Señalización y respuestas evolutivas a ambientes bacterianos en aves. El caso del estornino negro *(Sturnus unicolor)*.

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Diseño y dibujo de portada: D. Daza Selma

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Editor: Universidad de Granada. Tesis Doctorales
Autor: Manuel Azcárate García
ISBN: 978-84-1306-594-6
URI: http://hdl.handle.net/10481/63533

Este trabajo ha sido posible gracias a la concesión de una “Ayuda para contratos predoctorales para la formación de doctores 2014” del anterior Ministerio de Economía y Competitividad (BES-2014-068661), y a la financiación del Ministerio de Ciencia, Innovación y Universidades y fondos FEDER (CGL2013-48193-C3-1-P, CGL2017-83103-P). Esta Tesis ha sido desarrollada en la Estación Experimental de Zonas Áridas, perteneciente al Consejo Superior de Investigaciones Cientificas (EEZA-CSIC), en Almería.

A Mónica y Manuel

Por brindarme su cariño

y alegría

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RESUMEN

Los animales se enfrentan diariamente a la necesidad de tomar decisiones tales como elegir el alimento, con quien reproducirse, o donde criar; decisiones que repercutirán en su eficacia biológica y que, al menos en parte, vienen moduladas por la información que reciben del ambiente que los rodea, incluida aquella proveniente de pistas y señales emitidas por otros organismos. Las señales se emiten en contextos de comunicación social y son beneficiosas tanto para el emisor como para el receptor, mientas que la información proporcionada inadvertidamente por las pistas sólo acarrea beneficios al receptor. En ambos casos, la recepción de estas informaciones por organismos no deseados puede tener efectos negativos importantes sobre los emisores y, por tanto, sobre la evolución de los caracteres implicados.

En los últimos años se ha destacado el posible papel de los microorganismos en contextos de comunicación. Las bacterias están en continua interacción con los animales, y son, por ejemplo, productoras de muchos de los volátiles utilizados por ellos en comunicaciones químicas. Además, existen muchas especies causantes de graves enfermedades o incluso la muerte que, en un contexto de comunicación, podrían promover la evolución de señales y comportamientos orientados a indicar la capacidad de los individuos hospedadores de hacerles frente. Las bacterias patógenas no sólo afectan directa o indirectamente a las perspectivas de supervivencia de los organismos, sino que también podrían limitar la evolución de determinados rasgos que, como los ornamentos sexuales, impliquen por ejemplo un aumento de hormonas inmunosupresoras que, a su vez, aumentan la probabilidad de infecciones bacterianas. Entender la evolución de pistas y de señales, y por tanto las presiones selectivas que pueden haber favorecido, mantenido, o restringido su desarrollo, es esencial para entender el funcionamiento de estos

rasgos en particular, y de la comunicación animal en general. En este contexto de información y comunicación, mediado en algunos casos por microorganismos, se enmarcan los principales objetivos de la tesis.

Trabajamos principalmente sobre distintas pistas y señales del estornino negro *(Sturnus unicolor*), directa o indirectamente relacionadas con microorganismos, en contextos de parasitismo, depredación y comunicación social, incluida la sexual. Concretamente, estudiamos experimentalmente (i) el uso de pistas visuales de ectoparásitos en nidos de estornino por hembras parásitas de cría en la elección de nidos hospedadores (capítulo 1), y (ii) el uso de pistas químicas, asociadas a la rotura de sacos fecales de pollos de estornino en sus nidos, por depredadores y ectoparásitos (capítulo 2). También, nos planteamos (iii) poner de manifiesto variaciones inter-específicas en las características de las plumas de distintas especies relativas al estado de degradación y a su susceptibilidad a la degradación bacteriana (capítulo 4), y (iv) comprobamos la relación entre coloraciones de caracteres sexuales secundarios (pico, plumas y patas) y diferentes rasgos relacionados con las defensas antimicrobianas y éxito reproductor en hembras (capítulo 3). Por último, (v) estudiamos experimentalmente la relación entre un carácter sexual (la longitud de las plumas de la garganta de estorninos) e indicadores de calidad fenotípica o genética (la longitud y dinámica de los telómeros) (capítulo 5), y (vi) entre dos caracteres sexuales secundarios (longitud de las plumas de la garganta y color del pico de machos de estornino) en un marco teórico de señales múltiples (capítulo 6).

