Chroma	UV	**4.315**	**< 0.001**	36.4	1.689	0.061	19.1
	Yellow-Red	**3.827**	**< 0.001**	21.3	**2.251**	**0.009**	34.5
Carotenoid concentration		**8.369**	**< 0.001**	62.2	1.571	0.113	10.3

Plasma carotenoid concentration of VitE supplemented nestlings did not differ from that of control nestlings (Table 3), whereas those supplemented with antioxidant complex including carotenoids had a non-significantly lower concentration than control nestlings (Table 3). Moreover, plasma carotenoid concentration of nestlings from the first breeding attempt was higher than that of nestlings of second broods (Additional Table 4, and Fig. 3). The non-significant interactions between treatment and nest identity (Additional Table 4 and Additional Table 5) suggest consistent effects of this factor among nests. In summary, our results indicated that although carotenoid concentration in the blood is associated with colouration of begging related traits, experimental feeding with antioxidants does not affect carotenoid concentration in the blood, but colouration predicting their phenotypic condition. These results therefore point at the elevated environmental component of colouration of begging related traits, by which nestlings communicate anti-oxidative capacity to their parents.

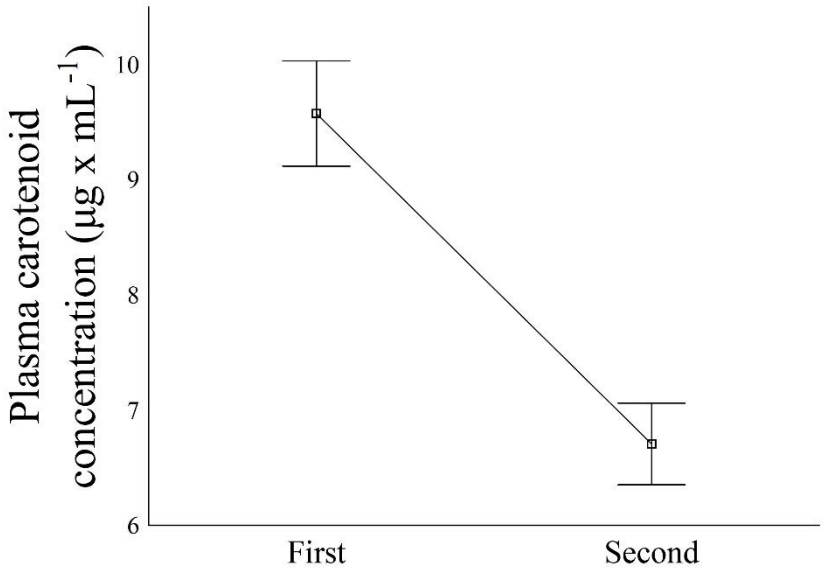


Figure 3. Average (±95 CI) values of plasma carotenoid concentration of first and second broods of nestlings under the VitE supplementation experiment.

Table 3. Results from Mixed model ANOVAs exploring the effect of antioxidant supplementation on colouration of begging-related characters (mouth, flanges and skin) and plasma carotenoid concentration of spotless starling nestlings after controlling for the fixed effect of breeding attempt, the random effect of nest identity, and the interaction between nest identity and experimental treatment to account for the repeated measure approach within nests. Colour variables used include the achromatic component (Brightness), as well as hue and chroma of the total (Total) or particular wavelength intervals (Yellow-red and UV). Statistical effects of the experimental treatment with associated two-tailed alpha-values lower than 0.1 are highlighted in bold font. Results from complete models are shown in the Additional Table 4 and Additional Table 5, while here we only show the effects of experimental food supplementation.

		Experimental Effects									
		VitE Supplementation					**Nutratom Supplementation**				
Dependent Factors		F	df	Means (SE)		P	F	df	Means (SE)		P
Mouth				Control	Experimental				Control	Experimental	
Brightness		1.93	1, 71.4	13.6 (0.3)	14.5 (0.4)	0.168					
Hue	Total	2.51	1, 98.0	325.7 (6.2)	338.5 (7.8)	0.116					
	UV	**4.99**	**1, 65.8**	**324.3 (1.1)**	**327.9 (1.4)**	**0.028**					
	Yellow-Red	0.19	1, 70.2	622.5 (1.7)	622.2 (1.7)	0.667					
Chroma	UV	1.15	1, 66.4	0.3 (0.0)	0.3 (0.0)	0.287					
	Yellow-Red	0.25	1, 65.7	0.4 (0.0)	0.4 (0.0)	0.622					
	Carotenoid	**3.79**	**1, 69.9**	**0.7 (0.0)**	**0.8 (0.0)**	**0.056**					
Flanges											
Brightness		**3.67**	**1, 68.5**	**25.5 (0.5)**	**26.6 (0.6)**	**0.059**	**5.459**	**1, 14.0**	**39.9 (1.7)**	**43.4 (1.7)**	**0.034**
Hue	Total	0.17	1, 73.6	336.0 (6.8)	346.7 (7.2)	0.680	**3.885**	**1, 16.1**	**470.4 (17.9)**	**430.3 (21.7)**	**0.066**
	UV	**4.16**	**1, 67.3**	**346.3 (1.4)**	**349.2 (1.2)**	**0.045**	0.081	1, 27.7	348.5 (2.5)	349.5(2.4)	0.777
	Yellow-Red	0.23	1, 76.1	618.9 (2.3)	618.0 (2.4)	0.635	0.058	1, 13.61	626.9 (5.8)	622.1 (5.49)	0.813
Chroma	UV	0.52	1, 81.3	0.3 (0.0)	0.3 (0.0)	0.473	**9.974**	**1, 14.81**	**0.2 (0.00)**	**0.2 (0.00)**	**0.006**
	Yellow-Red	0.52	1, 75.4	0.4 (0.0)	0.4 (0.0)	0.472	**6.347**	**1, 14.86**	**0.4 (0.00)**	**0.4 (0.00)**	**0.023**
	Carotenoid	0.87	1, 70.2	0.8 (0.0)	0.8 (0.0)	0.354	1.083	1, 15.81	0.9 (0.02)	0.9 (0.02)	0.313
Skin											
Brightness		0.25	1, 70.7	12.5 (0.4)	12.5 (0.4)	0.617	0.008	1, 13.85	17.5 (0.7)	16.8 (0.9)	0.929
Hue	Total	0.35	1, 56.5	315.1 (4.4)	317.4 (5.3)	0.557	0.498	1, 15.38	308.2 (0.1)	308.3 (0.09)	0.491
	UV	**5.20**	**1, 67.5**	**318.7 (0.4)**	**320.44 (0.9)**	**0.026**	0.729	1, 14.83	320.8 (0.6)	320.2 (0.6)	0.406
	Yellow-Red	0.04	1, 75.8	629.8 (2.2)	630.6 (2.2)	0.850	1.685	1, 18.04	646.0 (5.3)	638.2 (5.2)	0.211
Chroma	UV	0.72	1, 65.2	0.4 (0.0)	0.4 (0.0)	0.398	0.244	1, 14.35	0.4 (0.0)	0.4 (0.0)	0.629
	Yellow-Red	0.37	1, 66.0	0.2 (0.0)	0.2 (0.0)	0.544	0.253	1, 15.72	0.2 (0.0)	0.2 (0.0)	0.622
Carotenoid concentration		0.24	1, 59.9	8.5 (0.3)	8.7 (0.3)	0.628	**4.51**	**1, 14.4**	**9.0 (0.4)**	**7.7 (0.4)**	**0.050**

Discussion

Our experimental approaches allowed us to discern between environmental and genetic influences determining phenotype of begging related traits of starling nestlings, and to explore the effects of antioxidant-rich diets. We detected significant genetic effects for chromatic components of mouth and skin colouration of nestlings, but not for colouration of flanges, which largely depend on environmental factors including experimental diets. These results might suggest that colouration of flanges mainly functions as reflecting short term physiological needs and, in accordance, we found that flange colour predicted plasma carotenoid concentration and body condition of nestlings. Additionally, colouration of mouth and skin of nestlings would inform parents on other genetically determined characteristics of their offspring. Below, we discuss these possibilities as well as the importance of the detected experimental evidence for genetic and environmental components of coloration of begging related traits.

Characteristics of the rearing environment are assumed to account for intraspecific variation in colouration of begging related traits of nestling birds since they were, for instance, affected by experimental food supplementation/deprivation (Kilner 1997; Thorogood *et al.* 2008; Jacob & Heeb 2013) or immune system activation (Romano *et al.* 2011). Nevertheless, colouration of mouth, flanges and skin greatly vary among species (Avilés *et al.* 2008; Avilés & Soler 2009; Soler & Avilés 2010), and, thus, some of these colourations should have a genetic component allowing additive genetic variance and its evolution by natural selection. However, the genetic component of these nestling traits has rarely been explored. By means of cross-fostering experiments, Casagrande *et al.* (2009) evaluated the importance of nest of origin explaining colouration of tarsal skin of kestrel *Falco tinnunculus* nestlings, but did not find statistically significant effects. In a similar experiment, Dugas (2012) found statistically significant influences of nest of origin explaining chromatic component of flanges of house sparrow *Passer domesticus* nestlings, which until now was the only evidence of a genetic component determining colouration of begging related traits. We did not find evidence suggesting a genetic component of colouration of mouth flanges of starling nestlings, but on mouth and skin colouration, which is crucial for understanding their evolution and the large interspecific variability.

In agreement with previous work and general assumptions of the function of begging related traits, our cross-fostering experiment revealed that most variation in nestling colouration had an environmental component. Diet is one of the main environmental components determining nestling growth rates, and, thus, their colouration would reveal

nutritional condition that nestlings have experienced during development. Accordingly, colouration of nestlings predicts their body condition and immunity at the end of the nesting period (Saino *et al.* 2000b; Jourdie *et al.* 2004; Bize *et al.* 2006; Soler *et al.* 2007), and allows parents to infer short-term nestling needs and adjust their feeding effort (Kilner 1997; Ewen *et al.* 2008; Thorogood *et al.* 2008). Antioxidant carotenoids are particularly interesting in this context because they cannot be synthesized by animals. Carotenoids are therefore incorporated with the diet and are responsible for the typical yellow-red colouration of mouth and flanges of nestlings of many bird species (Wetherbee 1961; Kilner 2006), reflecting their oxidative condition (Hartley & Kennedy 2004). According to this scenario, previous experiments of supplementation with non-pigmentary antioxidants such as VitE and VitC detected differences in the carotenoid pigmentation between control and experimental individuals in the nuptial colouration of sticklebacks *Gasterosteus aculeatus* (Pike *et al.* 2007) and the red bill spot of yellow legged gulls *Larus michahellis* (Pérez, Lores & Velando 2008). Our experiment add evidence to this association since supplementation with either VitE or vitamin complex affected the chromatic component of mouth, flanges and skin colouration of starling nestlings, which was otherwise related to body condition.

Plasma carotenoid concentration was to some extent positively related to body condition and, although supplemental feeding with non-pigmentary antioxidants affected colouration of begging related traits, concentration of plasma carotenoids was similar in experimental and control nestlings. These results suggest that supplementation of non-pigmentary antioxidants allows the use of plasma carotenoids as pigments to colour carotenoid based signals (Hartley & Kennedy 2004; Bertrand, Faivre & Sorci 2006; Leclaire *et al.* 2011). In line with this argument, plasma carotenoid concentration of nestlings from second broods was significantly lower than that of nestlings from the first breeding attempt, which may be due to the typically reduced food availability experienced by late-second broods (Sorci, Soler & Møller 1997; De Neve *et al.* 2004). More importantly, since colouration of begging related traits of nestlings from first and second breeding attempts was similar, those from second clutches are likely using similar amount of carotenoids to colour their begging related traits as nestlings from first clutches, which would explain the lower concentration of plasma carotenoids shown by the second-brood nestlings. Curiously, control nestlings showed a non-significant higher plasma carotenoid concentration than nestlings supplemented with antioxidant complex in 2016, when only nestlings from second breeding attempts were sampled. It is therefore possible that antioxidant supplementation allowed nestlings dedicating more pigmented antioxidants to colour their flanges (see results in Table

3), while control nestlings kept pigmented antioxidants in the blood due to relatively scarce of antioxidants during the late breeding season. However, our experimental design does not allow discerning between mechanisms and, thus, future research should be directed to test this possibility.

Our antioxidants supplementation experiment mainly affected UV colouration of nestlings, while plasma carotenoid concentration was positively related to brightness and yellow-red hue of mouth flanges. From previous work, we know that UV-chroma of carotenoid dependent signals decreases as redness increases (Mougeot *et al.* 2007; Rajchard 2009), which may reconciliate these results. Moreover, and in accordance with this interpretation, Thorogood *et al.* (2008) found that carotenoid supplementation of Hihi *Notiomystis cincta* nestlings augmented intensity of carotenoid pigmentation and reduced UV colouration of their mouth flanges. Furthermore, UV colouration, particularly that of the skin, is known to predict body size (Bize *et al.* 2006; Pirrello *et al.* 2017) or immunocompetence (Soler *et al.* 2007) of nestlings, and our experimental results also suggest that it reflects oxidative status of nestlings (see also Leshchinsky & Klasing 2001; De Ayala, Martinelli & Saino 2006). Thus, it is likely that the combination of these UV and non-UV colourations of flanges, mouth and skin provides parents with more complete information on the phenotypic quality of their nestlings and adjust their responses in terms of parental effort (Kilner 1997; Jourdie *et al.* 2004; De Ayala *et al.* 2007; Wegrzyn 2013).

The genetic component of most colour variables affected by the antioxidant supplementation experiment were not significant, while those for which we detected a significant genetic component were unaffected by the food supplementation experiments. The only exception is skin UV-hue. Most literature dealing with colouration of begging related traits of nestlings that affect parental feeding behaviour focuses on mouth flanges of nestlings (Kilner 2006; De Ayala *et al.* 2007; Pirrello *et al.* 2017); a trait with no apparent genetic component, but directly affected by experimental antioxidants supplementation, plasma carotenoid concentration, and body condition of nestlings. Thus, flanges would inform parents on the short-term level of need of nestlings in the nest. Our results, therefore, open the possibility that to adjust feeding effort, parents not only use nestling colouration informing their short-term level of needs, but also colouration of particular characters informing on their genetic quality (Soler *et al.* 2008). Skin colouration (UV hue, yellow-red hue and yellow-red chroma) was the trait with the clearest genetic component and it was also affected by the antioxidant supplementation (UV hue). Skin colouration is also related to the nestling capacity of mounting an immune response as shown by previous studies (Jourdie *et*

al. 2004; Soler *et al.* 2007), which in some species, also has a significant genetic component (Brinkhof *et al.* 1999; Erin, Daniel & Ethan 2009; Morrison, Ardia & Clotfelter 2009; Drobniak *et al.* 2010). Our results might therefore suggest that skin coloration reflects the small genetic component of immune response, as well as the direct influences of antioxidants on immunity.

In conclusion, our results suggest that the colouration of begging related traits of nestlings is a multiple signal that parents could use to extract information about genetic characteristics of nestlings and their short-term needs. Future research should focus on determining feeding effort and preferences of parents toward nestlings that vary in colourations as determined both/either genetically and/or environmentally.

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Supplementary material

Additional Methods. Extended Details of Material and Methods.

Fieldwork

Cross-fostering experiment

We marked the nestlings by trimming different parts of the down for individual identification and, ten days later, we ringed them and measured their tarsus length with a digital calliper (precision 0.01 mm), and wing maximum-length with a metal ruler (precision 0.1 mm) following procedures described in Svensson (1992). We also measured body mass with a digital balance (Ascher CS, precision 0.01 g), and colouration of begging-related traits with a spectrophotometer (see below). On day 14, we collected blood samples by puncturing the brachial vein and filling heparinized capillaries that were emptied in microfuge tubes and kept at 4ºC in a portable fridge until arriving to the lab at the end of the day, when blood samples were centrifuged (18,000 x g RCF during 5 minutes) and plasma isolated from the cells. Plasma was stored at -20ºC for a maximum period of one week and then kept at -80ºC until the analyses.

Antioxidant supplementation experiment

In 2019, the food supplementation experiment was performed with tocopherol (VitE) diluted in corn oil at a density of 0.089 mg/ml. To calculate doses of supplemented experimental food, we assumed a dose of 50 mg of antioxidants per kg of nestling. Dose volume was adjusted to individual body mass and, for instance, two days-old nestlings of approximately 12 g of weight received a 0.6 mg dose of tocopherol. Since the used tocopherol (DL-α-tocopherol acetate (Sigma-Adrich T 3376-256)) was not as active as natural tocopherol, we applied a diet correction factor of 1.49 (De Ayala, Martinelli & Saino 2006). Therefore, nestlings that weighted 12 g received 0.89 mg of tocopherol diluted in 10 µl of corn oil.

In 2016, instead of tocopherol we used Nutratom (FARDI, S.A.), that, apart from sugar (9 g) and non-coloured antioxidants (VitE (10 mg) and VitC (60 mg)) also included 5 mg of coloured β-carotenes. For experimental treatment, each antioxidant-complex envelop (84 g) was diluted in 15 ml of mineral water, while 9 g of sugar was diluted in the same water volume as a control treatment. Doses were calculated assuming 50 mg of antioxidants per kg of nestling. Thus, two days-old nestlings of approximately 10 g that were assigned to

experimental and control treatment were supplemented with 0.1 ml of antioxidant or sugar solution, respectively.

In 2019, recently hatched nestlings were marked for individual recognition and, ten days later, we measured them as described above. Then, nestlings within each nest were ranked depending on body mass, the heaviest nestling randomly assigned to one of the experimental treatments (Antioxidants or Control), and the rest of nestlings with alternate treatment depending on their position in the body mass ranking. Every other day, we visited experimental nests to feed each nestling depending on the assigned experimental treatment. Ten days after hatching, we ringed nestlings and measured their tarsus and wing lengths, body mass, and colour of begging related traits. At this visit, we also fed the nestlings depending of their assigned experimental treatment. On day 14, we also collected blood samples by puncturing the brachial vein.

Colour measurements and estimation of colour variables

We used a spectrophotometer (Ocean Optics S2000) connected to a halogen deuterium lamp (D2-W, mini) through an optical fibre (QR-400-7-UV-vis) to measure coloration, after calibration to standard white (Ocean Optics WS-2) and to the dark (i.e., within the neck-warmer where we took all measures). Colour measurements were taken as spectral reflectance at 1 nm intervals between wavelengths of 300 to 700 nm. In nestlings, we measured colourations of mouth, flanges and breast skin. The mouth was measured by gently keeping the gape open and introducing carefully the probe to the centre of the upper mouthpart. Flanges were measured by maintaining nestlings with the mouth almost closed, but with the upper mandible slightly laterally displaced over the lower mandible, and placing the probe on the angle of the mouth-flange, thus avoiding confusion with mouth colouration (for further details on nestling colour measurements see Soler and Avilés (2010)).

To avoid the influence of ambient light on colour measurements we used an opaque and black neck-warmer. Briefly, one of the ends of the neck-collar warmer containing the probe was closed preventing the penetration of light. Nestlings or eggs were then included through the open end of the neck-warmer and completely covered with it once the probe was located on the structure we wanted to measure. We collected three measures perpendicularly to the surface to estimate repeatability.

We estimated brightness of eggshells and nestling traits as the mean reflectance value over the entire wavelength range (300-700 nm). Spectra of mouth and flanges present two different peaks, one at the UV region (300-400 nm) and another one between 580 and 680

nm, within the yellow-red wavelengths (Fig. 1). The skin was mainly UV coloured and, thus, the spectrum shows a clear peak in the UV-blue and small hill at the red regions (Fig. 1). Thus, for each trait, we estimated hue as the wavelength at which the reflectance is maximal for UV and visible range (i.e., yellow-red) separately (Doucet & Montgomerie 2003). We also estimated hue for the entire spectra as the wavelength at which the positive slope reaches its maximum. Moreover, we calculated chroma as the proportion of total reflectance due to that at the UV (300-400 nm), yellow-red (580-680 nm) and carotenoid (reflectance value at 700 nm minus that at the 450 nm wavelengths (Cuthill 2006; Isaksson *et al.* 2008; Charmantier *et al.* 2017)). Coloration of nestling skin is not carotenoid-based and, because skin reflectance at 450 nm is typically higher than that at 700 nm (Fig. 1), values of carotenoid chroma were negative and, thus, were not calculated for this body part. For eggshell colouration, we estimated the UV chroma and the blue-green chroma (400-575 nm).

Statistical analysis

Repeatability of colour measurements: Eggshell colouration: $R > 0.83$, $F_{(618,1242)} > 4.6$, $P < 0.0001$; cross-fostering experiment: Mouth $R > 0.89$, $F_{(107, 216)} > 7.48$, $P < 0.0001$; Flanges $R > 0.70$, $F_{(107, 216)} > 1.96$, $P < 0.0001$; Skin $R > 0.80$, $F_{(107, 215)} > 3.47$, $P < 0.0001$; VitE supplementation experiment: Mouth $R > 0.72$, $F_{(109, 220)} > 2.13$, $P < 0.001$; Flanges $R > 0.84$; $F_{(109, 219)} > 5.01$; $P < 0.001$; Skin $R > 0.79$, $F_{(109, 218)} > 3.46$, $P < 0.001$; Nutratom supplementation experiment: Flanges $R > 0.86$, $F_{(46, 94)} > 6.08$, $P < 0.001$; Skin $R > 0.71$, $F_{(46, 94)} > 2.06$; $P < 0.001$.

We explored the association between carotenoid concentration and nestling colouration by means of mixed model ANOVAs that only considered nestlings that were not antioxidant supplemented. The models included colouration of begging related traits as dependent variables, plasma carotenoid concentration as the fixed continuous independent factor, study year and breeding attempt nested within study year as fixed discrete factors, and the nest identity nested within breeding attempt (in turn, nested within study year) as random factor. In separate models that also included the main effects described above, we also estimated the interactions between breeding attempt and carotenoid concentration, and between study year and carotenoid concentration.

We estimated the genetic and environmental components of colouration of begging related traits and the concentration of carotenoids in plasma of cross-fostered nestlings by means of hierarchized nested ANOVAs with identity of nest of rearing as the variable explaining the environmental component, and nest-of-origin identity nested within nest of

rearing as the factor dealing with the genetic component. Both factors were considered as random factors. A significant effect of nest of origin will be interpreted as significant genetic component because siblings reared in separate nests would be more similar to each other than non-sibling. However, possible maternal effects, which by definition should be considered as environmental effects determining nestling phenotypes, might be confounded with variance explained by nest of origin or nest of rearing (Soler, Moreno & Potti 2003). Nestlings that are experimentally exchanged between nests share possible maternal effects with their genetic siblings that nest of origin will account for in our statistical models. We, thus, tried to control the estimates of genetic factors for maternal effects by estimating residuals of colour variables after controlling for eggshell colouration; a characteristic that indicates female condition at laying (Moreno *et al.* 2006), and it is related to antioxidants, hormones, and antibodies concentration of egg contents (Morales, Sanz & Moreno 2006; Siefferman, Navara & Hill 2006; López-Rull, Miksik & Gil 2008; Navarro *et al.* 2011).

To estimate the effects of the VitE and Nutratom supplementation on colouration and plasma carotenoid concentration, we also used mixed model ANOVAs. In this case, the model included colouration or carotenoid concentration of each nestling as dependent variables, experimental treatment and breeding attempt (i.e., first or second clutches, only for the VitE experiment) as fixed factors, and nest identity as the first random factor. The interaction between nest identity and experimental treatment was the second random factor to account for the repeated measures nature of our experimental approach (Quinn & Keough 2002; Schielzeth & Forstmeier 2009). Experimental effects on different body parts and colour variables were explored in separate statistical models.

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Additional Table 1. Results from Linear Regression Model exploring the association between body condition and colouration of begging-related traits (mouth, flanges and skin) or plasma carotenoid concentration of starling nestlings. Colour variables used include the achromatic component (Brightness), as well as hue and chroma of the total (Total) or particular wavelength intervals (UV, Yellow-red and carotenoid). Only nestlings from cross-fostering experiment and control-non-feeding nestlings were used to test these associations. Statistical effects of the experimental treatment with associated alpha-values lower than 0.05 are highlighted in bold fonts.

			Body condition			
			F	df	P	β
Mouth	Brightness		2.77	1, 220	0.097	-0.11
	Hue	Total	0.00	1, 220	0.996	0.00
		UV	0.60	1, 220	0.438	-0.05
		Yellow-Red	1.40	1, 220	0.237	0.07
	Chroma	UV	0.22	1, 220	0.638	0.03
		Yellow-Red	0.12	1, 220	0.725	0.02
		Carotenoid	0.12	1, 220	0.729	-0.02
Flanges	**Brightness**		**7.14**	**1, 220**	**0.008**	**0.17**
	Hue	Total	1.02	1, 220	0.314	-0.06
		UV	0.78	1, 220	0.378	0.05
		Yellow-Red	0.23	1, 220	0.626	0.03
	Chroma	UV	1.97	1, 220	0.162	0.09
		Yellow-Red	2.43	1, 220	0.120	-0.10
		Carotenoid	2.01	1, 220	0.156	-0.09
Skin	Brightness		2.05	1, 215	0.153	-0.09
	Hue	Total	0.37	1, 215	0.542	-0.04
		UV	3.02	1, 215	0.083	0.11
		Yellow-Red	0.28	1, 215	0.598	0.03
	Chroma	UV	0.08	1, 215	0.778	0.01
		Yellow-Red	0.72	1, 215	0.394	-0.05
Carotenoid concentration			3.73	1, 185	0.054	0.14

Additional Table 2. Results from Mixed model ANOVAs exploring the associations between carotenoid concentration in plasma and colouration of begging-related traits (mouth, flanges and skin) of spotless starling nestlings after controlling for the effect of year, breeding attempt (nested within study year) and the random effect of nest identity (nested within breeding attempt and study year). Interactions between carotenoid concentration and study year or breeding attempt were calculated in separated models that also included the main effects. Colour variables used include the achromatic component (Brightness), as well as hue and chroma of the total (Total) or particular wavelength intervals (UV, Yellow-red and carotenoid). We only have information on mouth colouration from the 2019 breeding season. Only nestling from cross-fostering experiment and control, non-feeding, nestlings were used to test these associations. Statistical effects of the experimental treatment with associated alpha-values lower than 0.05 are highlighted in bold fonts.

		Independent variables																		
		Carotenoid concentration				Study year			Breeding attempt (study year)			Nest ID (Breeding attempt (study year))			Study year * Carotenoid concentration			Breeding attempt * Carotenoid concentration		
Dependent variables		F	df	P	β	F	df	P	F	df	P	F	df	P	F	df	P	F	df	P
Mouth																				
Brightness		0.01	1, 95	0.972	0.00				1.16	1, 178.6	0.283	**1.61**	**84, 95**	**0.012**				0.82	1, 94	0.368
Hue	Total	1.01	1, 95	0.317	-0.10				3.49	1, 141.5	0.064	**5.30**	**84, 95**	**0.000**				0.19	1, 94	0.664
	UV	0.01	1, 95	0.904	0.15				1.15	1, 178.9	0.285	**1.70**	**84, 95**	**0.006**				2.58	1, 94	0.112
	Yellow-Red	0.11	1, 95	0.739	-0.04				**5.49**	**1, 170.2**	**0.020**	**2.80**	**84, 95**	**0.000**				0.00	1, 94	0.988
Chroma	UV	0.24	1, 95	0.625	-0.04				**4.78**	**1, 128.7**	**0.031**	**7.14**	**84, 95**	**0.000**				0.67	1, 94	0.414
	Yellow-Red	1.17	1, 95	0.283	0.13				1.99	1, 170.3	0.160	**2.78**	**84, 95**	**0.000**				0.20	1, 94	0.659
	Carotenoid	0.03	1, 95	0.870	-0.02				2.57	1, 177.1	0.111	**1.43**	**84, 95**	**0.044**				0.06	1, 94	0.804
Flanges																				
Brightness		**6.26**	**1, 103**	**0.014**	**0.25**	**38.21**	**1, 131.7**	**0.000**	**11.64**	**1, 183.8**	**0.001**	**2.87**	**96, 103**	**0.000**	2.28	1, 102	0.134	1.97	2, 101	0.144
Hue	Total	0.15	1, 103	0.703	-0.05	**29.59**	**1, 170.7**	**0.000**	1.64	1, 195.1	0.202	1.20	96, 103	0.185	**6.78**	**1, 102**	**0.011**	**3.41**	**2, 101**	**0.037**
	UV	0.79	1, 103	0.376	-0.11	0.14	1, 150.0	0.709	2.64	1, 198.2	0.106	**1.81**	**96, 103**	**0.002**	0.00	1, 102	0.987	0.52	2, 101	0.598
	Yellow-Red	**4.71**	**1, 103**	**0.032**	**-0.21**	1.15	1, 120.5	0.285	2.71	1, 164.2	0.101	**4.4**	**96, 103**	**0.000**	0.02	1, 102	0.882	0.01	2, 101	0.989
Chroma	UV	1.30	1, 103	0.257	0.13	**17.28**	**1, 131.9**	**0.000**	1.79	1, 184.0	0.183	**2.85**	**96, 103**	**0.000**	3.07	1, 102	0.083	2.41	2, 101	0.095
	Yellow-Red	0.48	1, 103	0.489	-0.09	**18.38**	**1, 152.5**	**0.000**	**6.42**	**1, 198.7**	**0.012**	**1.72**	**96, 103**	**0.004**	**4.50**	**1, 102**	**0.036**	2.76	2, 101	0.068
	Carotenoid	0.03	1, 103	0.867	-0.02	**13.29**	**1, 147.7**	**0.000**	**16.88**	**1, 197.4**	**0.000**	**1.91**	**96, 103**	**0.001**	0.10	1, 102	0.321	0.64	2, 101	0.530
Skin																				
Brightness		0.359	1, 101	0.550	-0.08	**21.15**	**1, 167.2**	**0.000**	**5.85**	**1, 192.9**	**0.017**	1.21	95, 101	0.170	0.06	1, 100	0.813	0.04	2, 99	0.958
Hue	Total	1.455	1, 101	0.231	0.00	0.26	1, 95.0	0.611	0.01	1, 95.0	0.921	**4705.94**	**95, 101**	**0.000**	0.00	1, 100	1.000	0.25	2, 99	0.783
	UV	0.470	1, 101	0.494	0.07	0.34	1, 117.8	0.560	0.04	1, 158.6	0.837	**4.45**	**95, 101**	**0.000**	0.02	1, 100	0.881	0.32	2, 99	0.726
	Yellow-Red	1.286	1, 101	0.259	0.14	**8.78**	**1, 140.1**	**0.004**	0.57	1, 191.0	0.452	**2.16**	**95, 101**	**0.000**	0.18	1, 100	0.673	0.15	2, 99	0.861
Chroma	UV	0.032	1, 101	0.858	-0.02	0.11	1, 124.8	0.747	0.08	1, 172.4	0.777	**3.37**	**95, 101**	**0.000**	0.06	1, 100	0.801	0.07	2, 99	0.929
	Yellow-Red	0.410	1, 101	0.523	-0.09	0.21	1, 148.1	0.646	0.32	1, 195.1	0.575	**1.80**	**95, 101**	**0.002**	0.05	1, 100	0.824	0.03	2, 99	0.973

Additional Table 3. Results from hierarchized ANOVA exploring the effect of nest of rearing and nest of origin (nested within nest of rearing) on colouration of begging-related characters (mouth, flanges and skin) and plasma carotenoid concentration of spotless starling nestlings without controlling for maternal effects. Colour variables used include the achromatic component (Brightness), as well as hue and chroma of the total (Total) or particular wavelength intervals (UV, Yellow-red and Carotenoid). Statistical effects of the experimental treatment with associated two-tailed alpha-values lower than 0.1 are highlighted in bold fonts and degrees of freedom associated to comparisons (F) of carotenoids concentration are marked with *.

			Independent factors					
			Nest of rearing			Nest of origin (nest of		
Dependent variables			$F_{29, 46}$ $F_{26, 37*}$	P	Estimated variance (%)	$F_{30, 46}$ $F_{25, 37*}$	P	Estimated variance (%)
Mouth	Brightness		**4.288**	**<0.001**	35.6	**1.691**	**0.053**	18.9
	Hue	**Total**	**1.647**	**0.064**	0.0	**1.748**	**0.043**	31.0
		UV	**2.091**	**0.012**	24.8	0.996	0.495	0.0
		Yellow-Red	**5.034**	**<0.001**	52.0	1.142	0.336	3.8
	Chroma	UV	**10.261**	**<0.001**	73.6	0.994	0.497	0.0
		Yellow-Red	**4.259**	**<0.001**	49.6	0.99	0.502	0.0
		Carotenoid	1.419	0.142	21.0	0.538	0.962	0.0
Flanges	Brightness		**2.832**	**<0.001**	38.4	0.767	0.777	0.0
	Hue	Total	**1.825**	**0.033**	26.3	0.642	0.899	0.0
		UV	**3.380**	**<0.001**	35.0	1.287	0.217	9.6
		Yellow-Red	**4.723**	**<0.001**	51.6	1.059	0.422	1.7
	Chroma	UV	**2.699**	**0.001**	27.0	1.272	0.227	10.3
		Yellow-Red	**2.386**	**0.004**	26.4	1.114	0.364	4.7
		Carotenoid	**2.840**	**<0.001**	31.8	1.151	0.328	5.7
Skin	Brightness		**2.649**	**0.001**	23.1	1.408	0.145	15.1
	Hue	Total	**2.763**	**0.001**	28.8	1.234	0.256	8.8
		UV	**12.717**	**<0.001**	58.2	**2.884**	**<0.001**	22.2
		Yellow-Red	**6.795**	**<0.001**	52.7	**1.652**	**0.061**	13.3
	Chroma	UV	**4.215**	**<0.001**	38.4	1.514	0.100	14.5
		Yellow-Red	**3.501**	**<0.001**	32.6	1.456	0.123	14.5
Carotenoid concentration			**8455**	**<0.001**	66.5	1.263	0.254	4.8

Additional Table 4. Results from Mixed model ANOVAs exploring the effect of VitE supplementation on colouration of begging-related characters (mouth, flanges and skin) and plasma carotenoid concentration of spotless starling nestlings after controlling for the fixed effects of breeding attempt, and the random effect of nest identity. The interaction between nest identity and experimental treatment was also included in the model as a second random factor to account for the repeated measure approach within nests. Colour variables used include the achromatic component (Brightness), as well as hue and chroma of the total (Total) or particular wavelength intervals (UV, Yellow-red and carotenoids). Statistical effects of the experimental treatment with associated two-tailed alpha-values lower than 0.1 are highlighted in bold fonts.

		Independent factors													
		Exp. treatment					Nest Id			Breeding attempt			Exp. x Nest Id		
Dependent variables		F	df	Means (s.e.) Control	Means (s.e.) Treatment	P	F	df	P	F	df	P	F(df)	df	P
Mouth															
Brightness		1.93	1, 71.4	13.6 (0.3)	14.5 (0.4)	0.168	0.84	58, 5.0	0.676	1.59	1, 9.3	0.238	0.780	57, 109	0.849
Hue	Total	2.51	1, 98.0	325.7 (6.2)	338.5 (7.8)	0.116	**3.87**	**67, 67.4**	**0.000**	**3.17**	**1, 75.6**	**0.078**	0.551	66, 100	0.995
	UV	**4.99**	**1, 65.8**	**324.3 (1.1)**	**327.9 (1.4)**	**0.028**	0.73	58, 8.6	0.778	0.00	1, 9.2	0.950	1.261	57, 109	0.149
	Yellow-Red	0.19	1, 70.2	622.5 (1.7)	622.2 (1.7)	0.667	0.58	58, 8.3	0.889	2.04	1, 9.1	0.187	0.849	57, 109	0.749
Chroma	UV	1.15	1, 66.4	0.3 (0.0)	0.3 (0.0)	0.287	0.38	58, 8.9	0.987	0.10	1, 9.0	0.757	1.189	57, 109	0.219
	Yellow-Red	0.25	1, 65.7	0.4 (0.0)	0.4 (0.0)	0.622	0.50	58, 8.9	0.943	0.88	1, 9.0	0.373	1.277	57, 109	0.138
	Carotenoid	**3.79**	**1, 69.9**	**0.7 (0.0)**	**0.8 (0.0)**	**0.056**	0.89	58, 4.4	0.644	0.04	1, 9.5	0.844	0.866	57, 109	0.723
Flanges															
Brightness		**3.67**	**1, 68.5**	**25.5 (0.5)**	**26.6 (0.6)**	**0.059**	0.81	58, 7.7	0.700	1.12	1, 9.2	0.317	1.116	58, 110	0.307
Hue	Total	0.17	1, 73.6	336.0 (6.8)	346.7 (7.2)	0.679	2.00	58, 3.5	0.289	1.35	1, 9.5	0.274	0.758	58, 110	0.877
	UV	**4.16**	**1, 67.3**	**346.3 (1.4)**	**349.2 (1.2)**	**0.045**	0.69	58, 8.6	0.810	**5.95**	**1, 9.2**	**0.037**	1.265	58, 110	0.146
	Yellow-Red	0.23	1, 76.1	618.9 (2.3)	618.0 (2.4)	0.635	0.64	58, 7.7	0.844	0.79	1, 9.1	0.396	0.652	58, 110	0.963
Chroma	UV	0.52	1, 81.3	0.3 (0.0)	0.3 (0.0)	0.473	0.29	58, 8.1	0.997	0.19	1, 9.0	0.671	0.509	58, 110	0.997
	Yellow-Red	0.52	1, 75.4	0.4 (0.0)	0.4 (0.0)	0.472	0.53	58, 6.3	0.903	0.01	1, 9.2	0.911	0.678	58, 110	0.948
	Carotenoid	0.87	1, 70.2	0.8 (0.0)	0.8 (0.0)	0.354	1.67	58, 4.1	0.328	**3.92**	**1, 9.6**	**0.077**	0.966	58, 110	0.550
Skin															
Brightness		0.25	1, 70.7	12.49 (0.4)	12.50 (0.4)	0.617	1.62	58, 3.0	0.396	2.84	1, 9.5	0.125	0.703	55, 104	0.925
Hue	Total	0.35	1, 56.5	315.1 (4.4)	317.44	0.557	0.21	58, 18.3	0.999	1.04	1, 9.1	0.334	**7.332**	**55, 104**	**0.000**
	UV	**5.20**	**1, 67.5**	**318.7 (0.4)**	**320.44(0.9)**	**0.026**	1.78	58, 5.0	0.690	0.67	1, 9.4	0.432	0.883	55, 104	0.690
	Yellow-Red	0.04	1, 75.8	629.8 (2.2)	630.6 (2.2)	0.850	1.22	58, 5.7	0.445	0.14	1, 9.2	0.718	0.533	55, 104	0.994
Chroma	UV	0.72	1, 65.2	0.4 (0.0)	0.4 (0.0)	0.398	0.29	58, 8.8	0.997	0.35	1, 9.0	0.567	1.075	55, 104	0.370
	Yellow-Red	0.37	1, 66.0	0.2 (0.0)	0.21 (0.00)	0.544	0.75	58, 7.7	0.773	1.78	1, 9.2	0.215	0.995	55, 104	0.498
Carotenoid concentration		0.24	1,59.9	8.5 (0.3)	8.7 (0.3)	0.628	2.58	53,6.27	0.109	**33.85**	**1,8.17**	**0.000**	1.14	50,78.0	0.297

Additional Table 5. Results from Mixed model ANOVAs exploring the effect of Nutratom supplementation on colouration of begging-related characters (flanges and skin) and carotenoids concentration in plasma of spotless starling nestlings after controlling for the random effects of nest identity and the interaction between nest identity and experimental treatment to account for the repeated measure approach within nests. Colour variables used include the achromatic component (Brightness), as well as hue and chroma of the total (Total) or particular wavelength intervals (UV, Yellow-red and carotenoid). Statistical effects of the experimental treatment with associated two-tailed alpha-values lower than 0.1 are highlighted in bold fonts.

		Independent factors										
		Exp. treatment					Nest Id			Exp. x Nest Id		
		F	df	means (s.e.)		P	F	df	P	F	df	P
Dependent variables				Control	Treatment							
Flanges												
Brightness		**5.459**	**1, 14.0**	**39.18 (1.7)**	**43.37 (1.7)**	**0.034**	**3.824**	**13, 13**	**0.011**	**2.022**	**13, 17**	**0.086**
Hue	Total	**3.885**	**1, 16.1**	**470.43 (17.9)**	**430.27 (21.7)**	**0.066**	1.156	13, 13	0.399	0.631	13, 17	0.797
	UV	0.081	1, 27.7	348.48 (2.5)	349.45(2.4)	0.777	**19.676**	**13, 13**	**0.000**	0.103	13, 17	0.999
	Yellow-Red	0.058	1, 13.61	626.91 (5.8)	622.09 (5.5)	0.813	1.631	13, 13	0.195	**3.236**	**13, 17**	**0.013**
Chroma	UV	**9.974**	**1, 14.81**	**0.19 (0.0)**	**0.22 (0.0)**	**0.006**	**2.783**	**13, 13**	**0.038**	1.092	13, 17	0.424
	Yellow-Red	**6.347**	**1, 14.86**	**0.42 (0.0)**	**0.39 (0.0)**	**0.023**	**3.121**	**13, 13**	**0.025**	1.06	13, 17	0.447
	Carotenoid	1.083	1, 15.81	0.92 (0.0)	0.89 (0.0)	0.313	1.034	13, 13	0.476	0.705	13, 17	0.736
Skin												
Brightness		0.0081	1, 13.85	17.46 (0.7)	16.78 (0.9)	0.929	0.618	13, 13	0.801	**2.333**	**13, 17**	**0.051**
Hue	Total	0.498	1, 15.38	308.17 (0.1)	308.27 (0.09)	0.491	1.416	13, 13	0.269	0.832	13, 17	0.626
	UV	0.729	1, 14.83	320.78 (0.6)	320.18 (0.6)	0.406	1.573	13, 13	0.212	1.079	13, 17	0.433
	Yellow-Red	1.685	1, 18.04	645.96 (5.3)	638.18 (5.2)	0.211	**3.927**	**13, 13**	**0.010**	0.389	13, 17	0.955
Chroma	UV	0.244	1, 14.35	0.40 (0.0)	0.40 (0.0)	0.629	1.205	13, 13	0.371	1.468	13, 17	0.226
	Yellow-Red	0.253	1, 15.72	0.20 (0.0)	0.20 (0.0)	0.622	**3.335**	**13, 13**	**0.019**	0.726	13, 17	0.717
Carotenoid concentration		**4.51**	**1, 14.4**	**8.94 (0.4)**	**7.71 (0.4)**	**0.051**	**9.08**	**13,10.9**	**0.000**	0.641	12, 15	0.778

CAPÍTULO V

Made-up mouths with preen oil reveal genetic and phenotypic conditions of starling nestlings

Abstract

Animal colouration results from pigments, nanostructures, or to the cosmetic use of natural products, and plays a central role in social communication. The role of cosmetic colouration has traditionally been focussed in scenarios of sexual selection, but it could also take place in other contexts. Here, by using spotless starlings (*Sturnus unicolor*) as a model system, we explore the possibility that nestlings cosmetically use their intensely yellow coloured uropygial secretion to signal their genetic and/or phenotypic quality. In agreement with the hypothetical cosmetic use of the uropygial secretion, (i) video recorded nestlings collected secretion with the bill at the age of feathering, (ii) cotton swabs turned to the colour of secretion after rubbing with them nestlings' gape, and (iii) gape and skin colourations correlated positively with that of secretion. Furthermore, we found that (iv) secretion colouration has a genetic component, and (v) associated positively with Vitamin-E supplementation and (vi) with plasma carotenoid concentration, which highlights the informative value of nestling secretion. Finally, (vii) colouration of begging-related traits and of secretion of nestlings predicted parental feeding preferences. Consequently, all these results strongly suggest that the cosmetic use of coloured uropygial secretion might also play a role in parent-offspring communication, complementing or amplifying information provided by the flamboyant coloured gapes and skin of nestlings. The use of makeups by offspring for communication with relatives has been scarcely explored and we hope that these results will encourage further investigations in birds and other taxa with parental care.

Keywords: *Antioxidants, Begging, Genetic component, Makeup hypothesis, Parent-offspring communication, Signalling, Uropygium, Vitamin E*

Introduction

Colouration plays important roles mediating the relationship between animals and the environment (De Salle & Bachor 2020). Although colouration of particular animal characters are mainly due to pigments that accumulate within the trait structure during development, or to nanostructures that affect light reflection (Shawkey & D'Alba 2017), it may also result from the cosmetic use of natural products including own exocrine secretions. The interest in cosmetic colouration for evolutionary biologists is due to papers published at the end of the last century by Negro *et al.* (1999) and Piersma, Dekker and Sinninghe-Damste (1999), where they proposed a role of cosmetic functioning in scenarios of animal communication (i.e., the makeup hypothesis). Particularly, they suggested that the deposition of cosmetics might provide an alternative honesty-reinforcing mechanism linking phenotypic condition and colouration of animals. However, mainly due to the lack of experimental tests, we are only starting to understand possible roles of coloured cosmetic products explaining covariation between animal colourations and their phenotypic quality (but see, Piault *et al.* 2008).

Birds are suitable organisms to test the makeup hypothesis; first because most species produce uropygial secretion that is spread on feathers and other teguments, but also because environmental factors that affect body condition (e.g., parasites or food availability) also determine both preen wax production and intensity of preening behaviour (Delhey, Peters & Kempenaers 2007; Piault *et al.* 2008). We know that birds use colouration of their plumage, beaks, or any other teguments to signal their phenotypic or genetic condition to conspecifics in scenarios of social communication (Hill & McGraw 2006). However, through preening, most avian teguments are smeared with uropygial secretion, which would affect perception of coloured signals (Delhey, Peters & Kempenaers 2007). Smeared uropygial secretion might, for instance, filter UV reflectance (Shawkey *et al.* 2007), enhance tegument colouration (Lopez-Rull, Pagan & Macias Garcia 2010; Amat *et al.* 2011) or even paint white plumage or bills with flamboyant coloured secretions (Kemp 2001). Importantly, as we mentioned before, quantity of secretion and intensity of preening activity usually reflect phenotypic condition of birds and, thus, the deposition of cosmetics on coloured traits might honestly reinforce their signalling information (Negro *et al.* 1999; Piersma, Dekker & Sinninghe-Damste 1999). Moreover, the strikingly yellow-orange coloured uropygial secretion of some species (e.g., some hornbill species of the genera *Buceros*, *Aceros*, *Peneloipes* and *Rhinoplax*) might directly reflect phenotypic condition (i.e., antioxidant capacity) of birds, which is shown when birds spread the secretion on white feather patches or on the beak (Delhey, Peters & Kempenaers 2007).

Similar to extravagant colourations, functionality of cosmetic colouration mediated by uropygial secretion has mainly been described in scenarios of sexual selection. This is mainly because conspicuous colourations of plumage and of some other teguments mainly develop during the breeding season (Delhey, Peters & Kempenaers 2007), and they operate either, to favour mating success (Amat *et al.* 2011) or to enhance parental contribution of mates (Díaz-Lora *et al.* 2020). However, cosmetic colourations mediated by uropygial gland secretion might also occur in nestlings in contexts of parent-offspring communication; at least after development of the uropygial gland and once production of secretion starts (Piault *et al.* 2008). This possibility mainly relies on the assumption that, similar to adults, nestling birds spread their preen wax on their teguments (i.e., skin, feathers, beak and gape), which would affect their colouration. The signalling role of nestling colouration is strongly supported (Kilner 2006). We know for instance that the skin of most bird species are UV-coloured (Avilés *et al.* 2008), and that its intensity in nestlings might inform parents on nestling immune capacity (Jourdie *et al.* 2004; Soler *et al.* 2007). Moreover, most altricial nestlings have evolved flashy coloured gapes and rictal flanges that signal to parents their phenotypic and/or genetic condition (Kilner 1997; Soler *et al.* 2007; Ewen *et al.* 2008; Martín-Gálvez & Soler 2017; Martínez-Renau *et al.* 2021). Thus, if nestlings smear those coloured traits with their uropygial secretion, colours detected by parents will be those after the colouring, filtering or enhancing effect of the preen wax. Similarly, we know that parents use nestling colouration to adjust feeding effort and to decide which nestlings to feed (Jourdie *et al.* 2004; Bize *et al.* 2006; De Ayala *et al.* 2007; Dugas 2009) accordingly. Consequently, if nestling colourations are at least partially determined by the spread of uropygial secretion, its cosmetic use should play a role influencing parental decisions and food allocation.

Similar to the above described functioning of cosmetic colouration in adults, colour of the uropygial secretion of nestlings might, not only modify or intensify already coloured traits, but also signal phenotypic and/or genetic conditions of nestlings when smeared on begging related traits. If that was the case, environmental conditions should influence production and/or coloration of nestling preen-wax. This might be the case of tawny owls (*Strix aluco*) nestlings, in which immune stimulation impaired development of the uropygial gland and resulted in nestlings with brighter beaks (Piault *et al.* 2008). Although those results conform the first experimental test of the makeup hypothesis in scenarios of parent-offspring communication, no result supported the assumption of nestlings preening their beak, or the associations between colourations of uropygial secretion and beak. Thus, results could also be explained if immune stimulation affected both the production of preen wax and the beak

colouration. Moreover, predictions of the makeup hypothesis functioning in scenarios of parent-offspring communication (i.e., parental feeding preference in relation to nestling colouration) have never been tested. Here, we try to fill these gaps and test several predictions and assumptions of the makeup hypothesis in a population of spotless starling (*Sturnus unicolor*), a species with intensely yellow coloured uropygial secretion in nestlings, which turns to pale-beige in fledglings and adults (see Additional Figure 1). In particular, (i) we explore the possibility that the colouration of nestling uropygial secretion has a genetic component, thus being a heritable character able of reflecting genetic quality. In order to test this, we performed a cross-fostering experiment and estimated genetic and environmental components of uropygial colouration. We also (ii) study the possibility that colouration of uropygial secretion can reflect phenotypic condition of nestlings. We predict that the intensity of yellow colour in the secretion should: (iia) reflect the antioxidant state of nestlings (i.e., concentration of carotenoids in the blood); (iib) be affected by the quality of the environment where the nestlings developed (vary with breeding attempt); and (iic) depend on availability of antioxidants in the diet. In order to test this, we conducted a food supplementation experiment with Vitamin E (VitE). Finally, (iii) by swabbing gapes and inspecting swabs colour, we explore the possibility that nestlings cosmetically use their uropygial secretion to stain signalling traits directed to parents. Moreover, (iv) we compared the colouration of secretions with those of begging related traits such as mouth, flanges, and skin of nestlings, which in this species are known to reflect phenotypic and genetic quality of nestlings (Soler *et al.* 2007; Martínez-Renau *et al.* 2021). Finally, (v) we video-recorded parental and nestling behaviours to detect directly preening behaviour of nestlings at an age far before feathers are developed, and quantify parental food allocation depending on nestling colourations.

Methods

Study area and species

Fieldwork was carried out in 2019 in a spotless starling (hereafter starlings) population located in southern Spain, at the old railway station of La Calahorra (37°15’ N, 3°01’W), sited at the high altitude plateau of the semiarid Hoya de Guadix. Starlings breed there from April to June in 94 cork-made nest boxes (internal dimensions: 180 mm x 210 mm and 350 mm high, 240 mm from the bottom to the hole entrance) attached to tree trunks or walls.

The starling is a medium-sized, hole-nesting altricial passerine. In our study population, starlings start laying their eggs in mid-April, the clutch size is commonly of 4-5 eggs and they lay one egg per day. The incubation is mainly a female duty, starts before

laying the last eggs, and extends for 11 days (Azcárate-García *et al.* 2020). Nestling period is about 18 days, although it can extend up to 25 days (Veiga & Polo 2011; Soler *et al.* 2017).

Fieldwork

At the end of March, we visited nest boxes every three days, which allowed estimating the date of laying of the first egg (hereafter, laying date). We then visited nests every other day until detecting clutch completion, when we measured colouration of eggshells (see below). Twelve days after laying, we visited nest boxes again and then daily until hatching, when nests were randomly assigned to one of the two performed experiments (VitE or cross-fostering). This approach has previously been used to estimate genetic and environmental components of colouration of begging related traits in spotless starlings (Martínez-Renau *et al.* 2021), and we use it here to estimate those components of the uropygial secretion colouration.

For nests that were assigned to the cross-fostering experiment, one day after hatching, we exchanged two nestlings between two nests of equal hatching date and similar (± 1 egg) clutch size. The food supplementation experiment also started the day after hatching and consisted in oral administration of an age-dependent dose of VitE (DL-α-tocopherol acetate (Sigma-Adrich T 3376-256)) diluted in corn oil, or of only corn oil, to experimental and control nestlings respectively (for further details see Additional Methods, and for a similar experimental approach see De Ayala, Martinelli and Saino (2006)). For dose estimates we also followed Martínez-Renau *et al.* (2021). Ten days later, we ringed birds and collected biometrical measurements (tarsus length with a digital calliper (precision 0.01 mm), wing maximum-length with a metal ruler (precision 0.1 mm) following procedures described in Svensson (1992), and body mass with a digital balance (Ascher CS, precision 0.01 g)) of all nestlings in the nest. At this visit, we also sampled uropygial secretion and measured colouration of begging-related traits (mouth, flanges and skin) and of uropygial secretion with a spectrophotometer (see below). Uropygial secretion of nestlings was extracted by keeping in contact the gland opening and a sterile micro-capillary (32 mm, 10 μl), and slightly pressing the gland until emptying it.

Between 7 and 11 days after hatching, we video recorded the interior of the starling nest-boxes for two hours to detect whether nestlings used uropygial secretion for preening at these ages, and to quantify rates of allocation of food by parents (feeding rate of each nestling in every nest). For a detailed explanation of the equipment used, and the protocol followed for video recording, see Additional Methods. On day 14, we collected blood samples of

nestlings by puncturing the brachial vein and filling heparinized capillaries, that were emptied in microfuge tubes and kept at 4ºC in a portable fridge until arriving to the lab. Blood samples were centrifuged (18,000 x g RCF) for 5 minutes, and plasma separated from the cells. Plasma was stored at -20ºC for a maximum period of one week and then kept at -80ºC until the analyses**.** A total of 95 nestlings from 28 nests were included in the cross-fostering experiment whereas 146 nestlings from 56 additional nests were used in the experiment of food supplementation.

Colour measurements and estimation of colour variables

In order to measure colouration, we used an Ocean Optics S2000 spectrophotometer connected to a halogen deuterium lamp (D2-W, mini) through an optical fibre (QR-400-7-UV-vis), which was calibrated to standard white (Ocean Optics WS-2) and to the dark (i.e., within the black neck-gaiter in which we took all measures). Colour measurements were taken as spectral reflectance at 1 nm intervals between wavelengths of 300 to 700 nm. In addition to nestling colouration, in order to statistically controlling for possible maternal effects on nestlings colouration (see below), we also took three measures of reflectance of the eggshells at the pointed and blunt ends, and at the centre of the eggshell. In nestlings, we measured colourations of mouth, flanges and breast skin following protocols described elsewhere (Soler & Avilés 2010; Martínez-Renau *et al.* 2021). Colour of uropygial secretion was estimated on a piece of blotting paper after gently smearing approximately 4 µl of the collected secretion on a 1-cm-diameter circle (Soler *et al.* 2014). We collected three measures perpendicularly to the surface and, since repeatability resulted relatively high for all measured characters ($R > 0.70$), we used mean values in subsequent analyses.