Nuestros resultados mostraron que la existencia de motas experimentales en los huevos de estornino, simulando las producidas por el ectoparásito *Carnus hemapterus* – pistas indicativas del nivel de infestación por ectoparásitos –, afectó a la elección de nidos por hembras conespecíficas parásitas de cría. Estas prefirieron como nidos hospedadores aquellos en los que se eliminaban las motas de los huevos que, en condiciones naturales, serían

aquellos en los que sus hijos experimentarían un bajo riesgo de ectoparasitismo. También relacionado con pistas, pero en este caso mediadas por microorganismos, mostramos que la rotura experimental de sacos fecales producía un aumento en la carga bacteriana de los nidos que afectó negativamente al crecimiento de los pollos y positivamente a la probabilidad de depredación y, no significativamente, a la de ectoparasitismo. Estos resultados sugieren que comportamientos asociados a la retirada de excrementos de los nidos han evolucionado, al menos en parte, para evitar los efectos negativos de bacterias patógenas y de pistas que faciliten la detección del nido por depredadores y ectoparásitos.

El estudio comparativo en 16 especies de aves puso de manifiesto diferencias inter-específicas en los ambientes bacterianos de los nidos que covariaron con diferencias inter-específicas en susceptibilidad de las plumas a la degradación por bacterias queratinolíticas, y también con el deterioro de las plumas analizadas. Varias características de algunas plumas de distintas especies de aves, como son la coloración, el diseño y la longitud, actúan como señales en distintos contextos sociales y, por tanto, estos resultados evidencian que los ambientes bacterianos podrían determinar la integridad de las plumas y su capacidad de resistir la degradación bacteriana, así como influir en la información que pudieran transmitir en escenarios de comunicación.

En cuanto a los objetivos relacionados con el funcionamiento de señales en contextos de comunicación social y sexual, encontramos que la capacidad antimicrobiana del plasma mediada por anticuerpos naturales, y de la secreción uropigial de hembras de estorninos, se relacionó positivamente con su éxito reproductor. A su vez, estas variables defensivas se relacionan con la coloración de sus tegumentos (plumas, pico y patas). Por tanto, estos resultados sugieren que distintas coloraciones podrían actuar como señales sexuales indicativas de la calidad de las hembras en época de emparejamiento. Además, con respecto a la longitud de las plumas sexuales de la garganta de

los machos de estornino, nuestros resultados mostraron una relación negativa con indicadores de la calidad individual y de la esperanza de vida de los animales (longitud y la tasa de deterioro de los telómeros un año después de la medida de las plumas y/o experimento). Sin embargo, al considerar la longitud de las plumas después de una manipulación de acortamiento, esta asociación desaparecía, sugiriendo que la misma no se debía a un efecto directo de la longitud de las plumas. La edad podría explicar las relaciones encontradas ya que machos más viejos tendrían telómeros más cortos y plumas de la garganta más largas.

El experimento de acortar plumas produjo cambios significativos en la coloración de la base del pico al año siguiente. Esta coloración resultó positivamente relacionada con la condición corporal y con la longitud natural de las plumas ornamentales de la garganta, lo que sugiere un papel señalizador y sexual de este carácter. Estos resultados apoyan por primera vez la existencia de un vínculo causal entre las expresiones de dos caracteres sexualmente dimórficos, lo cual resulta esencial para comprender su funcionalidad en el marco de estudio de las señales múltiples.