We used AVICOL v.6 (Gomez 2006) for correcting all negative values of reflectance to zero, and to reduce noise by means of a triangular correction implemented in the software. Reflectance spectra of the uropygial secretion of nestlings have one clear peak at the UV wavelength, where the maximum slope typically appeared (Additional Figure 1). This peak is followed by a depression at the blue part of the spectrum. After wavelength of approximately 500 nm, reflectance steeply increases reaching its maximum at the yellow-red (600-700) wavelength (See Additional Figure 1). We estimated brightness as the proportion of total reflectance, chroma as the proportion of total reflectance due to UV (300-400 nm), Yellow-Red (580-680nm), and hue as the wavelength at which reflectance reached its maximum at each of the two wavelength intervals considered (Cuthill, Hiby & Lloyd 2006). We also estimated hue for the entire spectra (Total hue) (as the wavelength at which the positive slope reaches its maximum), and carotenoid chroma (reflectance value at 700 nm

minus that at the 450 nm wavelengths (Cuthill, Hiby & Lloyd 2006; Isaksson *et al.* 2008; Charmantier *et al.* 2017)) for eggshells and nestlings traits including secretion. Spectrum of each measured trait is shown in the Supplementary Material (Additional Figure 1). For details on considered colour variables and spectra characteristics of each measured trait, see Martínez-Renau et al. (2021).

Estimating blood plasma carotenoid concentration

We estimated carotenoid concentration in blood plasma by means of a spectrophotometric assay described elsewhere (Bertrand, Faivre & Sorci 2006). Briefly, after adding 135 µl of ethanol to 15 µl of plasma, we vortexed the mix and centrifuged at 4ºC and 1,500 x g RCF for 10 min, measuring absorbance of the supernatant at 450 nm in a spectrophotometer (Sunrise-basic Tecan, 16039400). We used lutein (CAYM10010811-1, VWR) to adjust calibration curves of absorbance at 450 nm (from 0 to 200 µg x mL^{-1}) (R^2 = 0.999), which allowed us to extrapolate absorbance values to those of lutein concentrations, which we used as a proxy of carotenoid concentration in blood plasma.

Statistical analyses

Briefly, in order to estimate genetic and environmental components of colouration of uropygial secretion from the cross-fostering experiment (Merilä 1996), we used hierarchized nested ANOVAs with identity of nest of rearing as the random factor explaining the environmental component, and nest-of-origin identity nested within nest of rearing as the random factor dealing with the genetic component (Merilä 1996; Soler, Moreno & Potti 2003). A significant effect of nest of origin will be interpreted as uropygial secretion of siblings reared in separate nests being more similarly coloured to each other than non-siblings are. However, possible maternal effects, which by definition should be considered as environmental effects determining nestling phenotypes, might be invariably confounded with variance explained by nest of origin or nest of rearing (Soler, Moreno & Potti 2003). Thus, trying to statistically controlling the estimates of genetic factors for maternal effects we estimated residuals of colour variables after controlling for eggshell colouration. Eggshell colouration indicates female condition at laying (Moreno *et al.* 2006) and antioxidants, hormones, and antibodies concentration of egg contents (Morales, Sanz & Moreno 2006; Siefferman, Navara & Hill 2006; López-Rull, Miksik & Gil 2008; Navarro *et al.* 2011) and, thus, those residuals should be appropriately controlled for maternal effects. In any case, we also analysed raw colour values of the uropygial secretion of cross-fostered nestlings to estimate amount of variance explained by nest of rearing and nest of origin.

We also used mixed model ANOVAs to estimate the effects of the VitE supplementation on colouration of uropygial secretion. The model included colour variables as dependent factors, experimental treatment and breeding attempt (i.e., first or second clutches) as fixed factors, and nest identity nested within breeding attempt as the first random factor. The interaction between nest identity and experimental treatment was the second random factor to account for the repeated measures nature of our experimental approach (Quinn & Keough 2002). Experimental effects on different colour variables were explored in separate statistical models.

The hypothetical association between plasma carotenoid concentration and colouration of the uropygial secretion was estimated by looking at best models based on AIC criteria. Variables describing colouration of uropygial gland secretion, together with breeding attempt, were used as independent variables and carotenoid concentration as the explanatory variable. Only nestlings that were not supplemented with VitE and those involved in cross-fostering experiments were considered in this analysis. We estimated AIC's values and variance explained by each model, and commented those that differ in less than two units with the AIC value of the best model. Later, in order to estimate the partial effect of each variable in the best model, we ran General Lineal Models and estimated associated P-values by adjusting degrees of freedom to the number of nests used in the analyses.

In order to explore the association between colouration of uropygial gland secretion and that of begging related traits, we used bivariate Pearson correlations between them for each of the colour components and each of the begging related traits (mouth, flanges and skin). Finally, the association between parental feeding preferences and colouration of nestling traits, including that of the uropygial secretion, was explored by means of General Regression Analyses. Briefly, for each nestling, we estimated observed (number of received feedings divided by total feedings recorded in a target nest) minus expected (total feedings divided by number of nestlings in a target nest) feeding rates (OBS-EXP), and used this information as dependent variable. As independent factors, we included nestling body mass and all colour components (see above) of nestling traits (mouth, flanges, skin, and uropygial secretion) and looked for the best models (i.e., Mallow's Cp criteria) explaining OBS-EXP feeding rates. All these statistical analyses were run in STATISTICA v.13 (Dell-Inc 2015).

Results

Genetic and environmental components of colouration of uropygial gland secretion

Colouration of the uropygial gland secretion of starling nestlings was mainly environmentally determined (Table 1, Additional Table 1). After controlling for possible maternal effects,

brightness, UV and yellow-red hues and UV chroma were mainly explained by environmental factors with nest of origin explaining a not significant proportion of variance (Table 1). On the contrary, total hue, and yellow-red and carotenoid chroma demonstrated a significant genetic component, while nest of rearing did not explain a significant proportion of variance (Table 1). These results therefore suggest that some colour components of the uropygial secretion are genetically determined while some others depend on environmental factors.

Table 1: Results from hierarchized ANOVA exploring the random effects of nest of rearing and nest of origin (nested within nest of rearing) on colouration of uropygial gland secretion of spotless starling nestlings after controlling for maternal effects (i.e., eggshell colouration). Percentage of variance explained by each factor is also shown. Statistical effects of experimental treatment associated with two-tailed alpha-values lower than 0.1 are in bold font.

Uropygial gland secretion		Nest of rearing			Nest of origin (nested within rearing)		
		$F_{27,26.0}$	P	Variance (%)	$F_{27,40}$	P	Variance (%)
Brightness		**2.83**	**0.005**	**39.3**	1.45	0.140	13.1
Hue	Total	1.07	0.431	2.7	**2.01**	**0.022**	**37.3**
	UV	**12.41**	**< 0.001**	**48.2**	0.28	0.999	0
	Yellow-Red	**3.28**	**0.002**	**38.6**	0.89	0.615	0
Chroma	UV	**7.12**	**< 0.001**	**66.1**	1.07	0.413	1.4
	Yellow-Red	1.24	0.294	8.4	**1.98**	**0.024**	**34.5**
	Carotenoid	1.36	0.217	12.3	**2.04**	**0.020**	**34.3**

Effects of experimental food supply on colouration of uropygial secretions

Experimental supplementation with VitE affected yellow-red hue in interaction with breeding attempt (Table 2, Additional Table 2). Secretion of experimental nestlings resulted redder than that of control nestlings in second breeding attempts, while it was not the case for nestlings of first breeding attempts (Fig 1A). Moreover, total hue of experimental nestlings tended to be of higher values (i.e., maximal slope appearing closer to the human visual range) than those of control siblings (Fig 1B). Finally, secretion of nestlings from first breeding attempts had lower values of UV-hue than those of second breeding attempts (Fig 1C). No other colour variable was affected by neither experimental treatment nor breeding attempt.

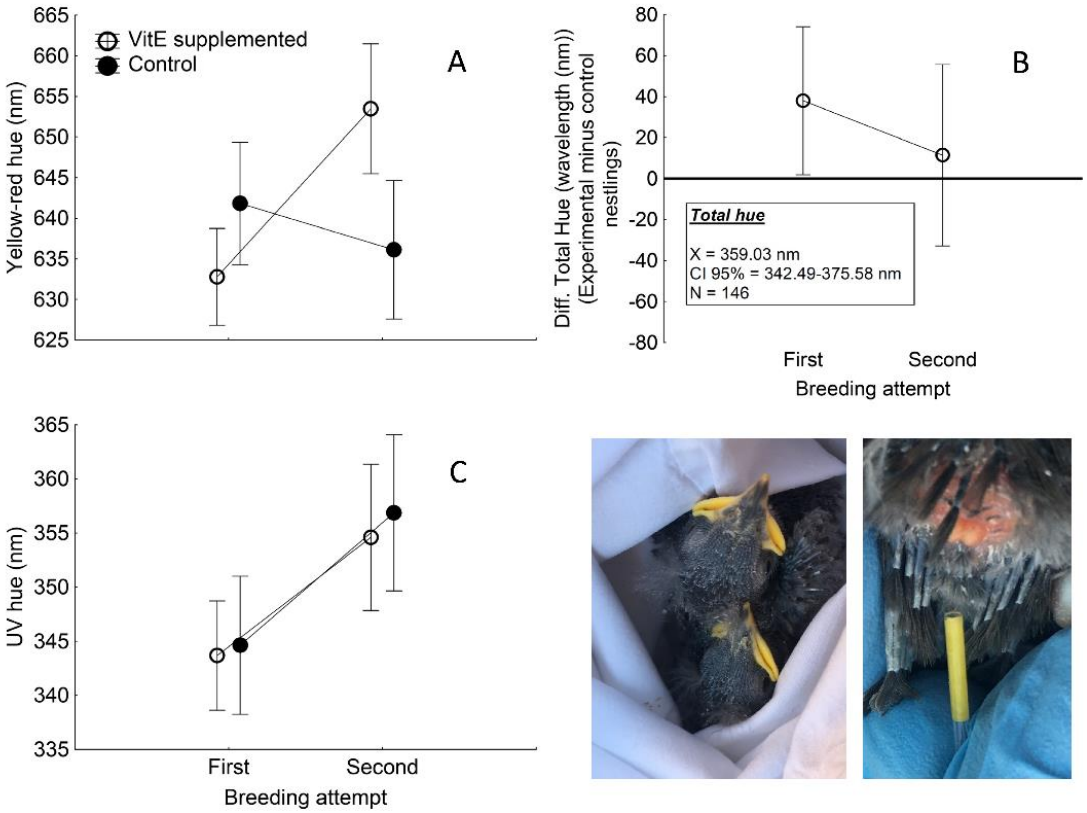

Figure 1: Least square means (± 95% CI) of uropygial gland colouration (Yellow-red hue, UV hue) of VitE food supplemented and control nestlings from first and second breeding attempts. Mean values of within-nest differences between experimental and control nestlings with respect to total hue values of uropygial secretions of first and second breeding attempt are shown in subfigure B. Finally, we show spotless starling nestlings within a cotton bag as well as the uropygial gland and the yellow secretion collected in a capillary.

Association between colourations of the uropygial secretion and plasma carotenoid concentration of starling nestlings

Colouration of uropygial gland secretion predicted carotenoid concentration in blood plasma. That was the case after correcting for the strong effect of breeding attempt on plasma carotenoid concentration (Fig 2). In fact, breeding attempt appeared in all 13 best models in the Additional Table 3 explaining plasma carotenoid concentration. Uropygial-secretion brightness also appeared in all best models (Additional Table 3); individuals with brighter uropygial secretion were those with lower carotenoid concentration (Fig. 2). UV-chroma is the other colour variable more commonly retained in best models (Additional Table 3); individuals with secretion of higher UV-chroma were those with higher carotenoid concentration in the blood (Fig. 2). These three variables comprise the models with lower AIC value (Additional Table 3) and, either, brightness ($F_{1,79}$ = 4.21, P = 0.043), UV-chroma ($F_{1,79}$ = 9.60, P = 0.003) or breeding attempt ($F_{1,79}$ = 57.9, P < 0.0001) explained significant proportion of variance. However, different colour variables of uropygial secretion are in most

cases related to each other and, thus, interpretation of results in terms of a specific colour variable is not straightforward. For instance, carotenoid and yellow-red chroma are negatively related to brightness (R = -0.44 and R = -0.43, respectively, $P < 0.0001$) and to UV-chroma (R = -0.40 and R = -0.73, respectively, $P < 0.0001$). Consequently, we can conclude that colouration of the uropygial secretion of starling nestlings predicts plasma carotenoids, variables retained in the final best models should be cautiously interpreted.

Table 2. Results from mixed-model ANOVAs exploring the effect of antioxidant supplementation (Exp Treatment) on colouration of the uropygial secretion of spotless starling nestlings after controlling for the fixed effect of breeding attempt (Breed attempt), the random effect of nest identity (nested within breeding attempt (Nest ID (Breed)), and the interaction between nest identity and experimental treatment to account for the repeated measure approach within nests. The random effects are shown in the Supplementary Material (Additional Table 2). Weighted means of first (column A) and second (column B) breeding attempts, as well as those of experimental (column A) and control (column B) nestlings are also showed. Statistical effects of the experimental treatment with associated two-tailed alpha-values lower than 0.1 are highlighted in bold font.

Dependent Factors	F	df	Weighted Means (SE)		P
			(A)	(B)	
BRIGHTNESS					
Breed attempt (1)	0.479	1, 61.4	41.77 (1.13)	42.72 (1.46)	0.491
Exp treatment (2)	0.903	1, 50.1	41.01 (1.27)	43.22 (1.25)	0.347
(1) * (2)	0.893	1, 49.8			0.349
TOTAL HUE					
Breed attempt (1)	2.57	1, 59.96	376.7 (12.1)	329.8 (8.2)	0.114
Exp treatment (2)	**3.58**	**1, 49.07**	**371.9 (13.4)**	**346.5 (10.1)**	**0.064**
(1) * (2)	0.709	1, 48.69			0.404
UV HUE					
Breed attempt (1)	**9.43**	**1, 61.36**	**342.0 (2.0)**	**355.1 (1.6)**	**0.003**
Exp treatment (2)	0.03	1, 53.18	347.5 (2.2)	346.4 (2.1)	0.862
(1) * (2)	0.00	1, 53.05			0.994
YELLOW-RED HUE					
Breed attempt (1)	0.85	1, 55.73	639.3 (4.3)	651.2 (4.7)	0.360
Exp treatment (2)	1.55	1, 46.82	647.5 (4.5)	640.2 (4.6)	0.219
(1) * (2)	**5.85**	**1, 46.49**			**0.019**
UV CHROMA					
Breed attempt (1)	0.26	1, 50.61	0.220 (0.004)	0.216 (0.007)	0.612
Exp treatment (2)	0.15	1, 50.88	0.214 (0.005)	0.223 (0.005)	0.698
(1) * (2)	0.40	1, 50.63			0.529
CAROTENOID CHROMA					
Breed attempt (1)	0.28	1, 60.77	0.412 (0.023)	0.382 (0.020)	0.600
Exp treatment (2)	1.44	1, 50.59	0.416 (0.023)	0.386 (0.022)	0.235
(1) * (2)	0.03	1, 50.24			0.865
YELLOW-RED CHROMA					
Breed attempt (1)	0.07	1, 60.38	0.309 (0.004)	0.309 (0.005)	0.796
Exp treatment (2)	1.09	1, 50.91	0.312 (0.005)	0.306 (0.004)	0.302
(1) * (2)	0.06	1, 50.60			0.809

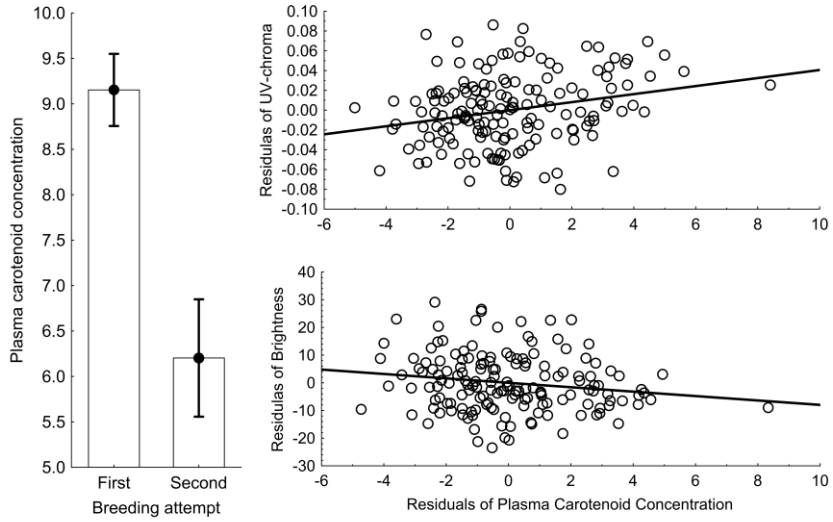

Figure 2: Least square means (± 95% CI) of plasma carotenoid concentration of nestlings from first and second breeding attempts, and its association with colouration (UV-chroma and brightness) of uropygial secretion after controlling for breeding attempt and the colour variable that is not included in each of the figure (residuals). Lines are regression lines.

Association between colouration of uropygial secretion and that of begging related traits

Variables describing colouration of uropygial secretion resulted in most cases related to the same colour component of mouth, flanges and skin of nestlings (Fig. 3). Particularly striking was the associations detected for UV and yellow-red chroma. In fact, to the human eye, yellow-red colouration of uropygial secretion is very similar to that of nestlings' mouth and flanges (Fig. 3).

Preening behaviour of nestlings and parental feeding preferences

In accordance with the possibility that nestlings use secretion to colour their body, in 34 of the 67 nests that were video recorded for two hours, we directly visualized nestlings collecting uropygial secretion with their bills and using it to spread in their body (see video in Additional Material 4). In addition, after rubbing the flanges and mouth of nestlings with cotton swabs, white swabs turned to yellow (Fig. 3). All these results strongly suggest that nestlings use uropygial secretion to colour their mouths and, thus, visual perception of parents might be partially determined by the colour of the uropygial secretion of nestlings.

Finally, nestling colouration explained parental feeding preferences (Additional Table 5). Nestlings with secretion of higher total hue values (*Beta(SE)* = 0.13(0.07), $F_{1,165}$ = 3.27, P = 0.07) and with mouth with higher values of UV hue (*Beta(SE)* = 0.23(0.09), $F_{1,165}$ = 6.83, P = 0.01), UV chroma (*Beta(SE)* = 0.44(0.16), $F_{1,165}$ = 7.76, P = 0.006) and yellow-red chroma (*Beta(SE)* = 0.30(0.15), $F_{1,165}$ = 4.32, P = 0.039), were preferentially fed by

parents after controlling for the effect of body mass (*Beta(SE)* = 0.16(0.08), $F_{1,165}$ = 3.85, *P* = 0.051).

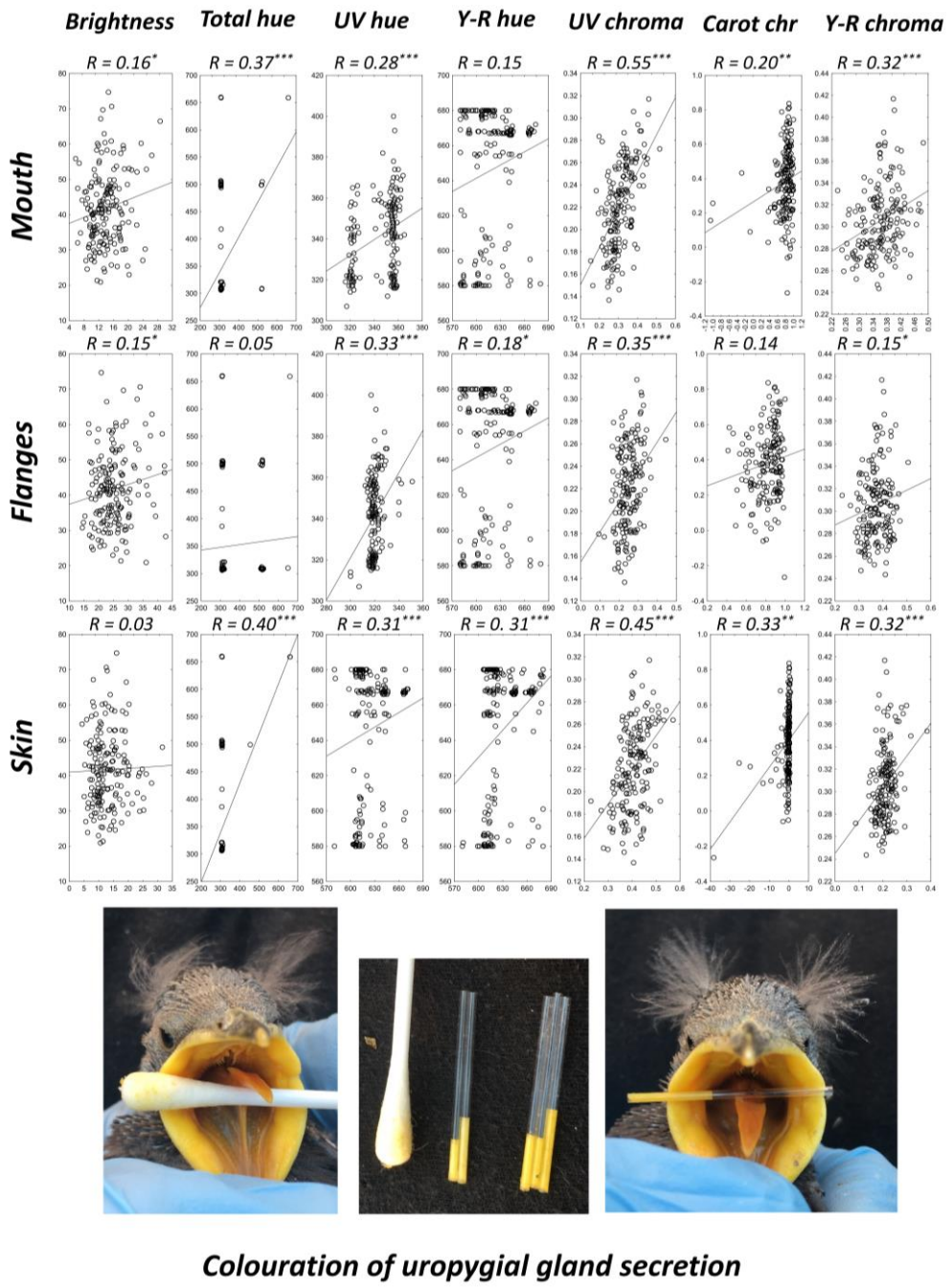

Figure 3: Associations between variables describing colouration of uropygial gland secretion (y-axes) and those describing colouration of mouth, flanges and skin (x-axes) of spotless starling nestlings. Correlation coefficients (R) and level of statistical significance (*: P < 0.05, **: P < 0.01; ***: P < 0.001)) are shown on the top of each plot. Photos at the bottom of the figures show similarity between colourations of open mouths of nestlings and a capillary containing yellow uropygial secretion (right) and a cotton swab that was used to clean mouth and flanges of the nestling (left). The photo in the centre shows uropygial secretion from two different nestlings that differed in colouration, also a swab that, after cleaning the mouth and flanges of nestlings, turned to yellow colouration.

Discussion

Our main results are two-fold. On the one hand, we evidenced a genetic component for some of the variables describing colouration of the uropygial secretion of starling nestlings, while some other colour variables were mainly environmentally determined. Effects of experimental VitE supplementation on secretion colour, and the association between secretion colour and plasma carotenoid concentration, demonstrate that one of the environmental factors affecting secretion colour is antioxidant availability. On the other hand, we show direct evidences of nestlings using uropygial secretion to colour their begging related traits, as well as significant covariations between colouration of secretion and that of mouth, flanges and skin. Thus, in accordance with the makeup hypothesis, when starling nestlings show their flamboyant coloured mouth to their parents, they are showing, not only information embedded in the colouration of their begging related traits, but also that related to secretion colour, which should reinforce or complement the message in scenarios of communication within the family. In accordance, we found that colouration of nestlings and that of their uropygial secretion predict parental food allocation in our starling population. Below we discuss the importance of our findings supporting the makeup hypothesis working in scenarios of offspring signalling their parents their phenotypic and genetic quality.

By means of a cross-fostering experiment, we found evidence suggesting genetic and environmental components of colouration of the uropygial secretion of 10 days-old starling nestlings. Curiously, colour variables that were significantly explained by environmental components (i.e., nest of rearing) differed from those explained by genetic factors (i.e., nest of birth), which suggests that some colour components of the uropygial secretion might inform on genetic factors and some other on environmentally determined characteristics of nestlings, including for instance antioxidant capacity. Interestingly, colouration of secretion of nestlings of ages between 6 to 12 days are intensely yellow coloured to the human eye, but turn to light beige in older nestlings, fledging and adults (Additional Figure 1). Yellow colouration might thus be a simple ontogenetic consequence with no adaptive value. However, changes in colouration of uropygial secretion coincide with changes in the way starling nestlings beg for food to their parents. While young nestlings beg for food by passively opening the mouth while standing up their heads, older nestlings move actively to the nest entrance when parents arrive. There, they beg for food more aggressively and are fed by parents, whom sometimes do not need to enter into the nest box (pers. obs.). This behaviour mainly occurs in nestlings older than 14 days when the colour of their secretion is frequently no longer yellow, but light beige (Additional Figure 1). Thus, it is possible that,

because of differences in begging display of young and old nestlings, mainly the former used uropygial secretion to showing their genetic and phenotypic aptitudes to parents.

In accordance with the possibility that colour of uropygial secretion reveals phenotypic condition of nestlings, we showed that it correlated to concentration of carotenoids in blood. Nestlings with less brighter and more UV coloured secretion were those with higher concentration of carotenoids. Moreover, the experimental feeding with VitE, a potent antioxidant, affected secretion colouration; mainly in interaction with breeding attempt, which is a good proxy of resource availability due to the typically reduced food availability experienced by late-second broods (Sorci, Soler & Møller 1997; De Neve *et al.* 2004). These two results strongly suggest that secretion colour could signal antioxidant capacities of nestlings. We found direct evidences of nestlings using uropygial secretion for preening, even at age when flight feathers are starting to open from their protective sheath (see photos in Fig. 1) and of secretion arrival to begging-related traits. Thus, those visualizations support that colouration of mouth, flanges and skin was partially determined by coloured uropygial secretion staining these traits.

In agreement with the possibility that nestlings show characteristics of their uropygial secretion when begging for food to their parents, we found that colourations of mouth, flanges and skin are positively related to colouration of nestling secretion. More importantly, cotton swabs turned to yellow colour after rubbing with them mouth of nestlings (see photos in Fig. 3). Thus, since it is broadly accepted that colouration of begging related traits inform parents on phenotypic and genetic conditions of their offspring (Kilner 1997; Soler *et al.* 2007; Ewen *et al.* 2008; Martín-Gálvez & Soler 2017; Martínez-Renau *et al.* 2021), whom accordingly adjust feeding effort and decide which nestlings to feed (Jourdie *et al.* 2004; Bize *et al.* 2006; De Ayala *et al.* 2007; Dugas 2009), colouration due to uropygial secretion might complement information for parents as the makeup hypothesis posits (Negro *et al.* 1999; Piersma, Dekker & Sinninghe-Damste 1999). In accordance with this possibility, we found that parental allocation of food was associated with colouration of nestling traits, including that of the uropygial secretion or of nestling mouth.

Recently, a genetic component has been described for total hue of mouth, as well as UV, yellow-red hue and yellow-red chroma of skin of starling nestlings (Martínez-Renau *et al.* 2021). Curiously, we have here detected a genetic component of two of these three colour factors describing secretion colour. Since we have detected that begging related traits of starling nestlings are stained with coloured uropygial secretion, it is possible that the

previously detected genetic component of colouration of mouth and skin were partially explained by characteristics of their uropygial secretion.

Moreover, the experimental VitE supplementation affected UV hue of mouth, flanges and skin starling nestlings (Martínez-Renau *et al.* 2021), but different colour components of the uropygial gland secretion, namely, yellow-red hue and, at a lower level, total hue. It is therefore possible that different colour components of secretion and of begging related traits inform on the antioxidant capability of nestlings. In accordance with this inference, brightness and yellow-red hue of flanges, but total and UV hue of the uropygial secretion were the main predictors of plasma carotenoid concentration. Interestingly, total hue of the uropygial secretion was one of the variables conforming the best models explaining parental food allocation. All those results therefore are in accordance with the hypothesis that coloured uropygial secretion of starling nestlings offer additional information to that of begging related traits and, thus, secretion colour might play a role in parent-offspring communication.

The makeup hypothesis proposed, at the end of the 90's, that deposition of cosmetics could be an alternative honesty-reinforcing mechanism linking phenotypic quality and colouration in birds (Negro *et al.* 1999; Piersma, Dekker & Sinninghe-Damste 1999). Since then, all except one published papers on the cosmetic use of uropygial secretion have dealt with its role in sexual selection processes, either, pre- or post-mating (Hirao, Aoyama & Sugita 2009; Lopez-Rull, Pagan & Macias Garcia 2010; Amat *et al.* 2011; Díaz-Lora *et al.* 2020). Our results strongly suggest that uropygial secretion could work in scenarios of parent-offspring communication. We have shown, not only that VitE supplementation and carotenoid concentration in the blood of starling nestlings influenced secretion colour, but also, that yellow coloured secretion stains nestling gapes and that parents use colouration of these traits for food allocation decisions. Particularities and functions of uropygial secretion of nestlings are poorly studied and our results support a cosmetic functioning in scenarios of parent-offspring communication.

Our results therefore open the possibility of future explorations of nestling makeup in scenarios of parent-offspring communication. We hope that the novelty of our findings encourage further research directed to further understand cosmetic colouration functioning and evolution in nestling birds. For instance, it would be interesting to assert partial contribution of uropygial secretion determining final colouration of begging related traits, which can be achieved by measuring colours before and after removing cosmetic colouration. It would also be of interest to explore how nestling colourations change during growth, and its association with changes in both colouration of their uropygial secretion and in parental

feeding rules. Moreover, since at least in starling, carotenoids seem to play a central role in cosmetic and tegument colourations, it would be interesting to know whether carotenoid concentration associates with colouration of the uropygial secretion and of begging-related traits along the nestling period.

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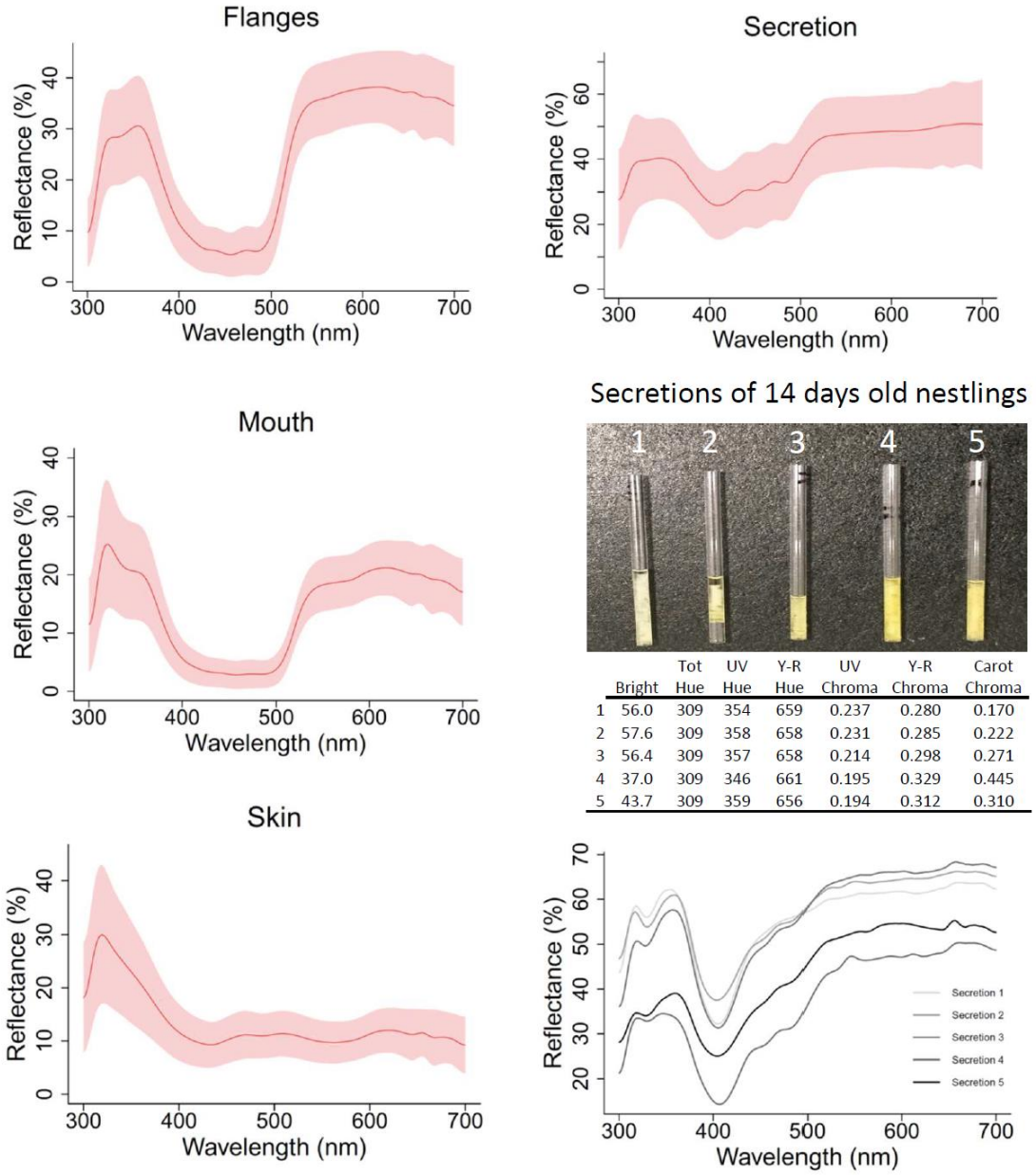
Supplementary material



	Bright	Tot Hue	UV Hue	Y-R Hue	UV Chroma	Y-R Chroma	Carot Chroma
1	56.0	309	354	659	0.237	0.280	0.170
2	57.6	309	358	658	0.231	0.285	0.222
3	56.4	309	357	658	0.214	0.298	0.271
4	37.0	309	346	661	0.195	0.329	0.445
5	43.7	309	359	656	0.194	0.312	0.310

Additional Figure 1: Reflectance spectra (± 95% CI) of gape (flanges and mouth), skin and secretion of ten days old nestlings. Below the secretion spectrum, we show an example of uropygial secretion of five 14th days old nestlings varying in colour as perceived by human vision, from light beige (1) to intense yellow (5). We also show values of considered colour variables (brightness (Bright), total hue (T Hue), UV hue, yellow-red (Y-R) hue, UV chroma, yellow-red (Y-R) chroma and carotenoid (Carot) chroma) and reflectance spectra of the five secretions.

Additional Methods. Extended Details of Material and Methods.

Food supplementation experiment

Nestlings within each brood were ranked depending on body mass, the heaviest randomly assigned to one of the experimental treatments (Antioxidants or Control), and the rest of nestlings with alternate treatment depending on their position in the body mass ranking. Every other day, we visited nests to feed each nestling depending on the assigned experimental treatment. Experimental nestlings were fed with a dose of VitE dissolved in corn oil and control nestlings only with corn oil as control of manipulation.

For individual identification, we trimmed the down of nestlings within the same nest in different body parts (i.e., left and right sides of head and back).

Video recording

Briefly, we installed the recording equipment by tying the mini video-camera (JCHENG Mini Hidder surveillance camera) to a rigid wire, and centred the camera at the top of the nest box. The infrared filter was removed by the technique services of the EEZA-CSIC, which allowed recording under dim light and with an external source of infrared light (i.e., not detected by starlings nor human eyes) connected to a small battery that was camouflaged and sited on the top of the nest box. The camera was connected to an external recorder (mini_dvr, eBoTrade) with a 32MB SD card that allowed recording approximately 10 hours in high quality. Both the camera and the recorder were connected to an external 12V battery (12AH). Moreover, we also used an external screen (KKMoon 3,5" TFT LED, OWSOO-EU) that was plugged to the video recorder and allowed us to watch what was going on inside the nest boxes, and to check whether the camera was properly sited and the recorder functional. The batteries, the recorder and the power relay module were included in a plastic bag that, with the help of leaves, stones and other natural materials was hidden on the floor, under the nest boxes. The cables connecting the camera to the recorder were also camouflaged around the tree trunk or wall.

Additional Table 1: Results from hierarchized ANOVA exploring the random effects of nest of rearing and nest of origin (nested within nest of rearing) on raw colouration values of uropygial gland secretion of spotless starling nestlings. Percentage of variance explained by each factor is also shown. Colour variables used include the achromatic component (Brightness), as well as hue and chroma of the total (Total) or particular wavelength intervals (Yellow-red and UV). Statistical effects of experimental treatment associated with two-tailed alpha-values lower than 0.1 are in bold font.

Raw colour values							
		Nest of rearing			Nest of origin (nested within rearing)		
Uropygial gland secretion		$F_{28, 29.1}$	P	Variance (%)	$F_{29, 44}$	P	Variance (%)
Brightness		**3.89**	**< 0.001**	**50.0**	1.51	0.105	11.7
Hue	Total	**1.67**	**0.088**	**19.3**	**1.69**	**0.057**	**23.5**
	UV	**9.55**	**< 0.001**	**48.9**	0.37	0.997	0
	Yellow-Red	**4.39**	**< 0.001**	**47.1**	0.88	0.640	0
Chroma	UV	**12.78**	**< 0.001**	**74.1**	0.81	0.722	0
	Yellow-Red	**3.08**	**0.002**	**42.3**	**1.61**	**0.075**	**15.4**
	Carotenoid	**2.44**	**0.010**	**30.5**	**1.98**	**0.020**	**24.1**

Additional Table 2. Results from Mixed model ANOVAs exploring the effect of antioxidant supplementation (Exp treatment) on colouration of the uropygial secretion of spotless starling nestlings after controlling for the fixed effect of breeding attempt (Breed attempt), the random effect of nest identity (nested within breeding attempt (Nest ID (Breed)), and the interaction between nest identity and experimental treatment to account for the repeated measure approach within nests. The interaction between breeding attempt and experimental treatment was estimated in models that include main and random effects, while main effects were estimated in models that do not include this interaction. Colour variables used include the achromatic component (Brightness), as well as hue and chroma of the total (Total) or particular wavelength intervals (Yellow-red and UV). Weighted means of first (column A) and second (column B) breeding attempts, as well as those of experimental (column A) and control (column B) nestlings are also showed. Statistical effects of the experimental treatment with associated two-tailed alpha-values lower than 0.1 are highlighted in bold font.

Dependent Factors	F	df	Weighted Means (SE)		P
			(A)	(B)	
BRIGHTNESS					
Breed attempt (1)	0.479	1, 61.4	41.77 (1.13)	42.72 (1.46)	0.491
Exp treatment (2)	0.903	1, 50.1	41.01 (1.27)	43.22 (1.25)	0.347
(1) * (2)	0.893	1, 49.8			0.349
Nest ID (Breed) (3)	**2.254**	**54, 44.2**			**0.003**
(2) * (3)	0.925	43, 46			0.601
TOTAL HUE					
Breed attempt (1)	2.57	1, 59.96	376.7 (12.1)	329.8 (8.2)	0.114
Exp treatment (2)	**3.58**	**1, 49.07**	**371.9 (13.4)**	**346.5 (10.1)**	**0.064**
(1) * (2)	0.709	1, 48.69			0.404
Nest ID (Breed) (3)	**2.46**	**54, 44.1**			**0.001**
(2) * (3)	1.09	43, 46			0.385
UV HUE					
Breed attempt (1)	**9.43**	**1, 61.36**	**342.0 (2.0)**	**355.1 (1.6)**	**0.003**
Exp treatment (2)	0.03	1, 53.18	347.5 (2.2)	346.4 (2.1)	0.862
(1) * (2)	0.00	1, 53.05			0.994
Nest ID (Breed) (3)	**3.09**	**54, 44.8**			**< 0.001**
(2) * (3)	0.65	43, 46			0.924
YELLOW-RED HUE					
Breed attempt (1)	0.85	1, 55.73	639.3 (4.3)	651.2 (4.7)	0.360
Exp treatment (2)	1.55	1, 46.82	647.5 (4.5)	640.2 (4.6)	0.219
(1) * (2)	**5.85**	**1, 46.49**			**0.019**
Nest ID (Breed) (3)	**5.91**	**54, 43.67**			**< 0.001**
(2) * (3)	**1.74**	**43, 46**			**0.034**
UV CHROMA					
Breed attempt (1)	0.26	1, 50.61	0.220 (0.004)	0.216 (0.007)	0.612
Exp treatment (2)	0.15	1, 50.88	0.214 (0.005)	0.223 (0.005)	0.698
(1) * (2)	0.40	1, 50.63			0.529
Nest ID (Breed) (3)	**6.99**	**54, 44.38**			**< 0.001**
(2) * (3)	0.84	43, 46			0.719
CAROTENOID CHROMA					
Breed attempt (1)	0.28	1, 60.77	0.412 (0.023)	0.382 (0.020)	0.600
Exp treatment (2)	1.44	1, 50.59	0.416 (0.023)	0.386 (0.022)	0.235
(1) * (2)	0.03	1, 50.24			0.865
Nest ID (Breed) (3)	**2.61**	**54, 44.33**			**< 0.001**
(2) * (3)	0.87	43, 46			0.675
YELLOW-RED CHROMA					
Breed attempt (1)	0.07	1, 60.38	0.309 (0.004)	0.309 (0.005)	0.796
Exp treatment (2)	1.09	1, 50.91	0.312 (0.005)	0.306 (0.004)	0.302
(1) * (2)	0.06	1, 50.60			0.809
Nest ID (Breed) (3)	**2.87**	**54, 44.39**			**< 0.001**
(2) * (3)	0.72	43, 46			0.723

Additional Table 3: Best models based on AIC criteria explaining concentration of plasma carotenoid depending on colouration of uropygial secretion (Brightness (1), Total hue (2), UV hue (3), Yellow-red hue (4), UV chroma (5), Carotenoid chroma (6), Yellow-red chroma (7)) and breeding attempt (8). Variance explained by each model is also shown.

	Included variables	AIC	R^2
1	1, 5, 8	690.0	0.287
2	1, 2, 5, 8	690.4	0.293
3	1, 3, 4, 5, 8,	691.0	0.294
4	1, 2, 3, 4, 5, 8	691.2	0.301
5	1, 3, 5, 8	691.3	0.288
6	1, 3, 4, 7, 8	691.3	0.296
7	1, 3, 7, 8	691.4	0.291
8	1, 3, 5, 8	691.4	0.288
9	1, 2, 3, 5, 8	691.4	0.296
10	1, 5, 6, 8	691.5	0.289
11	1, 5, 7, 8	691.7	0.289
12	1, 3, 5, 6, 8	691.8	0.295
13	1, 2, 4, 5, 8	691.9	0.294

Additional Material 4 . **(**Video of preening behaviour of nestlings): The video shows spotless starling nestlings collecting uropygial secretion with their bills and using it to spread in their body.

Additional Table 5. Association between parental feeding preferences in relation to nestling coloration: (i) Best models based on Mallow's Cp criteria explaining parental feeding preferences depending on colouration of **uropygial secretion** (Brightness (1), Total hue (2), UV hue (3), Yellow-red hue (4), UV chroma (5), Carotenoid chroma (6), Yellow-red chroma (7)), **flanges** (Brightness (8), Total hue (9), UV hue (10), Yellow-red hue (11), UV chroma (12), Carotenoid chroma (13), Yellow-red chroma (14)), **mouth** (Brightness (15), Total Hue (16), UV hue (17), Yellow-red hue (18), UV chroma (19), Carotenoid chroma (20), Yellow-red chroma (21)), **skin** (Brightness (22), Total Hue (23), UV hue (24), Yellow-red hue (25), UV chroma (26), Carotenoid chroma (27), Yellow-red chroma (28)), and **body mass** (29). Variables that appeared in the best model are highlighted in bold.

	Included variables	Mallow's Cp
1	**2, 17, 19, 21, 29**	-2.09
2	**2, 17, 19, 21**, 23, **29**	-1.70
3	**2, 17, 19, 21**, 24, **29**	-1.64
4	**17, 19, 21**, 24, **29**	-1.54
5	**17, 19, 21**, 23, **29**	-1.51
6	**2, 17, 19, 21,** 23, 24, 25, 28, **29**	-1.40
7	**17, 19, 21**, 23, 24, 25, 28, **29**	-1.36
8	**2, 17, 19, 21**, 23, 25, 28, **29**	-1.33
9	**17, 19, 21**, 23, 24, **29**	-1.27
10	**2, 17,** 18, **19, 21, 29**	-1.19

CAPÍTULO VI

Colouration of begging related traits in starling nestlings: a possible role of bacteria in parent-offspring communication

Abstract

There is evidence that animal communication can include cosmetic colourations. In this process, symbiotic bacteria may play a role in these signals by for instance, synthetizing associated pigments. Some birds use their uropygial secretion as cosmetics in a sexual selection context and, recently, it has been suggested that nestlings could also use uropygial secretions containing bacteria to make up the traits that they use (mouths and skin) to beg for food to their parents. Here, we explore the possible role of bacteria in producing the yellow colouration of the secretion of spotless starling (*Sturnus unicolor*) nestlings. To this end we have characterized the secretion colour and the 16S rRNA gene of secretion microbiota. We also performed an antioxidant supplementation experiment that affected secretion colours. Our results show associations between secretion colouration (brightness, UV hue and yellow-red chroma) and several characteristics of its bacterial community, including alpha and beta diversities, as well as the abundance of two bacterial genera (*Parabacteroides* and *Pseudogracilibacillus*). Moreover, the antioxidant supplementation increased the richness and diversity of the microbiota. These results show a link between the secretion microbiota and the colouration that reflects nestling antioxidant condition. Future experimental manipulation experiments of the microbiota will be needed to determine whether the detected empirical association is directly caused by bacteria or whether a third variable (e.g., phenotypic quality) is responsible for the detected association between bacteria and colour of the uropygial secretion.

Keywords*: bacterial community, microbiota, parent-offspring communication, preen gland, secretion colouration, signalling, uropygial secretion colouration*

Introduction

Flamboyant colourations of animals inform individuals of the same or different species on its physiological and/or genetic characteristics. For instance in interspecific communication, aposematic colourations of potential prey announce predators of their unpalatability or toxicity (Mappes, Marples & Endler 2005; Stevens & Ruxton 2012). At the intraspecific level, some exaggerate colourations of particular characters correlate with genetic and/or phenotypic quality of individuals and are used in contexts of sexual selection (Hill 1991; Seehausen & van Alphen 1998; Amundsen & Forsgren 2001) or parent-offspring communication (Jourdie *et al.* 2004; Wierucka *et al.* 2017; Soler *et al.* 2022). Reliability of colour signals depends on its associated production or maintenance costs (Grafen 1990), which include for instance the use of antioxidants or other limited resources that animals could otherwise use in different physiological processes. Thus, elucidating the mechanisms and strategies used by animals for colouring particular integuments, as well as the associated fitness consequences, have attracted the attention of behavioural ecologists for many years.

Animal colourations are mainly caused by the synthesis and/or accumulation of pigments (Blount *et al.* 2003; Logan, Burn & Jackson 2006; McGraw 2006; McGraw & Hill 2006), or by physical properties of animal integuments that affect reflectance of light when interacting with biological nanostructures (structural colouration) (Shawkey & D'Alba 2017). Colourations may also be modified by certain animal behaviours. For instance, some animal behaviours might prevent tegument degradation (Ruiz-Rodríguez *et al.* 2009); enhance the acquisition of pigments from the diet; or directly make up particular traits with coloured substances obtained from the environment (Nesbitt 1975; Negro *et al.* 1999) or produced in exocrine glands (Zamzow & Losey 2002; Saikawa *et al.* 2004; Amat *et al.* 2011; Díaz-Lora *et al.* 2020). The use of cosmetic colouration has been proposed to operate in some vertebrate taxa such as fish or mammals (Zamzow & Losey 2002; Saikawa *et al.* 2004), but it is in birds where cosmetic coloration is more phylogenetically widespread with examples in raptors (Negro *et al.* 1999; Piault *et al.* 2008), cranes (Nesbitt 1975), ibises (Wingfield *et al.* 2000) or hornbills (Kemp 2001), or passerines (Lopez-Rull, Pagan & Macias Garcia 2010).

Cosmetic colouration in birds is mainly associated with their exclusive uropygial gland, which produces a secretion composed by waxes, fatty acids and hydrocarbons (Jacob & Ziswiler 1982), that they spread onto the feathers and teguments by using their bills. This secretion can be transparent or coloured to the human eye and, when birds preen their feathers or other integuments, it might alter their colouration and. Thus, the secretion might influence,

to some extent, the colour signal received by con- or hetero-specific individuals. Interestingly, environmental factors determining body condition also affect the amount and the chemical composition of the uropygial secretion, as well as the rate of preening (Delhey, Peters & Kempenaers 2007; Piault *et al.* 2008). Therefore, secretion colour might inform on phenotypic characteristics of birds. Moreover, uropygial secretion is likely costly to produce (Piault *et al.* 2008; Magallanes *et al.* 2016) and preening is a time-consuming activity (Goldstein 1988; Walther & Clayton 2005), which suggest that cosmetic colouration mediated by uropygial secretion is a reliable honest signal (Moreno-Rueda 2016; Amat *et al.* 2018).

Colouration of the uropygial secretion might result from accumulation of pigments in greater flamingos *Phoenicopterus roseus*, in which accumulation of carotenoids in the uropygial secretion correlated with more colourful plumages (Amat *et al.* 2011; Amat *et al.* 2018). Pigments in the uropygial secretion might be synthesised *de novo* in the gland cells (Jacob & Ziswiler 1982) or in other body parts and then transported to the gland (Sparks 2011). They might also come from pigments synthesised by plants that birds incorporate through diet in the uropygium (McGraw & Hill 2006). Finally, pigments in the uropygial secretion may be also derived from the metabolism of symbiotic microorganisms growing within the uropygial gland of birds. A possible role of bacterial symbionts on animal signalling has been recently proposed and, although the hypothesis has been mainly explored for chemical signals (Mazorra-Alonso, Tomás & Soler 2021), there are also examples regarding visual traits. For instance, it is known that bacteria change the structural colour of bird feathers (Shawkey *et al.* 2007), that the integrity of ornamental feathers of spotless starling males shows off their antimicrobial capacity to females (Ruiz-Rodríguez *et al.* 2015), and that European hoopoe (*Upupa epops*) females cover their eggs with bacterial-mediated coloured secretion (Martín-Vivaldi *et al.* 2009) signalling the antimicrobial properties of their microbial symbionts to males (Soler *et al.* 2014). Therefore, to unravel the source of cosmetic substances involved on visual communication of animals requires exploring the role of bacteria in such substances.

The use of uropygial secretion as cosmetic to modify pigment- or structural-based colouration has mainly been described in contexts of sexual selection (Amat *et al.* 2011; Díaz-Lora *et al.* 2020), and it has recently started to be explored in other contexts, such as parent-offspring communication (Piault *et al.* 2008; Soler *et al.* 2022). Colouration of skin and gapes of nestling birds signals phenotypic and genetic quality to their parents (Kilner 1997; Martín-Gálvez & Soler 2017; Martínez-Renau *et al.* 2021), and, along with movement

and auditory signals, are key begging displays involved in sibling competition for parent attention (Kilner 2002). Parents assess the colouration of nestling traits to modulate their behaviour, adjusting the provisioning effort and deciding which nestling/s to feed (Jourdie *et al.* 2004; De Ayala *et al.* 2007; Dugas 2009; Soler *et al.* 2022). Interestingly, some days after hatching, nestlings of many species start collecting their uropygial secretion, which contains bacteria, and spread it into the skin, down and gapes (Piault *et al.* 2008; Soler *et al.* 2022). Moreover, at least for spotless starling (*Sturnus unicolor*) nestlings, the colour of the secretion is related to their phenotypic quality (Soler *et al.* 2022) and, thus, thanks to preening (i.e., making-up) behaviour of nestlings, the secretion colour may be part of the information that nestlings send to their parents (García-Núñez et al. in prep.). Thus, if the colouration of the secretion that the nestlings spread into the begging-related traits is mediated by bacteria, cultivating symbiotic bacteria in the uropygial gland would be an additional undescribed mechanism explaining colouration of nestling gapes.

Here, we test this hypothesis in spotless starlings, whose nestlings show a striking yellow-coloured uropygial secretion that reflect their genetic and phenotypic quality (Martínez-Renau *et al.* 2021) and that they use to make up their already flamboyant yellow-coloured gapes (Soler *et al.* 2022). Moreover, at the end of the nestling stage, the secretion colour changes from conspicuous yellow to a paler beige, resembling the colour of adult secretions (Soler et al. 2022). Then, since symbiotic bacteria have been found in the uropygial gland of multiple species (Law-Brown & Meyers 2003; Braun *et al.* 2016; Whittaker & Theis 2016; Braun *et al.* 2018a; Braun *et al.* 2018b; Braun *et al.* 2018c; Bodawatta *et al.* 2020; Grieves *et al.* 2021), starling nestlings are an ideal model system to test the role of bacteria in cosmetic colourations involved in parent-offspring communication.

If bacterial community of the uropygial secretion is responsible for the secretion colour, they should be related to each other. To this aim, we characterised the bacterial community present in nestlings uropygial secretion by amplicon sequencing of the bacterial 16S rRNA gene, characterized the secretion colour by spectrophotometry, and compared bacterial communities of the uropygial secretion of nestlings that greatly differed in colour due to nestling age. Moreover, as secretion colour reflects phenotypic condition in starlings, bacterial community of the uropygial secretion might also associate with nestling condition. We explore this last prediction experimentally by performing a Vitamin E (hereafter VitE) supplementation experiment that affect antioxidant status of nestlings (Martínez-Renau *et al.* 2021).

Methods

Study area and species

The fieldwork was carried out during the breeding seasons of 2019 in a spotless starling (hereafter starling) population located in southern Spain, at the old railway station of La Calahorra (37°15' N, 3°01'W). The area is a high altitude plateau of the semiarid Hoya de Guadix, where starlings breed in 94 cork-made nest boxes (internal dimensions: 180 mm [width] x 210 mm [depth] and 350 mm [high], 240 mm from the bottom to the hole entrance) attached to tree trunks or walls.

The starling is a medium-sized, hole-nesting altricial passerine. In our study population, starlings start laying their eggs in mid-April, and they lay one egg per day up to complete a clutch of 4-5 eggs on average. The incubation period lasts 11 days and they begin the incubation before laying the last egg, which result in last nestling hatching one day later than their siblings (Azcárate-García *et al.* 2020). Incubation is mainly performed by females, while both parents feed nestlings during development (Veiga & Polo 2016). The nestling period is about 18 days, although it can extend up to 25 days, and second clutches are common (Veiga & Polo 2016; Soler *et al.* 2017).