Todos estos resultados contribuyen a entender la importancia de la información contenida en pistas y señales, directa o indirectamente relacionada con ambientes bacterianos y de ectoparasitismo, en la evolución de caracteres y comportamientos aviares en contextos de parasitismo, depredación y comunicación social. La novedad de algunos de ellos abre las puertas a nuevas líneas de investigación que, por ejemplo, pongan de manifiesto el papel directo de los microorganismos en estos contextos en aproximaciones experimentales.

SUMMARY

Animals are continuously making decisions; the food to eat, the partner to mate, the place to breed, or the host to parasitize. Decision-making has enormous fitness consequences and, at least partially, are modulated by the information received from their environment, including that from clues and signals emitted by other organisms. By definition, the signals function in contexts of social communication and are beneficial for both senders and receivers, while the inadvertently information provided by clues, only benefits to receivers. In both cases, eavesdropping on these cues by undesirable organisms may have significant negative effects on the emitters and, thus, constraint the evolution of related traits.

The possible role of microorganisms in contexts of communication has been highlighted in recent years. Bacteria are in continuous interaction with animals, and might, for instance, produce volatile components used in chemical communication. Also, there are many species that inflict severe negative effects to their hosts, including death. Therefore, in a communication context, the evolution of signals and behaviours aimed to reliably showing the ability of individuals to cope with microorganisms will be favoured in host populations. Pathogenic bacteria may also limit the evolution of certain traits that for instance imply elevate level of immunosuppressive hormones that increase the bacterial infections probability. Understanding the evolution of clues and signals, and therefore the selective pressures that may have favoured, maintained, or restricted their development, is essential to understanding the functioning of these traits in particular, and of animal communication in general. The main objectives of the Thesis are embodied within this context of information and communication mediated, in some cases, by microorganisms.

We mainly worked on different clues and signals of the spotless starlings (*Sturnus unicolor*). These clues and signals may be directly or indirectly related to microorganisms, in contexts of parasitism, predation and social (including intersexual) communication. Specifically, we experimentally studied (i) the use of visual clues of ectoparasitism load in starling nests by brood parasite females when choosing the host nest to parasitize (chapter 1), and (ii) the use of chemical clues, associated with the breaking of faecal sacs of starling nestlings in their nests, by predators and ectoparasites (chapter 2). Moreover, we (iv) showed interspecific variations in wear and susceptibility to bacterial degradation of nestling feathers that covaried with nest bacterial environment (chapter 4). In addition, (iii) we checked the relationships between secondary sexual traits (beak, feathers and legs) and variables related to antimicrobial defences and reproductive success in females (chapter 3). Finally, (v) we experimentally studied the relationship between a sexually selected character, the length of starling throat feathers, and indicators of phenotypic and genetic quality (the length and dynamics of telomeres) (chapter 5), and (vi) between two secondary sexual characteristics (length of throat feathers and colour of starling male beak) in a theoretical framework of multiple signalling (chapter 6).

Our results showed that the presence of experimental spots in starling eggshells, simulating those produced by the ectoparasite *Carnus hemapterus* - clues indicating the level of ectoparasite infestation - affected nest choice by conspecific brood parasite females. The preferred host nests for brood parasitism were those without spots, i.e., they prefer nests with a low risk of ectoparasitism. Also related to starling clues, but in this case mediated by microorganisms, we showed that the experimental breakage of faecal sacs produced an increase in nest bacterial loads, and negatively affected nestlings development. On the other hand, the experimental breakage of faeces in starling nests increased the probability of depredation and, although not

significantly, that of ectoparasitism. These results suggest that behaviours associated with the removal by parents of faecal sacs from nests have evolved, at least partially, to avoid the negative effects of pathogenic bacteria, and to eliminate the clues that may facilitate the nest detection by predators and ectoparasites.

The comparative study performed in 16 bird species showed interspecific differences in nest bacterial loads that covaried with interspecific differences in the susceptibility of feathers to degradation by keratinolytic bacteria, and also with feather wear. Several characteristics of feathers from different bird species, such as coloration, design and length, act as signals in different social contexts. Therefore, our results suggest that species-specific bacterial environments could determine the integrity of the feathers and their capacity to resist bacterial degradation, and, consequently, the transmitted information in communication contexts.