Fieldwork

Since late March, we visited the nest boxes every three days to estimate the laying date of the first egg (hereafter, laying date). From there, we visited nest boxes every other day until clutch completion. Finally, 12 days after laying, we started to visit the nest boxes daily until hatching. The food supplementation experiment started the day after hatching and consisted of an oral administration every other day of an age-dependent dose of VitE (DL-α-tocopherol acetate (Sigma-Adrich T 3376-256)) estimated as 2.5 mg/ 50 g of nestling weight, diluted in corn oil to experimental nestlings (half of the brood), whereas only corn oil was administrated to the rest of the brood (control nestlings; for further details of the experimental protocol, see De Ayala, Martinelli and Saino (2006); Martínez-Renau *et al.* (2021); Soler *et al.* (2022)). Ten days after hatching, we ringed the nestlings, and collected biometric measurements such as tarsus length with a digital calliper (precision 0.01 mm), wing maximum-length with a metal ruler (precision 0.1 mm), and body mass with a digital balance (Ascher CS, precision 0.01 g). At that visit, we also collected the uropygial secretion of nestlings by keeping a sterile microcapillary tube (32 mm, 10 µl) in contact with the gland opening and slightly pressing the gland until extracting the secretion. We repeated the process twice; one capillary tube was kept in a sterile microfuge vial and stored at -18ºC until bacterial DNA extraction,

whereas approximately 4 µl of secretion from the second capillary tube was smeared on a piece of 1-cm-diameter circle of blotting paper to take the colour measurements immediately after collection (see below). On day 12 after hatching, we supplemented nestlings with their last VitE dose, and on day 14 we took biometric measures of the nestlings and collected the uropygial secretion of one randomly chosen nestling as mentioned above. The secretion was kept in a sterile microfuge vial and stored at -18^{o}C until DNA bacterial extraction, as above.

Colour measurements and estimation of colour variables

Colouration was measured with a spectrophotometer (Ocean Optics S2000) connected to a halogen deuterium lamp (D2-W, mini) through an optical fibre (QR-400-7-UV-vis), which was calibrated to standard white (Ocean Optics WS-2) and to the dark (i.e., within the black neck-gaiter in which we took all measurements). Reflectance was measured at 1 nm intervals between the wavelengths of 300 to 700 nm. We measured the colour of the secretion on the blotting paper three times, placing the optical fibre perpendicularly to the surface at a distance of 2 mm. Since repeatability was relatively high ($R > 0.70$), we used mean values per nestling in subsequent analyses.

To analyse the colour variables, we used AVICOL v.6 (Gomez 2006) and corrected all negative values of reflectance to zero, while the noise was reduced by means of a triangular correction. The reflectance spectra of the uropygial secretion of starling nestlings is bimodal, showing typically one clear peak at the UV wavelength, and a second peak at the yellow-red part of the spectrum (600-700 nm) (see Soler *et al.* (2022)). We estimated brightness (achromatic component of the colouration) as the proportion of total reflectance of the whole spectrum, the chroma of the UV (300-400 nm) and Yellow-Red (580-680 nm) as the proportion of total reflectance in those intervals, and hue of the UV and Yellow-Red parts of the spectrum as the wavelength at which the reflectance reached its maximum at the specific intervals considered (Cuthill 2006). The wavelength at which the positive slope reached its maximum was considered as the hue of the entire spectra (Total hue). We also estimated the carotenoid chroma following previous articles (Cuthill 2006; Charmantier *et al.* 2017) as the reflectance at 700nm minus the reflectance at 450nm divided by the reflectance at 700nm ((R700-R450)/R700).

DNA extraction and high-throughput sequencing

DNA from the bacterial community was extracted following the protocol described in Boom *et al.* (2000) with modifications to optimize cell lysis. First, we resuspended the sample in 30

µl of lysis solution, which was later incubated for 10 minutes at 75ºC. Then, we added 30 µl of neutralization solution to the sample. To verify the presence of bacterial DNA, 5 µl of the DNA extraction were used to amplify by PCR the V6-V8 region of the 16 ribosomal RNA gene. For that PCR, we used the primers B969F (ACGCGHNRAACCTTACC) and BA1406R (ACGGGCRGTGWGTRCAA) (Comeau *et al.* 2011), and the PCR products were visualized on a 1% agarose gel with electrophoresis. These primers were also used to construct Illumina libraries for MiSeq sequencing. Library construction and sequencing in the MiSeq (Illumina) platform was carried out in the facilities of the Institute of Parasitology and Biomedicine "López-Neyra" (IPBLN, Granada, Spain).

MiSeq sequencing analyses

We processed the amplicon sequences obtained from Illumina MiSeq using QIIME v2020.6 (Bolyen *et al.* 2019). We first used the DADA2 plugin (Callahan *et al.* 2016) for primer trimming, sequencing quality filtering and ASV (Amplicon Sequence Variant) clustering. Then, each ASV was assigned to taxonomy using the Silva 138 database (Quast *et al.* 2012). We used "Decontam" package in R v4.0.2 (Davis *et al.* 2018; R-Core-Team 2020) to identify and remove possible contaminant sequences, which were inferred by comparing bacterial samples and field (empty capillaries processed as samples) and laboratory (extraction and sequencing blanks) negative controls, and using the prevalence method with a threshold of 0.4. A rooted bacterial phylogeny was generated after aligning the ASVs using the method *align-to-tree-mafft-fasttree* in QIIME2.

Statistical analyses

Estimating alpha and beta diversities

To estimate alpha diversity, we first rarefied the ASV table 100 times to the minimum sampling depth (6,246) using the "phyloseq" package in R v4.0.2 (R-Core-Team 2020). Then, we calculated the alpha diversity indexes for each of the 100 rarefied ASV tables, estimating Chao1 index and Shannon's diversity index using the "microbiome" package (Lahti & Shetty 2017), and Faith's phylogenetic diversity (PD) using "picante" package (Kembel *et al.* 2010) within the R environment (R-Core-Team 2020). Then, we computed the mean value per sample of the three alpha diversity indexes and used them in subsequent analyses. To correctly account for the compositional nature of microbiome data, we first filtered samples with less than 6,246 ASVs from the raw (unrarefied) ASV table, and ASV abundances were transformed using the center-log ratio (CLR) transformation using the

"microbiome" v1.20.0 package (Lahti & Shetty 2017), which results in values that are scale invariant (Gloor *et al.* 2017). The distances were computed using Aitchison's distances (i.e., Euclidean distances on CLR-transformed data). We also applied a Phylogenetic Isometric Log Ratio (PhiLR) transformation to the unrarefied dataset using the "philr" v1.24.0 package (Silverman *et al.* 2017) to account for the information of phylogenetic relatedness between ASVs in compositional datasets, and calculated the distance matrices with the Euclidean metric.

Effect of treatment and breeding attempt on bacterial communities

The effect of the VitE supply and breeding attempt on alpha and beta diversity of bacterial community was explored separately in mixed model ANOVAs and PERMANOVAs with 10,000 permutations, respectively. The models included the experimental treatment (VitE or control) and the breeding attempt (first or second breeding attempt) as fixed factors, the nest identity (ID) nested within breeding attempt as the first random factor, and the interaction between nest identity (nested within breeding attempt) and experimental treatment as the second random factor. The interaction between treatment and breeding attempt was explored in separate models where the main effects and random factors were also included. The mixed effects ANOVAs were computed using TIBCO Statistica™ software (version 14), while the software PRIMER7 v.7.0.17 (PRIMER-e) was used for the PERMANOVA analyses. Principal Component Analyses (PCA) with Aitchison and PhiLR distances were used for 2D visualisation of the multidimensional space, and were computed using the "factoextra" v1.0.7 package (Kassambara 2016).

Association between the colouration of the uropygial secretion and the bacterial community

The relationship between alpha diversity indexes of the bacterial community and the colouration of the uropygial secretion was explored by mixed model ANOVAs as implemented in Statistica™ software (version 14). For each of the estimated colour variables and alpha diversity indexes, we performed separate models that included the colour variable as the dependent factor, while the alpha diversity index was specified as the continuous independent variable. The models also included the treatment and the breeding attempt as fixed factors, nest identity (ID) nested within breeding attempt as first random factor, and the interaction between experimental treatment and nest identity (nested within breeding attempt) as the second random factor. The interaction between treatment and breeding attempt was explored in separate models that also included the main factors.

The association between characteristics of the bacterial community and variables describing colouration of the uropygial secretion was analysed using Mantel tests. We first calculated the Euclidean distance matrices of the different colour variables in "vegan" package (Oksanen *et al.* 2022) within R environment. Those colour distance matrices were used as dependent variables in separate Mantel tests that included the following independent matrices: (1) the beta-diversity matrix of the bacterial community as the first independent matrix (Aitchison or PhiLR distances), (2) a binary matrix of nest identity (whether each cell in the matrix did (cell value = 1) or did not (cell value = 0) belong to the same nest-box identity), (3) a binary matrix of breeding attempt (whether each cell in the matrix did (cell value = 1) or did not (cell value = 0) belong to the same breeding attempt), and (4) a matrix of the experimental treatment (whether each cell in the matrix belonged to the same treatment (cell value = 0), to differences between experimental and a control nestlings (cell value = 1), or between control and experimental nestlings (cell value = -1). The Mantel tests were ran using the MRM function in the "ecodist" v2.0.9 package (Goslee & Urban 2007) within R environment, which allows to estimate partial correlation coefficients.

Detecting specific bacterial taxa that associated with colouration of the uropygial secretion

To search for specific bacterial strains related to colouration variables, we used sparce Partial Least Squares or Projection to Latent Structures (sPLS) models as implemented in "mixOmics" v6.22.0 package (Rohart *et al.* 2017) in R, integrating information of both datasets while handling multicollinearity (Chun & Keleş 2010). We first collapsed the ASV table to the genus level, and filtered those genera representing less than 0.008% or present in less than four samples across the dataset. The filtering resulted in 531 genera. For the sPLS implementation we used the regression mode, which fits a linear regression between high dimension datasets, assuming an asymmetric role of the dependent matrix (here colouration) and the explanatory matrix (here microbial data). We only used one latent component from the colouration matrix (response variables), as only the first one was near the established recommended threshold of Q^2 (0.0975) (Wold, Sjöström & Eriksson 2001). For the sPLS model, we used the Mfold cross-validation method with 50 repeats and 10 folds.

To further explore the resulting correlations with the first latent component of sPLS, we performed multiple regression analyses with the bacterial genera and the colouration variables selected in sPLS. First, we used the Akaike information criterion (AIC) to search for the best model explaining the colour variable when considering all bacterial genera associated with a Pearson's correlation coefficient larger than 0.25 as independent continuous variables. Second, from the best models (those with lower AIC values that differed less than

two units from the minimum value), we selected that with smallest number of independent variables and an AIC value close to the minimum.

In order to explore how the VitE supplementation affected the abundance of *Parabacteroides* and *Pseudogracilibacillus* (the two genera that the best models retained), we performed mixed effects ANOVAs. These models included one of the bacterial genus as the dependent variable, and the experimental treatment and the breeding attempt as the fixed factors. Nest ID nested within breeding attempt, and the interaction between treatment and nest ID (nested within breeding attempt) were set as random factors. Moreover, to explore the associations of both bacterial genera on the secretion colour variables that the sPLS models selected, we used mixed effects ANOVAs similar to the one described above. In this case, colouration variables were separately used as the dependent variable, while CLR-transformed abundances of bacterial genus were used as the independent variables. Since experimental treatment and breeding attempt affected colouration of the uropygial secretion (Martínez-Renau *et al.* 2021), these factors were also included in the models to control the expected association between coloration and particularities of the bacterial community of the secretion.

Association between bacterial communities of day 10 and 14 post-hatching and the influence of experimental Vit-E supply

To explore how the bacterial community of the uropygial secretion varied along the nestling period, we used information of the microbiota of individuals that were sampled both 10 and 14 days after hatching. By means of repeated measures ANOVAs that included breeding attempt as the between factor, we explored the associations between alpha diversity indexes of the microbiota of 10 and 14-days-old nestlings. To explore differences in the bacterial composition due to nestling age, we first created the CLR- and PhiLR-transformed distance matrices that included information of nestlings that were sampled when they were 10 and 14 days old, and used them as dependent matrices in PERMANOVAs in Primer7 v.7.0.17 (PRIMER-e) with 10,000 permutations. Nestling age (10 or 14) was used as the fixed factor, and the ring number (individual identity) as the random factor to account for the repeated measure nature of the dataset. We also ran Mantel tests using the "ecodist" package v2.0.9 package (Goslee & Urban 2007) to explore the association between the microbiota composition at both nestling periods. To do that, for nestlings with information at both ages, we created different distance matrices (CLR- and PhiLR-transformed) for information collected at days 10 and 14 after hatching, and used them as the independent and dependent

matrix, respectively. The model also included the binary matrix with information on breeding attempt to control the effect of this factor.

Results

We successfully sequenced the uropygial gland secretion of 193 starling nestlings belonging to 70 nests. We obtained 5,236,071 sequences classified into 31,087 ASVs assigned to 34 phyla and 982 genera. The average sequences per sample were 27,413.98 (SD=9,314.83), and after the rarefaction, the ASV table had an average of 29,011 ASVs (SD=38.93). The most abundant phyla were Proteobacteria (34.3 %), Actinobacteria (24.8 %), Firmicutes (16.4 %) and Bacteroidetes (16.2 %) (Fig 1).

Effect of VitE supplementation and age on the bacterial community of the secretion

VitE supplementation increased the richness (Chao1) and phylogenetic diversity (PD) of the bacterial community of the uropygial secretion of starling nestlings (Table 1, Fig. 2A), but did not affect its composition (i.e., beta-diversity) (i.e, no matter the distance matrix used (Table 1, Fig. 2B).

Table 1. Results of mixed model ANOVAs and PERMANOVAs exploring the effect of antioxidant supplementation on the alpha and beta-diversity indexes, respectively, of bacterial community of the uropygial secretion of starling nestlings, after controlling for the fixed effect of breeding attempt and the random effect of the nest ID (nested within breeding attempt). The interaction between the fixed factors (treatment*breeding attempt) was explored in separate models that also included the main effects. Values in bold are those with associate alpha value lower than 0.05.

	Independent variables														
	Treatment			Breeding attempt			Nest ID (Breeding attempt)			Treatment * Breeding attempt			Treatment * Nest ID (Breeding attempt)		
Dependent variables	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p
Alpha diversity															
Chao1	**5.44**	**1, 59.3**	**0.023**	**10.58**	**1, 74.2**	**0.002**	**2.30**	**70, 55.4**	**<0.001**	1.41	1, 58.2	0.240	**1.86**	**55, 63**	**0.010**
Shannon	0.26	1, 62	0.610	0.08	1, 78.8	0.772	**1.64**	**70, 55.3**	**0.029**	0.07	1, 60.6	0.786	1.13	55, 63	0.314
PD	**8.42**	**1, 62.2**	**0.005**	**4.98**	**1, 76.3**	**0.029**	**2.36**	**70, 55.8**	**<0.001**	3.10	1, 61.6	0.083	1.10	55, 63	0.361
Beta diversity															
Aitchison	1.04	1, 61.4	0.362	**3.22**	**1, 88.8**	**<0.001**	**1.31**	**69, 65**	**<0.001**	0.96	1, 61.7	0.590	1.04	54, 65	0.136
PhiLR	1.43	1, 62.1	0.148	**13.16**	**1, 78.2**	**<0.001**	**2.87**	**69, 65**	**<0.001**	1.23	1, 60.6	0.236	0.95	54, 65	0.680

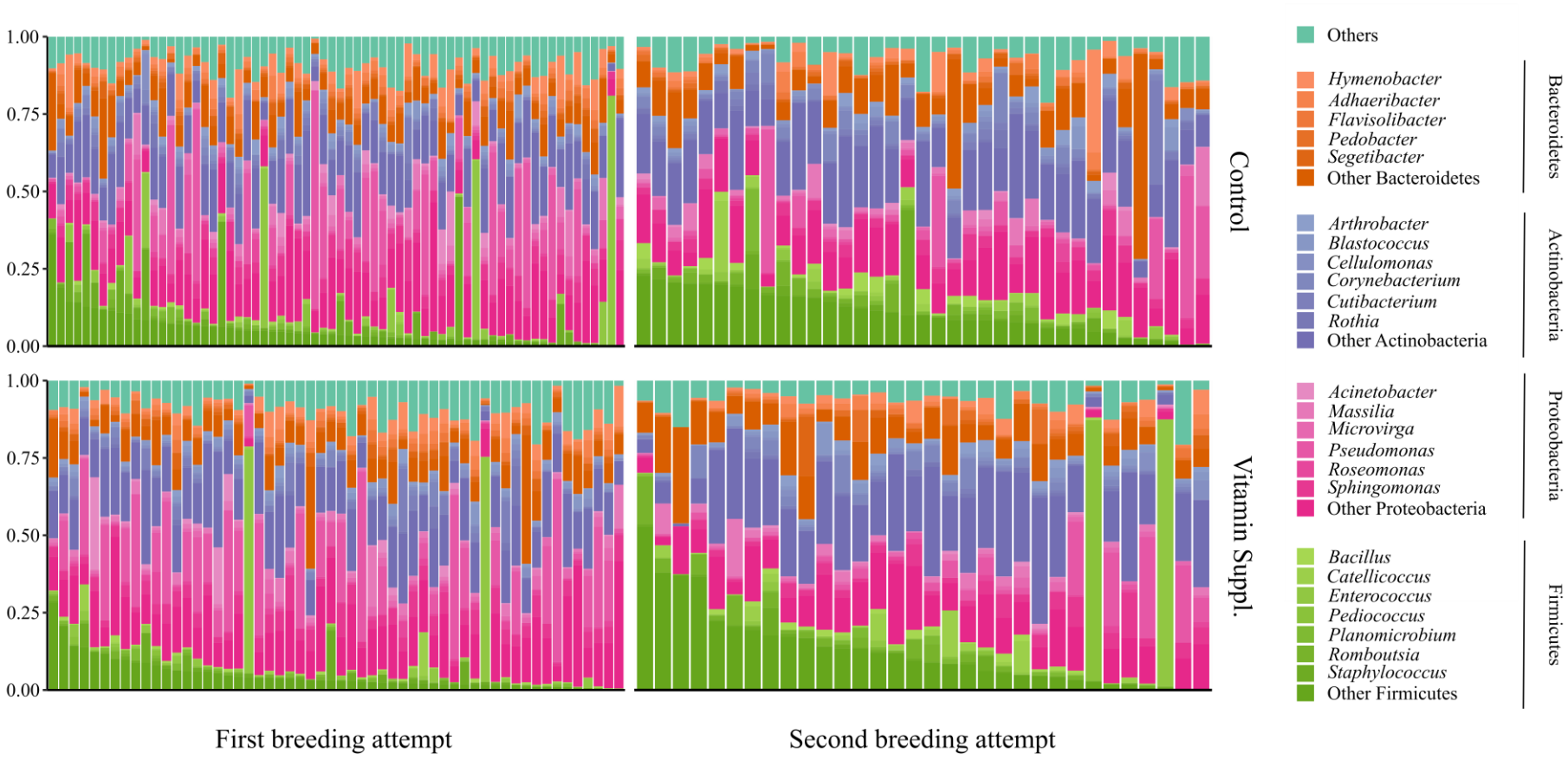


Figure 1. Stacked barplot showing the relative abundance of the most frequently detected genera for each individual. The figure provides information on the treatment (control vs vitamin supplementation) and the breeding attempt (first vs second).

The richness and diversity of bacterial communities were higher in samples of 10-days-old than in 14-days-old nestlings (Table 2). The composition of the bacterial communities of the uropygial gland secretion also differed between nestlings of different age (Table 2). Finally, the bacterial communities of 10 and 14-days-old nestlings did not associate to each other when using Mantel tests (Additional Table 1).

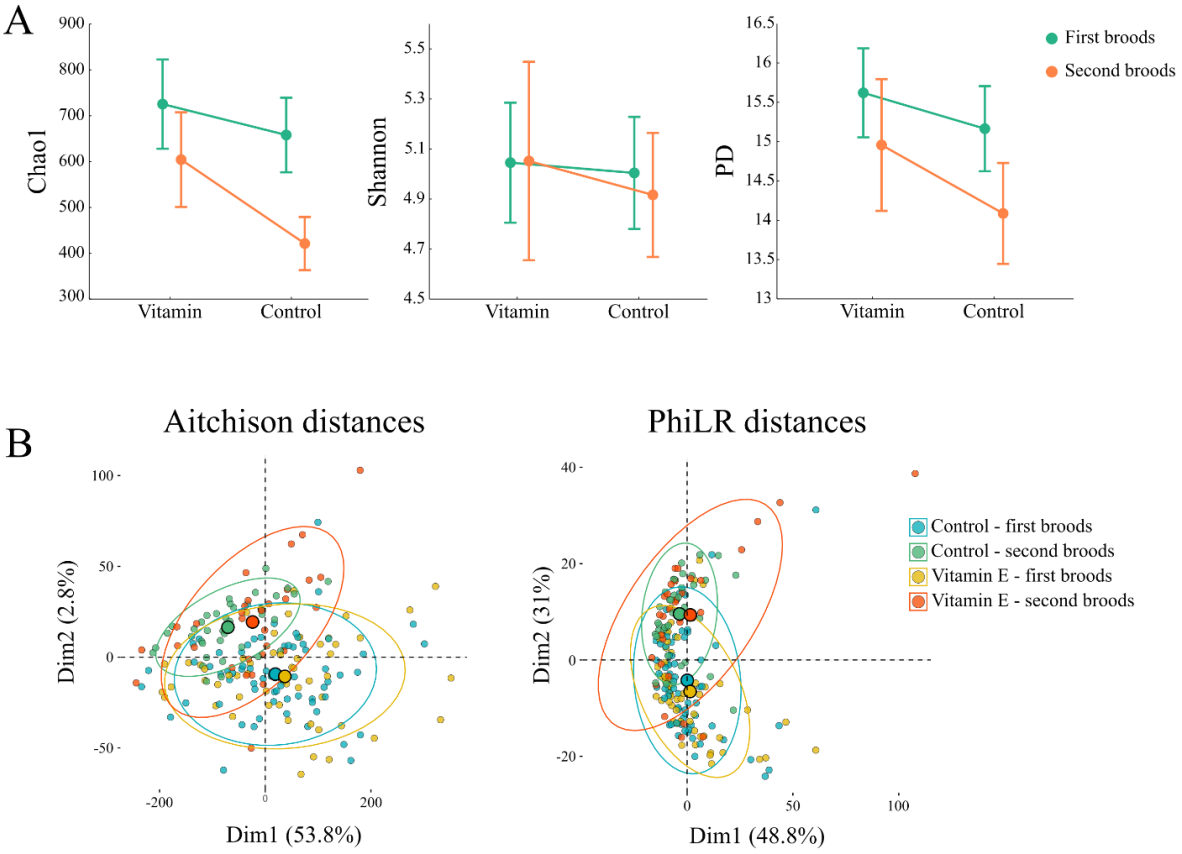


Figure 2. A. Least square means of alpha diversity indexes (Chao1, Shannon index and PD) for vitamin-supplemented and control nestlings from first and second breeding attempts, after controlling for nest identity. B. PCA ordination plots showing the diversity of bacterial communities of uropygial gland secretions of vitamin-supplemented and control nestlings from first and second breeding attempts. Ellipses correspond to 80% CI of each group.

Correlation between microbiota and colouration of the uropygial secretion

The UV hue and bacterial richness (Chao 1) of the uropygial secretion significantly covaried after controlling for the effects of treatment and breeding attempt ($F_{(1,48)}$ = 6.06, p = 0.017; Additional Table 2). Regarding the beta-diversity, both brightness and the UV hue correlated with the bacterial community composition of the uropygial gland secretion (Table 3) when respectively considering PhiLR and Aitchison distances, and after controlling for the effect of nest identity, experimental treatment and breeding attempt (Table 3).

Table 2. Results of repeated measures ANOVAs (RMAs) and PERMANOVAs exploring the effect of age on the alpha and beta diversity indexes, respectively, of bacterial community of the uropygial secretion of starling nestlings. The breeding attempt was included on the RMAs as the between factor. *F statistic is Pseudo-F in PERMANOVA analysis for beta diversity. Values in bold are those with associate alpha value lower than 0.05.

		F*	df	p
Alpha diversity				
Chao1	*Within-factor*			
	Difference day 10-14	**8.02**	**1, 48**	**0.007**
	Difference day 10-14* Breeding attempt	0.39	1, 48	0.535
	Between-factor			
	Breeding attempt	**4.48**	**1, 48**	**0.040**
Shannon	*Within-factor*			
	Difference day 10-14	**5.51**	**1, 48**	**0.023**
	Difference day 10-14* Breeding attempt	1.34	1, 48	0.253
	Between-factor			
	Breeding attempt	0.20	1, 48	0.660
PD	*Within-factor*			
	Difference day 10-14	**16.9**	**1, 48**	**<0.001**
	Difference day 10-14* Breeding attempt	0.01	1, 48	0.974
	Between-factor			
	Breeding attempt	1.01	1, 48	0.320
Beta diversity				
Aitchison	Age (10 vs 14)	**1.60**	**1, 50**	**0.002**
	Individual ID	**1.05**	**50, 50**	**0.005**
PhiLR	Age (10 vs 14)	**5.67**	**1, 50**	**<0.001**
	Individual ID	**1.31**	**50, 50**	**0.017**

Table 3. Results of Mantel test exploring the correlation between secretion colour variables and the bacterial community of the uropygial secretion of starling nestlings. Euclidean distances on CLR- and PhiLR-transformed abundances were used to build the distance matrices. Binary matrices with information on Nest ID, treatment and breeding attempt were also included to control for the effects of those factors. Number of permutations was set to 10,000. Values in bold are those with associate alpha value lower than 0.05.

	Aitchison		**Euclidean - PhiLR**	
	Coefficient of correlation	*p*	Coefficient of correlation	*p*
logBrightness	R^2 = 0.003, F = 11.17, p =0.449		R^2 = 0.012, F = 36.96, p =0.036	
Distance matrix	0.001	0.371	**0.011**	**0.028**
Nest ID	0.024	0.155	0.014	0.423
Treatment	-0.007	0.516	-0.008	0.415
Breeding attempt	0.011	0.231	0.003	0.770
UV hue	R^2 = 0.028, F = 86.23, p <0.001		R^2 = 0.010, F = 30.38, p = 0.025	
Distance matrix	**0.193**	**0.005**	0.124	0.706
Nest ID	**4.546**	**<0.001**	**5.396**	**<0.001**
Treatment	0.845	0.190	0.892	0.169
Breeding attempt	**2.525**	**<0.001**	2.299	0.002
Yellow-red hue	R^2 = 0.011, F = 32.75, p = 0.003		R^2 = 0.007 F = 21.76, p = 0.007	
Distance matrix	0.210	0.059	0.261	0.616
Nest ID	**23.40**	**<0.001**	**24.156**	**<0.001**
Treatment	-2.072	0.055	-2.040	0.058
Breeding attempt	-0.073	0.945	-0.395	0.683
Total hue	R^2 = 0.012, F = 36.53, p = 0.091		R^2 = 0.005, F = 14.10, p = 0.319	
Distance matrix	1.023	0.097	-2.577	0.366
Nest ID	**24.680**	**0.004**	**33.535**	**<0.001**
Treatment	-4.027	0.476	-3176	0.583
Breeding attempt	-8.080	0.070	-7.360	0.140
UV chroma	R^2 = 0.010, F = 31.26, p = 0.063		R^2 = 0.009, F = 27.25, p = 0.043	
Distance matrix	<0.001	0.237	<0.001	0.258
Nest ID	**0.018**	**<0.001**	**0.018**	**<0.001**
Treatment	0.002	0.258	0.002	0.270
Breeding attempt	**0.003**	**0.019**	0.003	0.077
logYellow-red chroma	R^2 = 0.007, F = 21.18, p = 0.138		R^2 = 0.002, F = 7.33, p = 0.440	
Distance matrix	<0.001	0.144	0.001	0.566
Nest ID	**0.016**	**0.026**	**0.018**	**0.016**
Treatment	0.005	0.251	0.004	0.236
Breeding attempt	-0.001	0.814	-0.002	0.584
Carotenoid chroma	R^2 = 0.012, F = 36.46, p = 0.068		R^2 = 0.002, F = 4.69, p = 0.688	
Distance matrix	0.002	0.058	-0.001	0.738
Nest ID	0.020	0.112	**0.030**	**0.019**
Treatment	-0.001	0.920	<0.001	0.992
Breeding attempt	-0.010	0.132	-0.010	0.153

The sPLS model resulted in a single latent component explaining 2.22% and 40.62% of the variance of microbiota and colouration respectively. The first latent component included 10 bacterial genera and 6 colour variables that were correlated to each other ($R \geq 0.25$). The genera *Parabacteroides*, *Pseudogracilibacillus* and *Dysgonomonas* correlated positively with the UV and negatively with the Yellow-Red chroma (Fig. 3), while only *Parabacteroides* was negatively correlated with the UV hue and the Yellow-Red hue (Fig. 3). Furthermore, the model that better explained the variance of the UV chroma included *Parabacteroides* and *Pseudogracilibacillus*, while the variance in Yellow-Red chroma was better explained by the relative abundance of *Parabacteroides* (Table 4, Fig. 4).

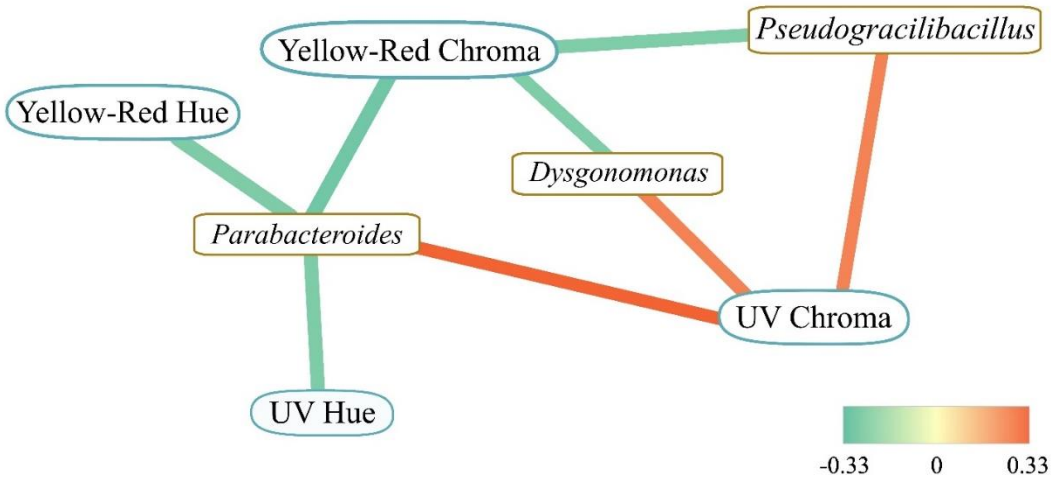


Figure 3. Bipartite network representation from sPLS models on bacterial genera and colour variables. Edges represent correlations (higher than 0.25 or lower than -0.25) between a genus and a colouration variable. Green edges represent negative correlations and red edges represent positive correlations. The intensity of green/red colour of edges relates with the correlation coefficient, from darker (higher correlations) to lighter (lower correlations).

Neither *Parabacteroides* nor *Pseudogracilibacillus* were significantly affected by the VitE supplementation after controlling for the effect of breeding attempt and nest identity (Parabacteroides: $F_{(1,53)} = 1.61$, $p = 0.211$; *Pseudogracilibacillus*: $F_{(1,49)} = 2.10$, $p = 0.153$). The association of both genera in the colouration variables was not significant after controlling for the VitE supplementation, breeding attempt and nest identity (Additional Table 3).

Table 4. Results of linear regressions exploring the correlation between CLR-transformed abundances of bacterial genera and secretion colour variables with correlations higher than 0.25 in the sPLS models. Values in bold are those with associate alpha value lower than 0.05.

	F	df	p	β
UV Chroma				
Parabacteroides	**5.25**	**1, 153**	**0.023**	**0.19**
Pseudogracilibacillus	**7.86**	**1, 153**	**0.005**	**0.23**
UV hue				
Parabacteroides	**7.26**	**1, 154**	**0.007**	**-0.21**
Yellow-Red hue				
Parabacteroides	**17,56**	**1, 154**	**<0.001**	**-0.32**
logYellow-Red Chroma				
Parabacteroides	**13.39**	**1, 154**	**<0.001**	**-0.28**

Discussion

Recently it has been shown that colour of the uropygial secretion of starling nestlings was partially explained by environmental and genetic factors, and that parents use this colour to guide food allocation (Soler *et al.* 2022). Secretions containing bacteria are used by other bird species as cosmetics (Martín-Vivaldi *et al.* 2009; Soler *et al.* 2014; Díaz-Lora *et al.* 2021) and we here explored the possibility that (i) the secretion used by starling nestlings also harbour symbiotic bacteria, and that (ii) these bacteria were partially responsible for secretion colour. In accordance with that possibility, we found complex bacterial communities in the uropygial gland of nestlings that varied with age, which parallels the known age-related differences in secretion colour. Our results also revealed a correlation between the bacterial community composition, as well as the abundance of particular taxa, and colouration of the uropygial gland secretion of nestlings, which further suggests a bacterial role determining secretion colour. More importantly, the antioxidant supplementation affected not only the colour (Soler *et al.* 2022), but also the richness and diversity of the bacterial community of the uropygial secretion. These results strongly suggest a link between the secretion microbiota and its colouration, highlighting the possibility that bacteria plays a role in parent-offspring communication. Here, we discuss alternative hypothesis and possible physiological mechanisms that could explain the detected associations.

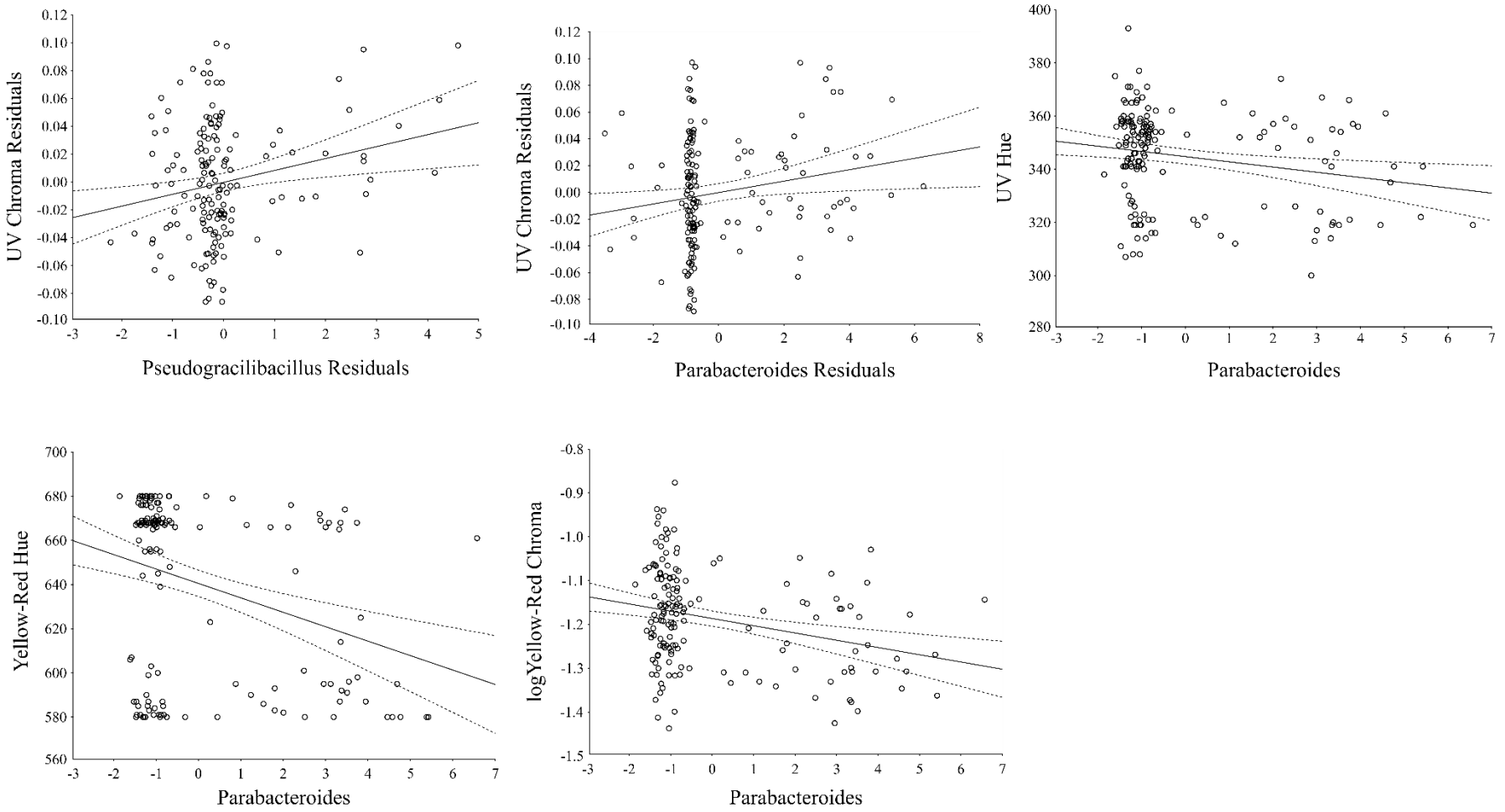


Figure 4. Regression plots showing the correlation between CLR-transformed abundance of bacterial genera that significantly explained a proportion of the variance of each colour variable and that colour variable. Since the best model for UV chroma included both *Parabacteroides* and *Pseudogracilibacillus*, the relative abundance of bacterial genera and the UV chroma values were controlled for the abundances of the other bacterial genera.

We found a complex symbiotic bacterial community in the yellow uropygial secretion of 10-days-old starling nestlings that differed from that sampled 4 days later when secretion colour turned to pale beige (Soler *et al.* 2022). This suggests that drastic ontogenetic changes in secretion composition of nestlings (Amo *et al.* 2012) might derive in changes on the microbiota, and then, on secretion colour. In accordance with that possibility the colour of the secretion of 10-days-old nestlings covaried with richness and composition of the bacterial community of the uropygial gland secretion. In particular, bacterial diversity predicted brightness and UV hue of secretion colour. We also found that relative abundance of genus *Parabacteroides* and *Pseudogracilibacillus* predicted UV and yellow-red components of secretion colour. Interestingly, all these colour components, when estimated on the nestling's mouth, associated with parental feeding preferences (Soler *et al.* 2022). Since we know that nestlings spread their uropygial secretion onto their begging related traits modifying their colourations (García-Núñez et al. in prep.), it might suggest that these bacteria are, at least partially, responsible for parental feeding preferences. Associations between bacteria and adaptive colourations of animals have rarely been described, but some examples exist in contexts of sexual selection (Díaz-Lora *et al.* 2020). Therefore, as far as we know, our results could be the first evidence of bacteria mediating parent-offspring communication.

The antioxidant supplementation experiment allowed us to conclude that both the bacterial community and the colouration of the secretion of nestlings are condition dependent traits (Soler et al. 2022). The experiment also affected carotenoid concentration in the blood (Martínez-Renau *et al.* 2021) and, thus, we cannot discard the possibility that the detected experimental effects on bacterial community and colouration (Martínez-Renau et al. 2021) of uropygial secretion were mediated by the antioxidant concentration in the blood. In other words, although the parallel experimental effects on bacteria and colour suggest that these two components are related to each other, experimental modification of the bacterial community of the uropygial gland is needed to demonstrate the role of bacteria on the uropygial secretion colour of starling nestlings.

Several mechanisms could explain the detected associations between uropygial secretion bacteria and colouration. It is known that some bacterial strains produce pigments as the result of their metabolism (Ram *et al.* 2020) and, then, one possibility is that the symbiotic bacteria directly synthetize carotenoids or other pigments implicated on secretion colour. However, bacteria could also degrade pigments in the secretion and then contribute to colour fading. A third possibility would be that specific bacterial taxa might determine

characteristics of the community through bacterial competition, including the presence and/or abundance of bacteria responsible for pigment synthesis or degradation. Whatever the underlying mechanisms, the detected associations broaden the hitherto known importance of bacterial symbionts on animal life and evolution in general (McFall-Ngai *et al.* 2013; Sherwin *et al.* 2019) and on signal evolution in particular (Ruiz-Rodríguez *et al.* 2015).

If bacteria were partially responsible for secretion colour, carotenoids incorporated through the diet into the blood stream could serve in other essential functions (Møller *et al.* 2000; Horak *et al.* 2006), or even be diverted to colour other bird integuments with signalling purposes (Navarro *et al.* 2010; Martínez-Renau *et al.* 2021). However, even if bacteria are directly responsible for the secretion colour, it is likely that, given the detected effect of VitE supplementation, the secretion colour still conveys information on nestling phenotypic quality. It seems plausible for instance that the chemical composition of the secretion determines the bacterial community developing inside the gland. Because trade-offs between mounting an immune response and the amount and composition of the secretion produced exist (Moreno-Rueda 2017), better-quality nestlings would produce secretions with certain characteristics that facilitate or avoid the establishment of specific bacterial taxa implicated in the production or fading of secretion colours.

Summarising, our results suggest a relevant role of the uropygial secretion microbiota on parent-offspring communication. This potential role is mainly supported by previous studies suggesting that parental food allocation rules are associated with the colour of nestlings mouth and secretion (Soler *et al.* 2022). Here we show that the latter is predicted by particularities of the secretion microbiota, and suggest that the colour of the secretion might be a by-product of the microbiota. However, covariation between colour and microbiota of the uropygial secretion does not imply that a direct relationship between both traits exists. For instance, a third variable (e.g., phenotypic condition) might be responsible for the detected covariation between both characteristics of the secretion. If that was the case, not only the colour, but characteristics of the microbiota detectable by adults (e.g., chemical volatiles), which are known to correlate with secretion colour (Martín-Vivaldi *et al.* 2010), could also explain food allocation rules of parents favouring nestlings with particular secretion colour (Soler *et al.* 2022). Therefore, future research manipulating the bacterial community of the uropygial gland secretion of starling nestlings in general, and *Parabacteroides* and *Pseudogracilibacillus* genera in particular, to evaluate its effect on secretion colouration, while recording food allocation is a much necessary next step to further

demonstrate a direct role of bacteria on the secretion colour, and thus on parent-offspring communication.

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Supplementary material

Additional Table 1. Result of Mantel test exploring the correlations between secretion bacterial communities at age 10 (independent matrix) and 14 (dependent matrix) post-hatching in starling nestlings. Breeding attempt was also included as a binary matrix. Values in bold are those with associate alpha value lower than 0.05.

	Distances day 14 post-hatching	
	Coef. of correlation	*p*
Aitchison distance	R^2 = 0.03, F = 17.22, p = 0.261	
Distances day 10 post-hatching	-0.218	0.236
Breeding attempt	0.182	0.904
PhiLR – Euclidean distance	R^2 = 0.08, F = 54.72, p <0.001	
Distances day 10 post-hatching	0.068	0.442
Breeding attempt	**1.952**	**<0.001**

Additional Table 2. Results of mixed effects ANOVAs exploring the effect of richness and diversity (alpha diversity indexes) of the bacterial community of the secretion on the secretion colouration. We also included the treatment and breeding attempt as fixed factors and Nest ID as random factor. The interaction between treatment and breeding attempt was explored in separate models. Values in bold are those with associate alpha value lower than 0.05.

	Chao1			**Shannon**			**PD**		
	F	df	p	F	df	p	F	df	p
logBrightness									
Alpha diversity index	1.04	1, 48	0.314	0.51	1, 48	0.480	0.83	1, 48	0.368
Treatment	1.21	1, 58.2	0.275	0.79	1, 49.7	0.379	1.19	1, 60.3	0.281
Breeding attempt	0.69	1, 95.1	0.409	0.14	1, 64.1	0.706	0.42	1, 76.9	0.520
Nest(breeding attempt)	**2.40**	**59, 41.3**	**0.002**	**2.35**	**59, 41.1**	**0.002**	**2.44**	**59, 41.4**	**0.002**
Treatment*Breeding attempt	0.22	1, 49.4	0.642	0.48	1, 50.5	0.493	0.23	1, 49.7	0.636
Treat*Nest(breeding attempt)	0.80	43, 48	0.764	0.80	43, 48	0.764	0.80	43, 48	0.776
UV hue									
Alpha diversity index	**6.06**	**1, 48**	**0.017**	0.01	1, 48	0.935	0.10	1, 48	0.754
Treatment	2.69	1, 56.1	0.106	1.00	1, 50	0.323	1.12	1, 60.9	0.294
Breeding attempt	**16.07**	**1, 80.6**	**0.001**	**10.83**	**1, 61.9**	**0.002**	**10.96**	**1, 70.1**	**0.001**
Nest(breeding attempt)	**3.91**	**59, 41.6**	**0.001**	**4.34**	**59, 41**	**0.001**	**4.21**	**59, 41.3**	**0.001**
Treatment*Breeding attempt	0.35	1, 48.4	0.557	0.75	1, 51	0.390	0.72	1, 50	0.400
Treat*Nest(breeding attempt)	0.94	43, 48	0.578	0.77	43, 48	0.810	0.77	43, 48	0.809
Yellow-red hue									
Alpha diversity index	0.17	1, 48	0.684	0.61	1, 48	0.438	1.70	1, 48	0.198
Treatment	0.27	1, 48.7	0.604	0.24	1, 45.4	0.626	0.50	1, 49	0.484
Breeding attempt	0.80	1, 68.2	0.374	1.05	1, 60	0.310	0.68	1, 62.9	0.413
Nest(breeding attempt)	**4.10**	**59, 42.4**	**0.001**	**4.13**	**59, 42.3**	**0.001**	**3.94**	**59, 42.4**	**0.001**
Treatment*Breeding attempt	3.16	1, 44.8	0.082	2.75	1, 45.2	0.104	3.31	1, 44.7	0.075
Treat*Nest(breeding attempt)	**2.19**	**43, 48**	**0.004**	**2.25**	**43, 48**	**0.003**	**2.34**	**43, 48**	**0.002**
Total hue									
Alpha diversity index	0.17	1, 48	0.686	0.25	1, 48	0.622	0.93	1, 48	0.339
Treatment	1.90	1, 49.3	0.175	1.84	1, 45.8	0.181	2.25	1, 50	0.140
Breeding attempt	2.35	1, 85.7	0.129	2.20	1, 62.2	0.143	2.85	1, 70.9	0.096
Nest(breeding attempt)	1.44	59, 42.3	0.107	1.49	59, 42.2	0.086	1.46	59, 42.4	0.098
Treatment*Breeding attempt	0.94	1, 45.2	0.338	0.01	1, 45.6	0.296	0.85	1, 45.1	0.362
Treat*Nest(breeding attempt)	**1.98**	**43, 48**	**0.011**	**1.96**	**43, 48**	**0.012**	**2.02**	**43, 48**	**0.009**
UV chroma									
Alpha diversity index	0.01	1, 48	0.961	0.06	1, 48	0.807	0.01	1, 48	0.937
Treatment	0.94	1, 57.5	0.338	1.10	1, 49.4	0.300	0.89	1, 59.2	0.349
Breeding attempt	0.50	1, 70.9	0.483	0.52	1, 60.4	0.474	0.53	1, 64.2	0.470
Nest(breeding attempt)	**8.25**	**59, 41.4**	**0.001**	**8.32**	**59, 41.2**	**0.001**	**8.17**	**59, 41.5**	**0.001**
Treatment*Breeding attempt	0.62	1, 49.2	0.435	0.57	1, 50.3	0.455	0.61	1, 49.3	0.439
Treat*Nest(breeding attempt)	0.85	43, 48	0.709	0.85	43, 48	0.709	0.85	43, 48	0.701
logYellow-red chroma									
Alpha diversity index	1.34	1, 48	0.254	0.21	1, 48	0.646	0.34	1, 48	0.562
Treatment	1.10	1, 53.6	0.298	0.67	1, 47.9	0.417	0.84	1, 55.4	0.362
Breeding attempt	0.01	1, 87.6	0.936	0.22	1, 62.7	0.637	0.08	1, 72.7	0.773
Nest(breeding attempt)	**2.25**	**59, 41.9**	**0.003**	**2.30**	**59, 41.7**	**0.003**	**2.29**	**59, 41.8**	**0.003**
Treatment*Breeding attempt	2.22	1, 47.1	0.644	0.09	1, 48.2	0.770	0.17	1, 47.5	0.678
Treat*Nest(breeding attempt)	1.17	43, 48	0.296	1.11	43, 48	0.356	1.12	43, 48	0.346
Carotenoid chroma									
Alpha diversity index	1.90	1, 48	0.174	0.12	1, 48	0.732	0.36	1, 48.0	0.551
Treatment	0.52	1, 52.9	0.473	0.17	1, 47.6	0.678	0.30	1, 54.8	0.588
Breeding attempt	1.21	1, 91.3	0.275	0.26	1, 63.4	0.609	0.46	1, 75.1	0.502
Nest(breeding attempt)	**1.79**	**59, 41.9**	**0.025**	**1.87**	**59, 41.7**	**0.018**	**1.83**	**59, 41.9**	**0.020**
Treatment*Breeding attempt	0.09	1, 46.8	0.762	0.18	1, 47.8	0.895	0.06	1, 47.2	0.812
Treat*Nest(breeding attempt)	1.26	43, 48	0.220	1.17	43, 48	0.296	1.18	43, 48.0	0.284

Additional Table 3. Results of mixed model ANOVAs exploring the effect of relative CLR-transformed abundances of *Parabacteroides* and *Pseudogracilibacillus* on the secretion colouration. We also included the treatment and breeding attempt as fixed factors and Nest ID as random factor. Values in bold are those with associate alpha value lower than 0.05.

	F	df	p
UV Chroma			
Parabacteroides	2.10	1, 49	0.153
Treatment	0.47	1, 51.4	0.497
Breeding attempt	0.08	1, 65.5	0.780
Nest ID (breeding attempt)	**7.89**	**59, 42.6**	**<0.001**
Treat*Nest (breeding attempt)	0.95	44, 49	0.574
Pseudogracilibacillus	0.41	1, 49	0.524
Treatment	0.62	1, 54.6	0.434
Breeding attempt	0.22	1, 63.4	0.642
Nest ID (breeding attempt)	**8.48**	**59, 42.2**	**<0.001**
Treat*Nest (breeding attempt)	0.82	44, 49	0.742
UV hue			
Parabacteroides	0.15	1, 49	0.698
Treatment	1.12	1, 53.2	0.295
Breeding attempt	9.31	1, 70.7	0.003
Nest ID (breeding attempt)	**4.60**	**59, 42.2**	**<0.001**
Treat*Nest (breeding attempt)	0.76	44, 49	0.817
Yellow-Red hue			
Parabacteroides	0.03	1, 49	0.875
Treatment	0.14	1, 47.2	0.714
Breeding attempt	0.91	1, 63.6	0.343
Nest ID (breeding attempt)	**3.88**	**59, 43.4**	**<0.001**
Treat*Nest (breeding attempt)	**2.22**	**44, 49**	**0.004**
logYellow-Red chroma			
Parabacteroides	1.24	1, 49	0.270
Treatment	0.34	1, 50	0.562
Breeding attempt	0.00	1, 75	0.987
Nest ID (breeding attempt)	**2.13**	**59, 42.8**	**0.005**
Treat*Nest (breeding attempt)	1.16	44, 49	0.307

Resultados generales y discusión integradora

Esta tesis se centra en estudiar el papel que las bacterias simbiontes desempeñan en funciones ya descritas de la glándula uropigial de las aves. En primer lugar, se busca entender su contribución en la producción de sustancias antimicrobianas que ayudan a las aves a defenderse frente a posibles infecciones patógenas. Además, se investiga cómo estas bacterias influyen en las señales relacionadas con la comunicación entre padres e hijos. Para ello, primero se estudia (i) la importancia del ambiente social durante las primeras etapas de la vida del ave en el establecimiento y ensamblaje de una comunidad especie-específica funcional. Después pasamos a estudiar (ii) la comunidad de bacterias asociada a la glándula uropigial, así como sus propiedades antimicrobianas, ya que, si juegan un papel importante en la lucha contra patógenos y enfermedades, deberían variar según las presiones selectivas debidas al riesgo de infección por microorganismos patógenos. Además, si las bacterias productoras de sustancias antibióticas se estuvieran cultivando en el interior de la glándula (secreción), (iii) la asociación entre las bacterias de la piel de la glándula y de la secreción será más fuerte en especies con mayores riesgos de infección. En relación a la posible función señalizadora de la secreción uropigial y de las bacterias asociadas, primero (iv) se estudia si la coloración de los rasgos de petición y de la secreción uropigial de los pollos de estornino señaliza tanto características genéticas como calidad fenotípica del individuo, para después analizar si (v) los pollos de estornino utilizan la secreción para maquillar sus rasgos de petición. Si esto ocurriera, ese maquillaje influiría en la información que trasmiten a los padres y, por tanto (vi) los padres deberían de responder de acuerdo al color tanto de estos rasgos como de la secreción, una predicción que también se analiza en la tesis. Por último, se estudia si (vii) la comunidad de bacterias de la secreción de la glándula uropigial de los pollos de estornino es, al menos en parte, responsable de su coloración, y por tanto estaría influyendo en la información que reciben los padres durante el reparto de cebas. A continuación, voy a exponer y discutir los principales resultados obtenidos en esta tesis enmarcados en los escenarios descritos: (1) ensamblaje de la comunidad bacteriana, (2) defensa contra microorganismos patógenos y (3) comunicación intraespecífica, para (4) acabar con algunas consideraciones generales sobre la importancia de bacterias simbiontes asociadas a la secreción uropigial de aves.

1. Ambiente social como factor importante para el ensamblaje de la comunidad de bacterias

En el **Capítulo I** de la tesis se utilizó el sistema de parasito de cría – hospedador formado por el críalo y la urraca, para explorar los efectos de las interacciones físicas entre individuos en la microbiota de la piel de la glándula uropigial de estas especies, separándolos de factores de confusión como las relaciones genéticas y familiares o el ambiente compartido. La composición de la comunidad de bacterias de los pollos de urraca y críalo que se desarrollaron en nidos mono-específicos fue significativamente distinta (Tabla 1). Los resultados obtenidos apuntan a una especificidad de la comunidad de bacterias de la piel de la glándula cuando ambas especies se desarrollan en nidos separados, con sólo individuos de su misma especie (siempre en nidos de urraca con progenitores urracas).

Tabla 1. Resultados de análisis PERMANOVA analizando el efecto de la especie en la beta diversidad de la comunidad bacteriana de la piel de la glándula uropigial de urracas y críalos cuando estos se desarrollan en nidos mono-específicos. Las matrices de distancias se han calculado usando los índices Bray-Curtis, Jaccard, y UniFrac ponderado y sin ponderar. Los valores alfa <0,05 se presentan en letra negrita.