Regarding the objectives related to signals functioning in social and sexual communication contexts, we found that the plasma antimicrobial capacity of females (mediated by natural antibodies), and the amount of uropigial secretion produced, were positively related to their reproductive success. Also, those defensive variables are related to the coloration of the integuments (feathers, beak and legs). Therefore, these colorations might function as sexually selected signals indicating immune capacity of females during mating. In addition, our results showed negative relationships between the male ornamental throat feathers and proxies of individual quality and probability of survival, such as telomere length and dynamic one year later. However, these associations disappeared when considering feather length after the experimental shortening, which suggests that the detected associations were not due to a direct effect of feather length. Age could explain the detected relationships since older males would have shorter telomeres and longer throat feathers.

The experiment of feathers shortening produced statistically significant effects on the coloration of the base of the beak the following year. This coloration was positively related to body condition and the natural length of ornamental throat feathers, suggesting a sexual signalling function of this character. These results support, for the first time, a causal link between the expressions of two sexually dimorphic traits, which is essential for understanding their functionality within the multiple signals framework.

All these results contribute to our understanding of the evolution of signalling and/or clueing avian characters, and the provided information in contexts of parasitism, depredation and social communication. The originality of some results opens new possibilities and lines of research, such as those highlighting the role of microorganisms in these contexts through experimental approaches.

INTRODUCCIÓN GENERAL

1. Información y comunicación animal

Los animales se enfrentan diariamente a la necesidad de tomar decisiones tales como elegir el alimento, con quien reproducirse, o donde criar; decisiones que repercutirán en su eficacia biológica. En el ambiente que rodea a los animales existen elementos bióticos y abióticos que proporcionan información sobre las características del mismo y, por tanto, utilizar esta información en la toma de decisiones ayuda a los animales a ajustar sus decisiones a las características del ambiente (Danchin et al. 2004). Parte de esta información puede obtenerse por experiencia propia (mediante el ensayo y error, interactuando con el medio), o por la observación de otros individuos (información social). Este último caso puede basarse en **pistas** (información proporcionada inadvertidamente por otros individuos), o en **señales** (rasgos específicamente seleccionados para transmitir información en escenarios de comunicación) (Danchin et al. 2004).

1.1 Las pistas como fuente de información

Las aves pueden producir diversos tipos de pistas en la naturaleza de manera no intencionada (Valone 1989, Danchin et al. 2004). Se denominan pistas porque otros individuos, ya sean miembros de la misma (Smith et al. 1999) o distinta (Danchin et al. 2001) especie, pueden obtener información de ellas. La información que encierran las pistas se puede considerar fiable debido al hecho de que no se producen deliberadamente, sino que son consecuencia directa de actividades biológicas (por ejemplo, desplazamiento, alimentación o reproducción) que, en cualquier caso, han sido seleccionadas por una función

distinta a la de comunicación. Estas pistas pueden ser visuales (Valone and Templeton 2002), auditivas (Naguib 2005) o químicas (Aragon et al. 2006) y, en algunos casos, pueden ocasionar costos al emisor debido, por ejemplo, a que depredadores y parásitos las pueden utilizar pare detectar a sus víctimas (Danchin et al. 2001).

Un ejemplo de estas pistas es el moteado de los huevos del estornino negro (*Sturnus unicolor*) (en adelante estornino). Durante la incubación, sus huevos adquieren unas motas que provienen de la actividad de un ectoparásito hematófago, *Carnus hemapterus*, en sus nidos (Grimaldi 1997, López-Rull et al. 2007b). *Carnus* es un díptero de 2 mm que parasita a una amplia diversidad de especies de aves en sus nidos (Brake 2011, Tomás et al. 2018) y, aunque se alimenta principalmente de los pollos, también lo hacen de los padres mientras incuban (López-Rull et al. 2007b, Tomás et al. 2018). Las motas de los huevos se corresponden con las heces de los parásitos, y restos de sangre de la hembra derramada a causa de las picaduras. Por ello, la densidad de motas que hay sobre la cáscara de los huevos se considera como una pista de la abundancia de *Carnus* en el nido que anticipa la intensidad de parasitismo durante la incubación, y la que van a sufrir los pollos cuando eclosionen (López-Rull et al. 2007b). Además, la actividad de este ectoparásito también aumenta la disponibilidad de nutrientes que pueden ser usados por las bacterias para su crecimiento en los nidos de aves y en la cáscara de los huevos (Tomás et al. 2018), lo que afectará negativamente al éxito de eclosión (Peralta-Sánchez et al. 2018) y al desarrollo de los pollos (Soler et al. 2017, Azcárate-García et al. 2019). Por lo tanto, utilizar esas pistas como indicativas del nivel de riesgo de ectoparasitismo y de infección bacteriana sería una clara ventaja selectiva para individuos que, como los machos, decidan su inversión parental en función del valor reproductivo de sus polluelos (Avilés et al. 2009), o para hembras parásitas de cría que busquen nidos libres de parásitos y de bacterias para poner sus huevos (Tomás et al. 2017, 2018).

El parasitismo de cría en aves es una estrategia reproductiva en la que las hembras parásitas ponen sus huevos en los nidos de otras hembras de la misma (intra-específico) o diferente (inter-específico) especie (Soler 2017), siendo estas quienes se ocupan del cuidado y la crianza de los huevos y pollos parásitos. Debido a que los parásitos deberían elegir hospedadores con características que resulten en mayores beneficios, se espera que el parasitismo de cría ocurra de manera no aleatoria dentro de las poblaciones de hospedadores, identificando y eligiendo características del huésped que aumenten su éxito reproductor. En esta tesis, exploramos el uso del moteado de los huevos de estornino por hembras conespecíficas parásitas, como pista sobre las condiciones del nido, esperando que prefirieran nidos con huevos inmaculados para depositar sus huevos.

1.2 Las señales como fuente de información

Las señales se definen como características comportamentales, morfológicas o fisiológicas que funcionan transfiriendo información intencionada entre organismos (Otte 1974), y han atraído la atención de los biólogos evolutivos durante décadas (Maynard-Smith and Harper 2003). Por definición, el uso de señales debe reportar beneficios tanto para el emisor como para el receptor o receptores y, para ello, la información transmitida debe ser fiable (Bradbury and Vehrencamp 1998, Searcy and Nowicki 2005). La hipótesis evolutiva que, en general, se utiliza para explicar la evolución y mantenimiento de los caracteres sexuales secundarios extravagantes, asume que éstos sean señales que reflejan diferentes componentes de su calidad genética y fenotípica (Andersson 1994). Aunque en un principio se sugirió que, en procesos de selección sexual, los rasgos sexuales extravagantes no necesariamente debían estar relacionados con la calidad fenotípica o genética de los poseedores (Fisher 1930), ahora se acepta que, principalmente debido a los costos

asociados a la competencia intra-sexual, estos rasgos evolucionarían rápidamente reflejando honestamente la calidad de los machos (Macías-García and Ramírez 2005). Por lo tanto, los rasgos sexualmente seleccionados deben reflejar de manera fiable la calidad fenotípica o genética del portador.

Las hembras, al elegir machos con rasgos que se correlacionan con una mejor calidad genética y/o fenotípica, obtendrán beneficios directos (mejores padres) o indirectos (buenos genes) y, por tanto, mayor eficacia biológica (Hamilton 1990, Andersson 1994, Møller 1994, Møller and Jennions 2001, Jones and Ratterman 2009). Por un lado, al exagerar los caracteres atractivos para las hembras, los machos obtendrían más oportunidades de reproducción (Darwin 1871). Por otro lado, las hembras que se emparejasen con machos de mayor calidad (expresada por la exageración de la señal) podrían realizar una mayor inversión en la reproducción (inversión diferencial (Burley 1988)), lo que debería resultar en un mayor éxito reproductor para los dos miembros de la pareja (Møller 1994, Sheldon 2000). Es decir, la evolución de rasgos seleccionados sexualmente en los machos, predice una asociación positiva entre la intensidad de expresión del rasgo y el éxito reproductor, tanto de machos como de hembras.