	Bray-curtis			**Jaccard**			**UniFrac no ponderado**			**UniFrac ponderado**		
	Pseudo-F	*gl*	p	Pseudo-F	*gl*	p	Pseudo-F	*gl*	p	Pseudo-F	*gl*	p
Especie	**1,90**	**1, 16,3**	**0,002**	**1,51**	**1, 16,9**	**0,009**	**2,35**	**1, 16,4**	**0,009**	2,62	1, 15,2	0,091
Nido (Especie)	**1,55**	**13, 13**	**<0,001**	**1,30**	**13, 13**	**<0,001**	**1,47**	**13, 13**	**<0,001**	2,33	13, 13	0,099

Puesto que la piel de la glándula se impregna regularmente con la secreción uropigial, y la composición de ésta varía entre especies, estas diferencias pueden ser debidas a las diferencias en las características fisicoquímicas de la secreción de pollos de urracas y de críalos (Jacob & Ziswiler 1982). Es improbable que estas diferencias vengan dadas por la transmisión vertical de simbiontes de padres a hijos por parte de los adultos de cada especie, puesto que los críalos no tienen contacto con sus progenitores a partir del momento de la puesta de los huevos (Soler et al. 1999; Soler & Soler 1999), y, por tanto, cualquier transferencia de bacterias debe darse durante la formación del huevo. Curiosamente, estas diferencias interespecíficas desaparecen cuando los pollos de urraca y críalo conviven en el mismo nido (Tabla 2).

Tabla 2. Resultados de análisis PERMANOVA analizando el efecto de la especie en la beta diversidad de la comunidad bacteriana de la piel de la glándula uropigial de urracas y críalos en nidos hetero-específicos. Las matrices de distancias se han calculado usando los índices Bray-Curtis, Jaccard, y UniFrac ponderado y sin ponderar. Los valores alfa <0,05 se presentan en letra negrita.

	Bray-curtis			**Jaccard**			**UniFrac no ponderado**			**UniFrac ponderado**		
	Pseudo-F	*gl*	p	Pseudo-F	*gl*	p	Pseudo-F	*gl*	p	Pseudo-F	*gl*	p
Especie	1,06	1, 9	0,430	1,02	1, 9	0,485	1,21	1, 9,1	0,282	2,96	1, 9,3	0,089
Nido	**2,78**	**8, 8**	**<0,001**	**1,99**	**8, 8**	**<0,001**	**1,74**	**8, 8**	**0,001**	1,45	8, 8	0,271
Especie*Nido	0,91	8, 8	0,730	0,96	8, 8	0,652	0,85	8, 8	0,850	0,69	8, 8	0,729

Esto no implica necesariamente que las similitudes se deban a un intercambio de bacterias entre los pollos de ambas especies, sino que, al estar comparando pollos dentro de un mismo nido, estas similitudes podrían también ser debidas al ambiente físico compartido, o a una interacción de ambos factores. Estos resultados contradicen estudios previos donde, utilizando el mismo sistema de parasitismo de cría, vieron que las diferencias interespecíficas de la microbiota de la cloaca eran retenidas incluso después de compartir nido (Ruiz-Rodríguez *et al.* 2018; Lee *et al.* 2020). Esos resultados aparentemente contradictorios pueden ser debidos a la exposición y vulnerabilidad de cada una de las comunidades bacterianas a factores ambientales externos y, por tanto, a la colonización por bacterias exógenas. Mientras la comunidad de la piel de la glándula está mucho más expuesta al ambiente (que incluye los individuos con los que interactúa) y a las bacterias que en él se desarrollan, la microbiota de la cloaca está menos expuesta y, por las características específicas del tracto digestivo de cada especie, puede ser más resistente a las perturbaciones (Ruiz-Rodríguez *et al.* 2018).

Tabla 3. Resultados de análisis PERMANOVA analizando el efecto del ambiente social en la beta diversidad de la comunidad bacteriana de la piel de la glándula uropigial de urracas que conviven o no conviven junto con críalos. Las matrices de distancias se han calculado usando los índices Bray-Curtis, Jaccard, y UniFrac ponderado y sin ponderar. Los valores alfa <0,05 se presentan en letra negrita.

	Bray-curtis			**Jaccard**			**UniFrac no ponderado**			**UniFrac ponderado**		
	Pseudo-F	*gl*	p	Pseudo-F	*gl*	p	Pseudo-F	*gl*	p	Pseudo-F	*gl*	p
Ambiente social	**2,19**	**1, 22,2**	**<0,001**	**2,46**	**1, 22,5**	**<0,001**	**4,20**	**1, 22,6**	**<0,001**	**5,47**	**1, 21,8**	**0,006**
Nido (Ambiente social)	**1,57**	**20, 18**	**<0,001**	**1,38**	**20, 18**	**<0,001**	**1,35**	**20, 18**	**<0,001**	1,99	20, 18	0,116

Finalmente, comparamos urracas que se desarrollaron en nidos hetero-específicos (junto con críalos), con urracas que se desarrollaron solo con individuos de su misma especie, lo que nos permitió controlar el efecto del ambiente compartido, ya que todos los nidos tenían las mismas características (nidos de urraca alimentados y cuidados por urracas). La microbiota de las urracas sometidas a los distintos ambientes sociales fue estadísticamente diferente (Tabla 3), y, además, las urracas que no compartían nido con críalos tenían mayores abundancias relativas de géneros bacterianos potencialmente patógenos comparados con las urracas que convivían con críalos (Fig. 1). Estos resultados sugieren que, efectivamente, el ambiente social es importante para establecer y determinar la comunidad de bacterias de la piel de la glándula uropigial. Sin embargo, en un experimento de intercambio de pollos entre dos especies de fringílidos en cautividad se vio que el efecto del ambiente social en el establecimiento de la microbiota intestinal de individuos juveniles pierde importancia a medida que avanza el desarrollo de los individuos (Maraci *et al.* 2022). Por tanto, sería

necesario monitorear los cambios de la comunidad bacteriana de la piel de la glándula uropigial de las urracas que se desarrollan en nidos mono- y hetero-específicos después de abandonar el nido para así determinar si los efectos del ambiente social detectados durante la etapa de estancia en el nido persisten una vez los individuos lo abandonan.

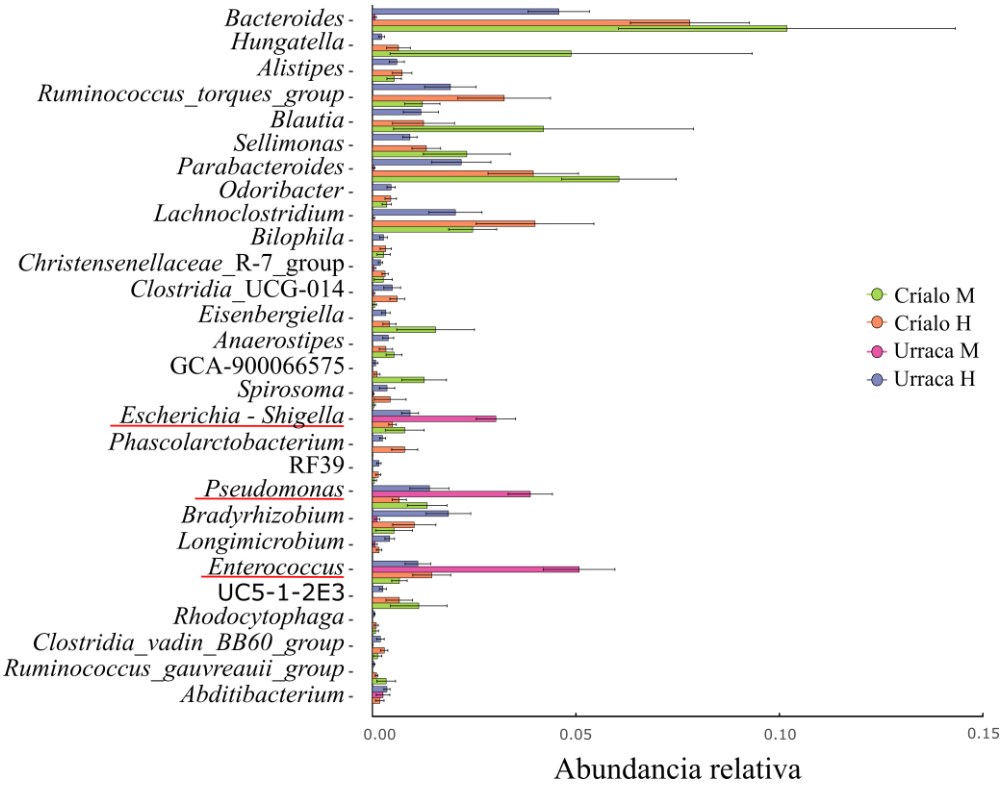

Figura 1. Géneros bacterianos con abundancias relativas estadísticamente distintas entre especies y ambiente social (pollos de nidos mono- (M) o hetero-específicos (H)). Resultados de análisis de Random Forest con valores alfa < 0,01. Los géneros subrayados en rojo son géneros considerados potencialmente patógenos.

El posible impacto negativo de la convivencia con críalos en la abundancia de los géneros potencialmente patógenos de su microbiota (Fig.1) podría ser debido al aumento de la riqueza y diversidad de la comunidad de bacterias detectado en los nidos hetero-específicos, ya que un aumento en la diversidad resulta en una mayor resistencia a la colonización por microorganismos patógenos (Lozupone *et al.* 2012; Kriss *et al.* 2018). Otra posible explicación a este resultado podría ser que existiera una mayor actividad del sistema inmune de los pollos, relacionada con la exposición a una amplia variedad de bacterias (Weiss, Maltz & Aksoy 2012), lo que derivaría en mayores defensas contra estos microorganismos. En cualquier caso, aunque este resultado implicaría una ventaja para las urracas que compartieran el nido con críalos, es muy poco probable que contrarreste los diversos efectos negativos que provoca el críalo en la eficacia biológica de su hospedador (Soler *et al.* 2017).

2. Uso de bacterias productoras de sustancias antibióticas como defensa contra microorganismos patógenos

En los **Capítulos II** y **III** caracterizamos las comunidades microbianas asociadas a la glándula uropigial de distintas aves y encontramos diferencias en la riqueza, diversidad, composición y propiedades antimicrobianas de las comunidades tanto de la piel de la glándula como de su secreción que variaron según la especie, la edad y sus características ecológicas en relación al riesgo de infección. De forma similar a lo que ocurre con algunas características del sistema inmunitario (Møller & Erritzøe 1996), las defensas mediadas por las bacterias mutualistas varían también según la fuerza de las presiones selectivas debidas al riesgo de infección por microorganismos patógenos, presentando más defensas (mayores capacidades antimicrobianas) las especies que experimentan mayores riesgos de infección debido al tipo de nido que utilizan durante la época de reproducción (Fig. 2A y 2B; **Capítulo II**), como son las especies que anidan en cavidades. Estas especies, además, albergan una mayor densidad de bacterias en su glándula (Fig. 2C; **Capítulo II**) y en las cáscaras de sus huevos (Peralta-Sánchez *et al.* 2012). Mayores densidades de bacterias en los nidos de las aves se asocian con un menor éxito de eclosión de los huevos (Peralta-Sánchez *et al.* 2018) y de vuelo de los pollos (Azcárate-García *et al.* 2019), por lo que nuestros resultados apuntan a que la selección natural podría estar actuando en características fenotípicas de las especies con mayores presiones selectivas (especies que anidan en cavidades) que favorecerían el establecimiento de bacterias productoras de sustancias antibióticas, y estarían ajustadas al riesgo de infección que experimentan las distintas especies de aves.

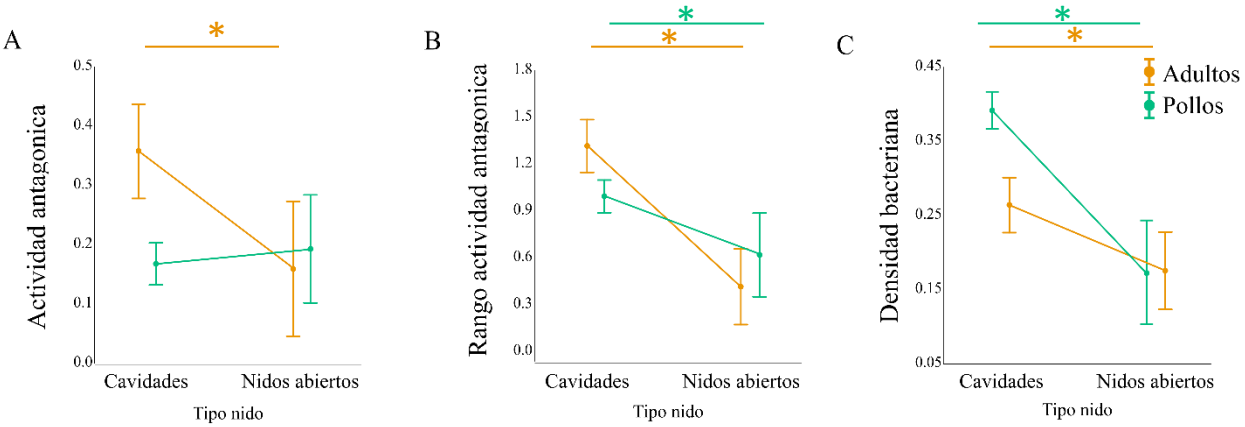

Figura 2. Medias de mínimos cuadrados (± 95% CI) mostrando el efecto del tipo de nido (cavidad frente a nido abierto) en la capacidad antimicrobiana y la densidad de bacterias de la comunidad de la piel de la glándula uropigial. Los gráficos muestran las distintas variables de capacidad antimicrobiana estimadas: **A**. actividad antimicrobiana (la media de los valores de los halos de antagonismo analizados frente a cada una de las bacterias indicadoras) y **B**. el rango de la actividad antimicrobiana (calculado como el índice de Shannon de la actividad antimicrobiana). **C**. muestra la densidad de bacterias.

Una de las características de las aves que podrían determinar los potenciales antimicrobianos de las comunidades bacterianas es la secreción uropigial, tanto dentro de la glándula como al ser extendida por el resto de partes del cuerpo. La secreción uropigial, una vez distribuida por los distintos tegumentos del ave, podría estar actuando como sustrato nutricional para que ciertas bacterias beneficiosas se desarrollasen sobre el cuerpo del ave. Por otro lado, si la secreción uropigial actúa como sustrato para bacterias beneficiosas, las aves podrían estar también cultivando en el interior de su glándula uropigial (donde se produce la secreción) bacterias productoras de sustancias antibióticas que, durante el acicalamiento, podrían dispersarse por el resto de partes del cuerpo donde actuarían. En ambos casos, la selección natural podría estar actuando, por ejemplo, en la composición química de la secreción, creando un ambiente que favorecería el establecimiento y crecimiento de unas bacterias frente a otras. La segunda posibilidad asumiría la presencia de una comunidad de bacterias simbionte establecida en la secreción de distintas especies de aves, que, en esta tesis se demuestra que está más extendida en la filogenia de las aves de lo que se conocía hasta el momento. Teniendo en cuenta todas las especies muestreadas (26, provenientes de diversos órdenes), nuestros resultados apuntan a que la microbiota de la glándula y de su secreción varía entre especies (Tabla 4; **Capítulo III**). Además, ambas comunidades de bacterias varían, al igual que sus propiedades antimicrobianas, dependiendo del riesgo de infección que experimentan (Tabla 4; **Capítulo III**).

Tabla 4. Resultados de análisis PERMANOVAs explorando el efecto del tipo de nido y la especie en la beta diversidad de las comunidades microbianas asociadas a la piel de la glándula uropigial y a su secreción en individuos adultos y pollos. Las matrices de distancia se han calculado usando Bray-Curtis, Jaccard y UniFrac ponderado y no ponderado. Valores de alfa < 0,05 están señalizados en letra negrita.

	Bray-Curtis			**Jaccard**			**UniFrac no ponderado**			**UniFrac ponderado**		
	Pseudo-F	*gl*	p	Pseudo-F	*gl*	p	Pseudo-F	*gl*	p	Pseudo-F	*gl*	p
Adultos												
Piel glándula												
Tipo nido	**4,02**	**1, 108**	**<0,001**	**2,64**	**1, 108**	**<0,001**	**7,53**	**1, 108**	**<0,001**	**10,77**	**1, 108**	**<0,001**
Especie (tipo nido)	**3,40**	**11, 108**	**<0,001**	**2,34**	**11, 108**	**<0,001**	**2,84**	**11, 108**	**<0,001**	**16,68**	**11, 108**	**<0,001**
Secreción glándula												
Tipo nido	**2,65**	**1, 125**	**<0,001**	**1,88**	**1, 125**	**<0,001**	**16,35**	**1, 125**	**<0,001**	**10,06**	**1, 125**	**<0,001**
Especie (tipo nido)	**1,31**	**14, 125**	**<0,001**	**1,18**	**14, 125**	**<0,001**	**1,93**	**14, 125**	**<0,001**	**1,56**	**14, 125**	**0,015**
Pollos												
Piel glándula												
Tipo nido	**2,59**	**1, 135,2**	**<0,001**	**1,83**	**1, 143,5**	**<0,001**	**2,92**	**1, 155,8**	**0,001**	**11,67**	**1, 141,1**	**<0,001**
Especie (tipo nido)	**4,75**	**12, 112,4**	**<0,001**	**3,15**	**12, 113,5**	**<0,001**	**6,96**	**12, 115,3**	**<0.001**	**9,46**	**12, 113,2**	**<0,001**
Nido (Especie (tipo nido))	**3,29**	**109, 95**	**<0,001**	**2,45**	**109, 95**	**<0,001**	**1,75**	**109, 95**	**<0.001**	**2,65**	**109, 95**	**<0,001**
Secreción glándula												
Tipo nido	**1,99**	**1, 148,1**	**<0,001**	**1,53**	**1, 158,9**	**<0,001**	1,73	1, 146,6	0.133	2,50	1, 159,46	0,090
Especie (tipo nido)	**2,36**	**11, 97,7**	**<0,001**	**1,78**	**11, 100,8**	**<0,001**	**3,80**	**11, 97,3**	**<0.001**	**3,10**	**11, 101**	**<0,001**
Nido (Especie (tipo nido))	**1,83**	**83, 115**	**<0,001**	**1,51**	**83, 115**	**<0,001**	**1,88**	**83, 115**	**<0.001**	**1,49**	**83, 115**	**<0,001**

Los resultados muestran, también, que las comunidades de la piel de la glándula y de la secreción están asociadas entre ellas (Tabla 5; **Capítulo III**), lo que apoyaría la hipótesis de que las bacterias de la piel de la glándula puedan derivar de la microbiota de la secreción. Este patrón de anidamiento se ha descrito antes para la abubilla, cuyas bacterias simbiontes establecidas en la secreción se esparcen mediante el acicalamiento por diversas partes del cuerpo hasta llegar a los huevos (Soler *et al.* 2016), donde previenen infecciones aumentando el éxito de eclosión (Martín-Vivaldi *et al.* 2014).

Tabla 5. Resultados de test de Mantel explorando las correlaciones entre la microbiota de la piel de la glándula uropigial (matriz dependiente) y la de la secreción (matriz independiente), tanto para todas las especies conjuntamente, como para las especies que anidan en cavidades y en nidos abiertos por separado. También se presentan los resultados de los análisis con la tabla ASV sin colapsar (a nivel de cepa) y colapsadas a nivel de género. Los índices usados para calcular las matrices de distancias fueron Bray-Curtis, Jaccard y UniFrac ponderado y no ponderado. Los valores de alfa < 0,05 se muestran en letra negrita.

		Bray-Curtis		**Jaccard**		**UniFrac no ponderado**		**UniFrac ponderado**	
		R	p	R	p	R	p	R	p
Adultos	Todas las especies								
	Sin colapsar (ASV)	**0,16**	**0,038**	**0,18**	**0,020**	-0,01	0,541	**0,25**	**0,013**
	Colapsada a nivel de género	0,02	0,378	0,02	0,386	-0,02	0,569	**0,19**	**0,049**
	Especies que anidan en cavidades								
	Sin colapsar (ASV)	-0,09	0,780	-0,04	0,624	**0,31**	**0,019**	**0,36**	**0,029**
	Colapsada a nivel de género	0,09	0,238	0,08	0,256	**0,42**	**0,002**	**0,42**	**0,021**
	Especies de nidos abiertos								
	Sin colapsar (ASV)	0,19	0,078	0,20	0,065	-0,02	0,565	0,18	0,119
	Colapsada a nivel de género	0,08	0,268	0,07	0,286	-0,08	0,784	-0,01	0,485
Pollos	Todas las especies								
	Sin colapsar (ASV)	**0,33**	**<0,001**	**0,35**	**<0,001**	0,04	0,299	**0,23**	**0,003**
	Colapsada a nivel de género	**0,35**	**<0,001**	**0,30**	**<0,001**	0,05	0,263	0,11	0,106
	Especies que anidan en cavidades								
	Sin colapsar (ASV)	**0,45**	**<0,001**	**0,43**	**<0,001**	0,02	0,381	**0,19**	**0,040**
	Colapsada a nivel de género	**0,35**	**<0,001**	**0,29**	**<0,001**	0,07	0,190	0,03	0,357
	Especies de nidos abiertos								
	Sin colapsar (ASV)	-0,17	0,884	-0,18	0,897	0,04	0,376	-0,07	0,602
	Colapsada a nivel de género	-0,12	0,725	-0,14	0,771	-0,01	0,422	-0,16	0,758

Curiosamente, la asociación entre las comunidades de la piel y de la secreción es más fuerte en las especies que anidan en cavidades (Tabla 5; **Capítulo III**). Si las bacterias productoras de sustancias antibióticas estuvieran cultivándose dentro de la glándula y dispersándose por el resto de tegumentos gracias al comportamiento de acicalamiento, cabría esperar que aquellas especies con mayores riesgos de infección por microorganismos patógenos se acicalaran más que las especies que experimentan un riesgo menor, y, por tanto, se predeciría una asociación más fuerte entre las comunidades bacterianas de su secreción y piel, predicción que concuerda con nuestros resultados. Un acicalamiento constante garantizaría la presencia de bacterias productoras de sustancias antibióticas en los tegumentos

más vulnerables, por lo que la selección natural podría estar actuando en la frecuencia de acicalamiento de las especies con presiones selectivas más fuertes. Sabemos que las aves que producen más secreción para acicalarse tienen unas glándulas de mayor tamaño y que aquellas que anidan en cavidades producen más secreción (Pap *et al.* 2010). Nosotros hemos encontrado que los adultos de esas especies también presentan tamaños de glándula mayores que las especies que anidan en nidos abiertos (después de controlar por el peso corporal y la identidad de la especie; tipo de nido: $F_{(1\ ,\ 14)} = 24{,}5$, $p < 0{,}001$; LS means ± SE especies que anidan en cavidades = 0,05 ± 0,03; LS means ± SE especies que anidan en nidos abiertos = -0,19 ± 0,04; **Capítulo III**), por lo que la mayor frecuencia de acicalamiento de esas especies podría explicar que en ellas la asociación entre comunidades de la glándula y de la secreción también sea mayor.

Tabla 6. Resultados de Modelos Lineares Generales y PERMANOVAs explorando el efecto de la especie y la edad en las capacidades antimicrobianas de los aislados bacterianos de la piel de la glándula, medidas como la actividad antimicrobiana (la media de los valores de los halos de antagonismo analizados frente a cada una de las bacterias indicadoras), el rango de la actividad antimicrobiana (calculado como el índice de Shannon de la actividad antimicrobiana), el perfil de antagonismo (valor del halo de antagonismo para cada bacteria indicadora) y la densidad de bacterias. Valores alfa < 0,05 se muestran en letra negrita.

	General Linear Models (GLM)									**PERMANOVA**		
	Actividad antimicrobiana			**Rango actividad antimicrobiana**			**Densidad bacteriana**			**Perfil de antagonismo**		
	F	df	p	F	df	p	F	df	p	Pseudo-F	df	p
Especie	**4,14**	**8, 173**	**<0,001**	**9,17**	**8, 173**	**<0,001**	**6,89**	**8, 164**	**<0,001**	**3,7**	**8, 165**	**<0,001**
Edad	**35,19**	**1, 173**	**<0,001**	**12,87**	**1, 173**	**<0,001**	**52,97**	**8, 164**	**<0,001**	**9,56**	**1, 165**	**<0,001**
Especie*Edad	1,86	8, 165	0,07	**3,7**	**8, 165**	**<0,001**	**2,89**	**8, 156**	**0,005**	**1,61**	**8, 165**	**0,003**

Tanto las propiedades antimicrobianas de las bacterias aisladas de la piel de la glándula, como la riqueza, diversidad y composición de las comunidades de la piel y la secreción varían también dependiendo de la edad (Tabla 6 y Tabla 7, ver Tabla 3 del Capítulo III; **Capítulos II** y **III**), y también varía con la edad el grado de asociación entre ambas comunidades (Fig. 3, ver Tabla 5 del **Capítulo III**). Los pollos, en general, presentan mayores densidades bacterianas en la piel de su glándula comparados con los adultos de su misma especie (ver Fig. 1 del **Capítulo II**), lo que podría explicar la mayor riqueza y diversidad encontrada en ambas comunidades (Tabla 7 y ver Figura Adicional 1 del **Capítulo III**).

Tabla 7. Resultados de Modelos Lineares Generales explorando el efecto de la especie y la edad en la alfa diversidad (Chao 1, índice de Shannon y Diversidad filogenética de Faith (PD)) de las comunidades de bacterias de la piel y la secreción de la glándula uropigial. Valores alfa < 0,05 se muestran en letra negrita.

	Chao1			**Índice de Shannon**			**PD**		
	F	*df*	p	F	*df*	p	F	*df*	p
Piel glándula									
Especie	**11,63**	**7, 195,4**	**<0,001**	**5,45**	**7, 198,5**	**<0,001**	**4,35**	**7, 252**	**<0,001**
Edad	**41,36**	**1, 196,3**	**<0,001**	**12,33**	**1, 199,7**	**<0,001**	0,01	1, 252	0,927
Especie*Edad	1,42	7, 193,5	0,200	**3,38**	**7, 199,4**	**0,002**	**3,31**	**7, 192,4**	**0,002**
	2,61	**182, 70**	**<0,001**	**2,09**	**182, 70**	**<0,001**	**2,97**	**182, 70**	**<0,001**
Secreción glándula									
Especie	**5,72**	**6, 126,8**	**<0,001**	5,14	**6, 131,9**	**<0,001**	**5,27**	**6, 126,1**	**<0,001**
Edad	**4,28**	**1, 129,1**	**0,040**	0,00	1, 135	1,000	0,02	1, 128,3	0,892
Especie*Edad	1,67	6, 118,8	0,135	**2,23**	**6, 123,9**	**0,045**	**3,04**	**6, 119,4**	**0,008**
Nido(Especie*Edad)	**3,45**	**113, 76**	**<0,001**	**2,50**	**113, 76**	**<0,001**	**3,65**	**113, 76**	**<0,001**

Estos resultados podrían ser debidos a factores ontogénicos puesto que, en el momento del muestreo de los pollos, aunque la glándula uropigial ya es funcional y produce secreción, puede que no esté completamente desarrollada y que, por tanto, las características de la secreción varíen entre pollos y adultos (Grieves, Bernards & MacDougall-Shackleton 2019), algo similar a lo que ocurre con ciertas características del sistema inmunitario (Apanius 1998; Palacios *et al.* 2009; Stambaugh *et al.* 2011). Estas diferencias en la composición podrían, al menos en parte, explicar las diferencias encontradas en las comunidades bacterianas de pollos y adultos en los **Capítulos II** y **III**. Encontramos que los potenciales antimicrobianos de las comunidades de pollos eran menores que en adultos (ver Fig. 1 del **Capítulo II**) y que el nivel de asociación entre las comunidades de la piel y la secreción también varió entre adultos y pollos, siendo mayor en los pollos (Fig. 3, ver Tabla 5 del **Capítulo III**), sobre todo en las especies que anidan en cavidades.