La fiabilidad de las señales está garantizada principalmente por los elevados costes que conllevan, y que solo individuos de buena calidad fenotípica y/o genética podrían abordar (Zahavi 1975, Zahavi 1977, Maynard-Smith and Harper 2003). Una señal puede por ejemplo implicar costes de producción (Hill 2000, Pardal et al. 2018), de mantenimiento (por ejemplo, en tiempo y energía (Blount et al. 2003, Ruiz-Rodríguez et al. 2015)), y sociales (Tibbetts and Dale 2004, Webster et al. 2018). Además, los caracteres sexuales ostentosos podrían ser utilizados como pistas por parásitos o depredadores (Stuart-Fox et al. 2003, Johnson and Candolin 2017, pero ver Webster et al. 2018), lo que suponen costos importantes en términos de aumentar la probabilidad de ser detectados por receptores no deseados. En general, la

detección e identificación de costes asociados a las señales, así como la caracterización de asociaciones entre la expresión del rasgo y las variables que reflejan la calidad fenotípica o genética del portador, es esencial para entender el funcionamiento de las señales en general, y de las señales sexuales en particular.

La evolución y funcionamiento de señales han sido intensamente estudiadas en aves, tanto por las ventajas y facilidades de estudiar distintos aspectos de la comunicación padres-hijos en sus nidos, como por lo llamativo de las coloraciones y formas de sus plumajes (Stoddard and Prum 2011, Bulluck et al. 2017), picos (Navarro et al. 2010) y otros tegumentos (Velando et al. 2005), que solo podrían explicarse como resultado de procesos de selección sexual. Por ello, y porque son el foco de esta tesis, vamos a profundizar en algunas de sus características.

1.3 Señales en aves

1.3.1 La coloración de partes desnudas

Las señales visuales tienen gran importancia en aves ya que en muchas especies determinan en gran medida el éxito de apareamiento (Hill et al. 2006a). Aunque los estudios tienden a centrarse en las plumas (Hill and Montgomerie 1994, Kose and Møller 1999, Badas et al. 2018), existen otros tegumentos en los que pueden evolucionar características señalizadoras. Un ejemplo lo encontramos en las coloraciones de la piel de las patas, de picos, o de otras partes desnudas, no recubiertas de plumas (Iverson and Karubian 2017), que actúan como señales sexuales (Torres and Velando 2005, Bonato et al. 2009, Schull et al. 2016). Aunque las partes desnudas y el plumaje suelen estar coloreados por los mismos pigmentos (Hill et al. 2006b, Hill et al. 2006a), existen diferencias entre ellos que pueden tener implicaciones importantes para

la función y la evolución de la señal. Dentro del contexto de la señalización relacionada con bacterias, las partes desnudas como pico y patas podrían ser señales fiables del estado de salud e inmunitario (Merrill et al. 2016) de los individuos en un momento dado y, por tanto, de la resistencia del individuo a las infecciones, ya que al contrario que las plumas, estas áreas pueden sufrir cambios en el color a muy corto plazo (en días u horas) (Iverson and Karubian 2017).

1.3.2 Plumas

La presencia de plumas es una de las características que definen a las aves, y ha atraído históricamente la atención de los biólogos evolutivos que tratan de entender la altísima variación en la morfología, estructura y coloración que presentan (Hanson 2011). Las funciones adaptativas clásicas que se han atribuido a las plumas son el aislamiento (es decir, la termorregulación o la impermeabilización) y el vuelo (Rayner 1988, Stettenheim 2000). Hoy sabemos que son también muy útiles en diferentes escenarios de comunicación social y selección sexual (Andersson 1994, Møller et al. 1998, Senar 2006, Hanson 2011, Roulin 2016). Por lo tanto, distintos factores que afectan a la integridad y desarrollo de las plumas son decisivos en su funcionalidad, e imprescindibles para comprender el funcionamiento de sus distintas morfologías, coloraciones, y por consiguiente, su evolución.

La integridad de las plumas puede, por ejemplo, depender de las condiciones fisiológicas de los individuos durante su formación; individuos en peor condición física formarían plumas de peor calidad y menor resistencia al deterioro y a la rotura (Jovani and Rohwer 2017). Factores que dañan directamente las plumas, como el desgaste físico debido a la fricción con el aire o el agua mientras el ave vuela o se zambulle, o el deterioro debido al contacto con distintos materiales mientras el ave incuba, camina o se posa, son

bien conocidos desde hace muchos años (Francis and Wood 1989, Swaddle et al. 1996). La abrasión química, especialmente aquella inducida por bacterias degradadoras de queratina, también se ha estudiado durante la última década (Gunderson 2008). La queratina es el principal componente de las plumas y existen una gran cantidad de microorganismos que son capaces de digerirla (Burtt and Ichida 1999). Por tanto, en contacto con las plumas de las aves, los microorganismos degradadores podrían modificar su coloración estructural (Shawkey et al. 2007), y aumentar la probabilidad de rotura (Ruiz-Rodríguez et al. 2015). Tanto el color de las plumas como su integridad son usadas como señales en distintas especies de aves indicando por ejemplo la capacidad antioxidativa (Galván and Alonso-Alvarez 2009) y/o inmunológica del portador (Hamilton and Zuk 1982, Clayton 1991, Zuk 1992, Møller et al. 1999, Zuk and Johnsen 2000). Por tanto, la actividad bacteriana podría estar modulando el mensaje de estas señales (Ruiz-Rodríguez et al. 2015).

2. Los microorganismos como agentes selectivos

Los microorganismos se caracterizan por su ubicuidad, estando presentes en todos los medios donde los animales se desarrollan y reproducen, por lo que interaccionan constantemente con ellos. Actualmente sabemos que han jugado un papel muy importante en la evolución del resto de organismos, y en particular en la de sus hospedadores animales, por lo que cada vez son más abundantes los estudios biológicos que los tienen en cuenta en marcos evolutivos y ecológicos muy dispares (Archie and Theis 2011, Ezenwa et al. 2012, McFall-Ngai et al. 2013).

Los microorganismos en general, y las bacterias en particular, también tienen una gran relevancia en los sistemas de comunicación entre animales, pudiendo influir en el desarrollo y producción de pistas y señales, así como en la información que éstas proporcionan. Por ejemplo, sabemos que las

sustancias químicas producidas por el metabolismo bacteriano influyen en la comunicación de los suricatas (*Suricata suricatta*) (Leclaire et al. 2017), y que algunos parásitos utilizan volátiles liberados por bacterias como pistas para encontrar y seleccionar a sus huéspedes (Verhulst et al. 2011, Busula et al. 2017). Las bacterias también pueden tener efectos positivos (Møller et al. 2012) o negativos (Polin et al. 2015) sobre la probabilidad de captura de sus hospedadores por los depredadores, o incluso disuadir a los parasitoides de utilizar potenciales huéspedes infectados (Costopoulos et al. 2014).

La patogenicidad es quizás la característica más conocida de las bacterias. Algunas cepas infringen importantes efectos negativos a sus hospedadores (Pinowski et al. 1994, Lombardo et al. 1996, Mills et al. 1999, Peralta-Sánchez et al. 2018), influyendo en el desarrollo de determinados rasgos. El fenotipo de estos rasgos, por tanto, podría informar de la carga bacteriana, o la capacidad del emisor de la señal de defenderse de las bacterias. Pueden incluso existir rasgos que por su forma o estructura estén más expuestos a la degradación bacteriana, siendo la información transmitida por estos rasgos más fiable en términos de capacidades antimicrobianas (Ruiz-Rodríguez et al. 2015).