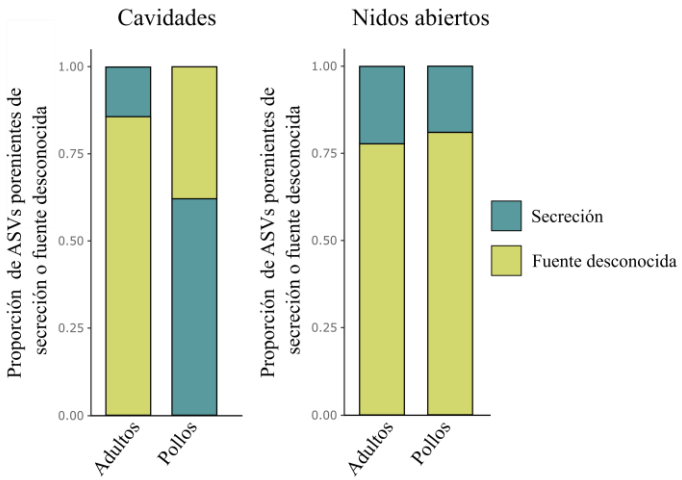

Figura 3. Resultados del análisis de "source-tracking" mostrando las proporciones de la microbiota de la piel de la glándula uropigial que se originan en la microbiota de la secreción o que provienen de una fuente desconocida.

Debido a que las comunidades de los pollos presentan propiedades antimicrobianas más débiles, es posible que necesiten acicalarse más frecuentemente que los adultos para mantener el nivel de defensas adecuado que les permitiera hacer frente a posibles infecciones, lo que podría derivar en la mayor asociación entre la microbiota de la piel de la glándula y la microbiota de la secreción que encontramos en los pollos respecto a los adultos. Los resultados del análisis de "source-tracking" del **Capítulo III** (Fig. 3) también ponen de manifiesto que una proporción alta de bacterias encontradas en la piel de la glándula uropigial de los pollos de especies que anidan en cavidades provienen de las bacterias de la secreción uropigial, lo que respaldaría la hipótesis de que las aves están cultivando bacterias beneficiosas en sus glándulas que, gracias al comportamiento de acicalamiento, se dispersan hasta los tegumentos donde actúan defendiendo a sus hospedadores frente a posibles infecciones patógenas.

Puesto que los hábitos de nidificación tienen un componente filogenético, cabría la posibilidad de que los efectos de este factor en la comunidad de bacterias y en sus propiedades antimicrobianas se explicaran por las relaciones filogenéticas entre las especies estudiadas. Sin embargo, en aves parece que las relaciones filogenéticas tienen un efecto débil sobre la variación de las comunidades bacterianas (Song *et al.* 2020; Trevelline *et al.* 2020; Bodawatta *et al.* 2021). En concordancia, en el **Capítulo III** no hemos encontrado una asociación fuerte entre la comunidad de bacterias y las relaciones filogenéticas de las aves (ver Tabla 6 del **Capítulo III**), y, además, el efecto de los hábitos de nidificación en las comunidades bacterianas de los adultos y sus propiedades antimicrobianas no varían después de corregir por el posible efecto de la filogenia (ver Tabla 4 del **Capítulo II** y Tabla Adicional 2 y Tabla Adicional 3 del **Capítulo III**). Por tanto, la explicación adaptativa a nuestros resultados sería la más probable, ya que sugiere que las presiones selectivas debida al riesgo de infección por bacterias patógenas estaría determinando las características de las comunidades microbianas de la piel y la secreción de la glándula uropigial.

3. <u>Bacterias y coloración. Posible papel de la microbiota en contextos de comunicación entre padres e hijos</u>

En la segunda parte de la tesis ponemos de manifiesto la importancia de la secreción uropigial de los pollos de estorninos en la comunicación con sus padres, y sugerimos un posible papel de la comunidad simbionte asociada a esta secreción en la producción o modulación de su coloración.

El intercambio de pollos entre nidos nos permitió detectar evidencias del efecto del ambiente en la coloración de los rasgos de petición, lo que sugiere que estos caracteres estarían reflejando necesidades a corto plazo y calidad fenotípica de los individuos (Tabla 8; **Capítulo IV)**. Uno de los factores ambientales que más afecta a la coloración de estos rasgos es la dieta (Kilner 1997; Thorogood *et al.* 2008; Jacob & Heeb 2013) y, mediante un experimento de suplemento de antioxidantes demostramos un efecto en la coloración de los rasgos de petición (ver Tabla 3 del **Capítulo IV**). Además, el experimento de intercambio de pollos entre nidos nos permitió comparar entre hermanos y hermanastros, poniendo de manifiesto un componente genético significativo en diversas variables de la coloración de la boca y la piel (Tabla 8), que explicarían la gran variabilidad interespecífica en el color de estos rasgos (Aviles *et al.* 2008; Avilés & Soler 2009; Soler & Aviles 2010).

Tabla 8. Resultados de ANOVAs jerarquizados del experimento de intercambio de pollos de estornino negro mostrando la influencia del nido de origen (componente genético) y el nido de cría (componente ambiental) en la coloración de los rasgos de petición (boca, boquera y piel). Los valores de alfa < 0,05 se muestran en letra negrita.

		Factores independientes					
		Nido de cría			Nido de origen (Nido de cría)		
Factores dependientes		$F_{28, 41}$	P	Varianza estimada (%)	$F_{28, 41}$	P	Varianza estimada (%)
Boca							
Brillo		**3,754**	**<0,001**	35,5	1,472	0,127	14,7
Tono	Total	**2,184**	**0,011**	44,9	**2,305**	**0,007**	0,0
	UV	**1,961**	**0,024**	13,2	1,357	0,183	15,8
	Amarillo-Rojo	**4,492**	**<0,001**	50,6	1,072	0,411	2,1
Saturación	UV	**8,364**	**<0,001**	68,9	1,054	0,431	1,0
	Amarillo-Rojo	**3,568**	**<0,001**	45,6	0,906	0,603	0,0
	Carotenos	1,212	0,282	18,2	0,516	0,966	0,0
Boqueras							
Brillo		**2,408**	**0,005**	32,9	0,850	0,671	0,0
Tono	Total	1,632	0,075	21,9	0,747	0,789	0,0
	UV	**4,186**	**<0,001**	47,1	1,125	0,360	3,8
	Amarillo-Rojo	**4,716**	**<0,001**	53,5	1,016	0,474	0,4
Saturación	UV	**2,399**	**0,005**	19,6	1,414	0,154	16,5
	Amarillo-Rojo	**2,132**	**0,013**	20,2	1,214	0,281	9,4
	Carotenos	**2,167**	**0,011**	22,9	1,136	0,349	6,0
Piel							
Brillo		**2,489**	**0,004**	22,5	1,351	0,186	14,0
Tono	Total	**2,356**	**0,006**	16,1	1,529	0,106	20,9
	UV	**14,524**	**<0,001**	56,8	**3,562**	**<0,001**	26,6
	Amarillo-Rojo	**8,588**	**<0,001**	51,9	**2,309**	**0,007**	21,7
Saturación	UV	**4,315**	**<0,001**	36,4	1,689	0,061	19,1
	Amarillo-Rojo	**3,827**	**<0,001**	21,3	**2,251**	**0,009**	34,5

El componente genético, sin embargo, no resultó significativo para ninguna variable de coloración de las boqueras ($p > 0,154$), algo que sí parece ocurrir en gorriones (Dugas 2012). Sin embargo, el color de este rasgo fue el más afectado por el suplemento de antioxidantes (ver Tabla 3 en **Capítulo IV**) y, además, fue el único que reflejó la condición corporal del pollo (Fig. 4), lo que puede indicar que el color de las boqueras es el carácter que con mayor fiabilidad puede reflejar la condición y necesidades de los pollos a corto plazo.

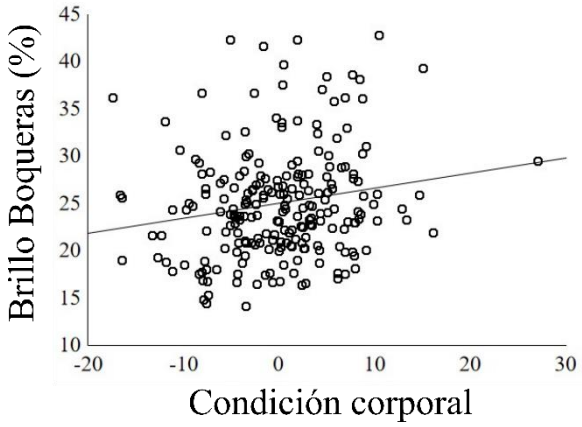

Fig. 4. Asociación entre la condición corporal de los pollos de estornino negro y el brillo de sus boqueras.

Por tanto, estos resultados en su conjunto sugieren que mientras la coloración de la boca y la piel informan tanto de características genéticas como fenotípicas, la coloración de las boqueras refleja la condición corporal y el estado oxidativo (nivel de antioxidantes en sangre) de los pollos, y, por tanto, los estorninos adultos al evaluar las distintas señales transmitidas por su descendencia, obtienen información variada acerca de las características de sus hijos que usaran para ajustar el reparto de cebas (Jourdie *et al.* 2004; Bize *et al.* 2006; De Ayala *et al.* 2007; Dugas 2009).

Los pollos de estornino presentan una secreción de color amarillo muy similar al color de la boca y las boqueras, que cambia hacia una tonalidad beige más clara y menos conspicua alrededor de los 14 días de edad. El cambio de coloración coincide, justamente con un cambio en el comportamiento de petición por parte de los pollos, que pasa de ser pasivo (mostrando la coloración de bocas y boqueras mientras mueven el cuello), a ser activo (los pollos se mueven hasta la entrada del nido por dónde sacan la cabeza para ser alimentados). Esas características podrían sugerir que la coloración amarilla de los pollos más pequeños no es una simple consecuencia de procesos ontogénicos, sino que podría estar implicada en la comunicación entre padres y pollos. Los resultados del experimento de intercambio de pollos revelaron que, al igual que la coloración de los rasgos de petición, la coloración de la secreción también dependía en parte de las características genéticas (ver

Tabla 2 del **Capítulo V**). Además, el experimento de antioxidantes afectó a la coloración de la secreción (Tabla 9), y ésta se relacionó con la concentración de carotenos en plasma (ver Fig. 2 del **Capítulo V**) y con la coloración de los rasgos de petición (boca, boquera y secreción) (ver Fig. 3 del **Capítulo V**). Por tanto, el color de la secreción uropigial también podría ayudar a señalizar la calidad genética, las necesidades a corto plazo y el estado oxidativo de los pollos.

Tabla 9. Resultados de ANOVAs mixtos explorando el efecto del suplemento de antioxidantes en la coloración de la secreción uropigial de los pollos de estornino negro después de controlar por el efecto fijo de la puesta y los efectos aleatorios del nido (anidado dentro de la puesta) y la interacción entre el nido y el tratamiento experimental. Solo se muestran las variables de color estadísticamente significativas. Las variables de color no significativas y los efectos aleatorios se muestran en la Tabla 2 y Tabla Adicional 2 del **Capítulo V**. También se muestran las medias de la primera (A) y segunda (B) puesta y de los tratamientos experimental (A) y control (B). Los estadísticos asociados al tratamiento experimental con un alfa de dos colas menor de 0,1 se muestran en negrita.

Factores dependientes	F	gl	Medias ponderadas (SE)		p
			(A)	(B)	
Tono total					
Puesta (1)	2,57	1, 59,96	376,7 (12,1)	329,8 (8,2)	0,114
Tratamiento (2)	**3,58**	**1, 49,07**	**371,9 (13,4)**	**346,5 (10,1)**	**0,064**
(1) * (2)	0,71	1, 48,69			0,404
Tono UV					
Puesta (1)	**9,43**	**1, 61,36**	**342,0 (2,0)**	**355,1 (1,6)**	**0,003**
Tratamiento (2)	0,03	1, 53,18	347,5 (2,2)	346,4 (2,1)	0,862
(1) * (2)	0,00	1, 53,05			0,994
Tono amarillo-rojo					
Puesta (1)	0,85	1, 55,73	639,3 (4,3)	651,2 (4,7)	0,360
Tratamiento (2)	1,55	1, 46,82	647,5 (4,5)	640,2 (4,6)	0,219
(1) * (2)	**5,85**	**1, 46,49**			**0,019**

Para que el color de la secreción uropigial interviniera en la señalización de los pollos a los adultos, los pollos tendrían que ser capaces de impregnarse las bocas con la secreción y, mediante la grabación del interior de las cajas nido pudimos detectar como los pollos de estornino colectan su secreción uropigial con el pico incluso antes de tener las plumas desarrolladas (ver Material Adicional 4 del **Capítulo V**). Además, en el momento del muestreo, al frotar el interior de las bocas y boqueras con un hisopo blanco, este se tornaba de color amarillo (Fig. 5), lo que sugiere que, efectivamente, los pollos de estornino impregnan los rasgos de petición con secreción uropigial.

Figura 5. Imágenes de pollos de estornino negro que muestran la coloración de las bocas y boqueras, así como los capilares que contienen secreción uropigial. Además, también se muestra un hisopo que, después de frotarlo por las boqueras del pollo, se tornó amarillo.

Finalmente, la tasa de cebas recibida por cada pollo se asoció con el color de la secreción y las bocas: pollos con el tono total de la secreción con valores más altos (Beta(SE) = 0,13(0,07), $F_{(1\ ,\ 165)}$ = 3,27, p = 0,07), y con valores más altos de tono de UV (Beta(SE) = 0,23(0,09), $F_{(1\ ,\ 165)}$ = 6,83, p = 0,01), saturación del UV (Beta(SE) = 0,44(0,16), $F_{(1\ ,\ 165)}$ = 7,76, p = 0,006), y saturación del amarillo-rojo (Beta(SE) = 0,30(0,15), $F_{(1\ ,\ 165)}$ = 4,32, p = 0,039) en sus bocas, fueron cebados preferentemente por los padres, lo que sugiere que los padres utilizan, no solo la información de la coloración de los rasgos de petición, sino también la coloración de la secreción para evaluar a su descendencia y decidir en qué pollo invertir la siguiente ceba. La teoría del maquillaje sugiere que teñir las estructuras con cosméticos es un mecanismo alternativo para señalizar honestamente la calidad fenotípica del individuo (Negro *et al.* 1999; Piersma, Dekker & Sinninghe Damsté 1999). Dado que la composición de la secreción varía según la calidad fenotípica y la condición corporal del individuo (Delhey, Peters & Kempenaers 2007; Piault *et al.* 2008), y que el tamaño de la glándula y la producción de secreción están relacionados con el sistema inmunitario (Moreno-Rueda 2015; Moreno-Rueda 2016), los pollos de estornino, al maquillar sus bocas, estarían reforzando la información que transmiten a sus progenitores.

En el **Capítulo VI** caracterizamos la comunidad de bacterias de la secreción y detectamos una correlación entre la riqueza y el tono UV (ANOVAs mixtas: $F_{(1,48)}$ = 6,06; p = 0,017; ver Tabla Adicional 2 del **Capítulo VI**) y entre la composición de la microbiota de la secreción y (i) el brillo (test de Mantel: R = 0,011; p = 0,028; ver Tabla 3 del **Capítulo VI**) y (ii) el tono UV (test de Mantel: R = 0,193; p = 0,005; ver Tabla 3 del **Capítulo VI**) de ésta. Además, mediante un estudio de redes de co-ocurrencia, vimos que los géneros de bacterias *Parabacteroides* y *Pseudogracilibacillus* también se correlacionaban positivamente con el componente UV y negativamente con el componente amarillo-rojo de la coloración de la

secreción (Fig. 6), variables de coloración que, medidas en la boca, se relacionan con las preferencias de ceba de los padres (**Capítulo V**). Aunque las correlaciones entre estos géneros bacterianos y la coloración no fueron muy altas, esta sería una primera evidencia de la posibilidad de que las bacterias se asocian con coloraciones animales que señalizan calidad fenotípica, una posibilidad que se ha estudiado muy poco en la literatura, con solo algún ejemplo en contextos de selección sexual (Díaz-Lora *et al.* 2020).

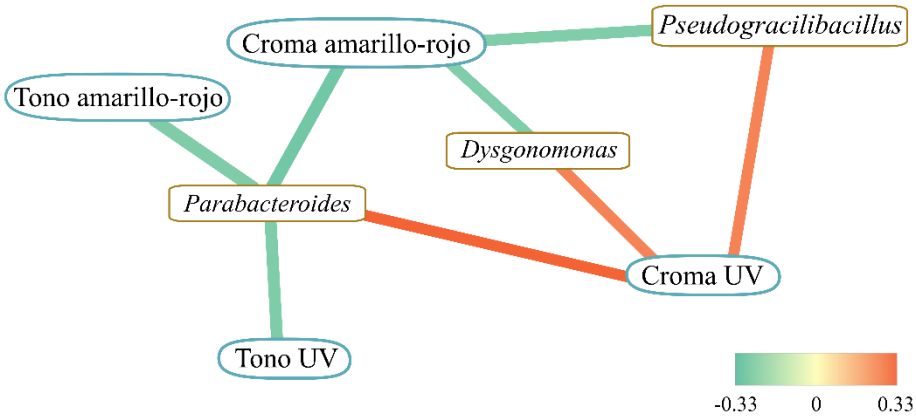

Figura 6. Representación de los resultados de una red bipartita de los análisis sPLS entre la comunidad de bacterias de la secreción uropigial de los pollos de estornino negro y la coloración de su secreción. Las correlaciones mostradas por las líneas son correlaciones de Pearson mayores de 0,25 o menores de -0,25 entre los géneros bacterianos y las variables de coloración. Las líneas verdes representan correlaciones negativas mientras que las líneas rojas representan correlaciones positivas. La intensidad del verde/rojo de las líneas se relaciona con el coeficiente de correlación, de más oscuro (mayores correlaciones) a más claro (menores correlaciones).

En este **Capítulo VI**, también encontramos un efecto del suplemento de antioxidantes en la riqueza y diversidad filogenética de la microbiota (Tabla 10). Como el tratamiento también afectó a la coloración de la secreción (Tabla 9; **Capítulo V**), estos resultados apoyarían la hipótesis de que ambos caracteres (coloración y microbiota) estarían correlacionados. Sin embargo, puesto que el tratamiento también afectó a la concentración de carotenos en plasma (ver Tabla 3 del **Capítulo IV**), cabría la posibilidad de que fueran los carotenos del plasma los responsables tanto del color de la secreción como de la riqueza y diversidad de la microbiota, sin que, necesariamente, las bacterias y la coloración tengan una relación directa. Curiosamente, la microbiota de la secreción cambió radicalmente entre los pollos de 10 y 14 días de edad, justo cuando la coloración de la secreción cambia de amarilla a beige claro. Este cambio en la composición de la microbiota refleja cambios ontogénicos en la composición de la secreción uropigial (Amo *et al.* 2012) y podrían ser, en parte, los responsables del cambio de coloración observado en la secreción.

Tabla 10. Resultados de ANOVAs mixtas explorando el efecto del suplemento de antioxidantes en los índices de alfa diversidad de la comunidad de bacterias de la secreción uropigial de los pollos de estornino negro, después de controlar por el factor fijo de la puesta y el factor aleatorio de la identidad del nido (anidada en la puesta). La interacción entre los factores fijos (tratamienito*puesta) se calculó en modelos separados que también incluían los efectos principales. Los valores de alfa < 0,05 se muestran en negrita.

	Variables independientes														
	Tratamiento			Puesta			Nido (Puesta)			Tratamiento*Puesta			Tratamiento * Nido (Puesta)		
Variables dependientes	F	gl	p	F	gl	p	F	gl	p	F	gl	p	F	gl	p
Chao1	**5,44**	**1, 59,3**	**0,023**	**10,58**	**1, 74,2**	**0,002**	**2,30**	**70, 55,4**	**<0,001**	1,41	1, 58,2	0,240	**1,86**	**55, 63**	**0,010**
Shannon	0,26	1, 62	0,610	0,08	1, 78,8	0,772	**1,64**	**70, 55,3**	**0,029**	0,07	1, 60,6	0,786	1,13	55, 63	0,314
PD	**8,42**	**1, 62,2**	**0,005**	**4,98**	**1, 76,3**	**0,029**	**2,36**	**70, 55,8**	**<0,001**	3,10	1, 61,6	0,083	1,10	55, 63	0,361

Teniendo en cuenta las asociaciones detectadas entre bacterias y coloración de la secreción, es posible que las características de las comunidades bacterianas ayuden a reflejar la condición fenotípica de los pollos cuando, durante el reparto de cebas, estos muestran a sus padres las bocas impregnadas con secreción uropigial. Los pollos con mayor calidad fenotípica podrían estar produciendo una secreción uropigial con características específicas que faciliten o impidan el crecimiento de las bacterias implicadas en la producción o degradación del color de la secreción. Todos estos resultados, sugerirían que la comunidad de bacterias de la secreción uropigial de los pollos de estorninos, mediante mecanismos de síntesis o incluso degradación de pigmentos, estarían produciendo o modificando la coloración de la secreción. De ser así, estas bacterias estarían mediando en la comunicación intraespecífica entre padres e hijos. En cualquier caso, estos resultados deben ser considerados preliminares, y, para acabar de concluir en favor de la hipótesis de que las bacterias son las responsables, al menos en parte, de la coloración de la secreción, habría que recurrir a diseños experimentales que nos permitan modificar la comunidad de bacterias y explorar así el efecto de este cambio en la coloración de la secreción.

4. <u>Consideraciones globales sobre la importancia de bacterias simbiontes asociadas a la secreción uropigial de las aves</u>

En su conjunto, los resultados de esta tesis ponen de manifiesto la importancia de la comunidad de bacterias asociada a la glándula uropigial de las aves, y sugieren que estas microbiotas pueden estar mediando en las funciones previamente atribuidas a la secreción uropigial. Por un lado, la comunidad de bacterias tanto de la secreción uropigial como de la piel de la glándula y sus propiedades antimicrobianas covarían con el riesgo de infección que experimentan las distintas especies estudiadas, y, por tanto, la comunidad de bacterias proporcionaría a las aves defensas adicionales que complementarían las defensas

comportamentales o inmunológicas para hacer frente a las posibles infecciones por microorganismos patógenos. Además, la asociación encontrada entre la comunidad de la piel y de la secreción apoyaría la hipótesis de que las bacterias productoras de sustancias antimicrobianas son cultivadas dentro de la glándula uropigial, y, dado que la asociación es mayor en las especies con un mayor riesgo de infección, estas especies estarían acicalándose más frecuentemente para dispersar las bacterias desde la glándula hasta los distintos tegumentos donde estas bacterias con propiedades antimicrobianas actuarían frente a posibles infecciones. Por otro lado, los resultados de la segunda parte de esta tesis sugieren que la secreción uropigial de los pollos de estornino negro interviene en la comunicación intraespecifica al ser usada como sustancia cosmética para maquillar los rasgos de petición, modificando o reforzando la información que los pollos transmiten a sus padres a través de la coloración de estos rasgos. Además, también mostramos evidencias de que, mediante la producción o modulación de la coloración de la secreción de los pollos, la comunidad de bacterias podría estar mediando en la información que los pollos proporcionan a sus padres durante el reparto de cebas, y que los padres utilizan para evaluar la condición de los pollos y decidir a cuál de ellos alimentar.

Conclusiones

1. La microbiota de la piel de la glándula uropigial de los pollos de urraca es producto del ambiente social en el que se desarrollan los individuos. Cuando las urracas se desarrollan en nidos junto con críalos, presentan comunidades microbianas más parecidas a sus hermanos hetero-específicos, con menor abundancia de géneros potencialmente patógenos.

2. Las propiedades antimicrobianas de las bacterias aisladas de la piel de la glándula uropigial de distintas especies dependen, al menos en parte, de las presiones selectivas debidas a parásitos y enfermedades. Las especies que anidan en cavidades (más vulnerables a las infecciones por microorganismos patógenos) presentan mayores propiedades antimicrobianas que las de las bacterias aisladas de la piel de especies con menor riesgo de infección.

3. Las comunidades bacterianas tanto de la piel de la glándula como de su secreción varían dependiendo de sus hábitos de nidificación y, por tanto, de las presiones selectivas debidas a parásitos y enfermedades.

4. Existe una asociación entre la comunidad bacteriana de la piel de la glándula y de la secreción, que es más fuerte en las especies que anidan en cavidades y sufren mayores riesgos de infección por bacterias patógenas. Esto sugiere que las bacterias productoras de sustancias antimicrobianas encontradas en la piel, pueden estar siendo cultivadas dentro de la glándula uropigial.

5. Mediante la coloración de los rasgos de petición que los pollos muestran a sus padres durante el reparto de cebas, los pollos de estornino transmiten información a sus padres relativa a sus características genéticas y su calidad fenotípica, incluyendo su estado oxidativo.

6. Los pollos de estornino utilizan la secreción uropigial como sustancia cosmética para maquillar sus bocas y boqueras. Así, los pollos podrían estar modificando la coloración de sus rasgos de petición y, por tanto, la información que transmiten a sus progenitores a través de esta.

7. La cantidad de cebas que proporcionan los estorninos adultos a cada pollo se ajusta a la coloración tanto de la secreción uropigial como de los rasgos de petición de cada hermano.

8. Existe una correlación entre la comunidad de bacterias de la secreción de los pollos de estornino y la coloración de esta secreción. Además, los géneros bacterianos *Parabacteroides* y *Pseudogracilibacillus* se correlacionan directamente con los componentes UV y amarillo-rojo de la secreción. Esto sugiere que estos géneros, o la comunidad de bacterias en general, pueden estar mediando, al menos en parte, en la coloración de la secreción uropigial y, por tanto, en la comunicación intraespecífica entre padres e hijos.

Conclusions

1. The uropygial gland skin microbiota of magpie nestlings is influenced by the social environment in which they develop. When magpies develop in heterospecific nests with great-spotted cuckoos, they exhibit bacterial communities more similar to their heterospecific siblings, with lower abundance of potentially pathogenic genera.

2. The antimicrobial properties of the bacterial strains isolated from the uropygial gland skin of different bird species are associated with the strength of the selection pressures due to parasitism and infection risk. Cavity-nester species, that are more vulnerable to pathogenic infection by microorganisms, show higher antimicrobial properties than the bacterial strains isolated from species with lower risk of pathogenic infection.

3. The bacterial communities of both, the uropygial gland skin and the secretion vary depending on the nesting habits and, thus, on the strength of the selection pressures due to parasitism and infection risk.

4. The bacterial communities of the uropygial gland skin and the uropygial secretion associate to each other, and the association is stronger in cavity-nester species with higher risk of pathogenic infection. These results suggest that the antibiotic-producing bacteria found in the skin might be cultivated inside the uropygial gland.

5. By showing their coloured begging related traits to their parents during parental provisioning, starling nestlings are signalling information about their genetic quality and phenotypic characteristics, including the antioxidant capacities.

6. Starling nestlings use their urgopygial secretion as cosmetics to stain their mouth and flanges. Consequently, nestlings are modifying the colouration of their begging-related traits, and hence, modulating the information they convey to their parents.

7. The feeding rate that adult starlings allocate to each of their offspring depends on the colouration of both, the uropygial secretion and that of the begging related traits of each sibling.

8. The bacterial community of the uropygial secretion correlates with its colouration. Moreover, the *Parabacteroides* and *Pseudogracilibacillus* genera correlate with the UV and yellow-red components of the secretion colour. These results suggest that these genera, or the bacterial community as a whole, might be mediating, at least partially, the colouration of the uropygial secretion of nestlings, and thus, the parent-offspring communication.

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