El ambiente bacteriano que rodea a los animales puede afectar directa o indirectamente a sus perspectivas de supervivencia, y por lo tanto podría limitar la evolución de características que aumenten la probabilidad de infecciones bacterianas. Este podría ser el caso de capacidades o personalidades exploratorias, o cualquier otra habilidad cognitiva que implique un aumento en la probabilidad de contacto y contaminación con bacterias patógenas (Soler et al. 2012b). En las aves, los efectos patógenos de las bacterias se habían explorado tradicionalmente en especies de corral y domésticas, aunque en los últimos años se están estudiando sus efectos en aves silvestres (Benskin et al. 2009). Se han detectado efectos negativos en términos de supervivencia o viabilidad en embriones (Soler et al. 2012a, Peralta-

Sánchez et al. 2018), pollos y aves silvestres adultas (Benskin et al. 2009). Todos estos efectos negativos hacen que las presiones selectivas que favorecen la evolución de estrategias defensivas y barreras contra infecciones bacterianas sean muy fuertes. Este es el caso de distintos tipos de respuestas inmunitarias (Soler et al. 2011), secreciones antimicrobianas (Soler et al. 2008a, Møller et al. 2009, Ruiz-Rodríguez et al. 2009, Soler et al. 2010, Soler et al. 2012a, Martín-Vivaldi et al. 2014), comportamientos de automedicación usando por ejemplo plantas aromáticas con capacidad antimicrobiana como materiales de nidificación (Gwinner and Berger 2005, Mennerat et al. 2009a, Ruiz-Castellano et al. 2016), o comportamientos de saneamiento de nidos para prevenir o limitar el crecimiento bacteriano (Ibáñez-Álamo et al. 2017).

En el caso de comportamientos de saneamiento de los nidos, las presiones selectivas ejercidas por las bacterias podrían ser las responsables de la evolución de los sacos fecales de los pollos presentes en muchas especies de aves (envolturas mucosas que envuelven los excrementos) (Guigueno and Sealy 2012), que facilitan la manipulación de los excrementos por los padres para retirarlos del nido sin riesgos de contaminación (Ibáñez-Álamo et al. 2014). Estos dos rasgos, la eliminación de las heces de los pollos y la cobertura de los excrementos con sacos fecales, están bastante extendidos en las aves y se correlacionan entre sí a lo largo de la filogenia aviar (Ibáñez-Álamo et al. 2017). Retirando los sacos, las aves consiguen evitar, por un lado, los efectos negativos que podrían causar bacterias intestinales potencialmente patógenas (por ejemplo enterobacterias) (Brittingham et al. 1988, Lombardo et al. 1996, Westneat and Rambo 2000) en los pollos, lo cual beneficiaría su desarrollo y aumentaría la probabilidad de supervivencia (Ibáñez-Álamo et al. 2014). Además, debido a que tanto la visualización de excrementos como la actividad bacteriana asociada (olores) podrían suponer pistas visuales o químicas que podrían atraer a depredadores (Petit et al. 1989, Lang et al. 2002) y/o ectoparásitos (Hart 1997, Quan et al. 2015, Ibáñez-Álamo et al. 2016), los

caracteres asociados a la fácil retirada de los excrementos del nido se han seleccionado en la filogenia de las aves y, actualmente, existen en la mayoría de las especies (Ibáñez-Álamo et al. 2017).

En esta tesis, estudiamos presiones selectivas, directa o indirectamente relacionadas con bacterias, que podrían afectar a la evolución y desarrollo de algunos de los caracteres más llamativos y típicos de las aves como son las coloraciones y morfologías de plumas, patas y pico. Estos caracteres funcionan como señales sexuales en muchas especies, dando información sobre la calidad del portador y, a su vez, están afectadas por las bacterias del medio (Shawkey et al. 2007, Ruiz-Rodríguez et al. 2015, Iverson and Karubian 2017).

3. Mecanismos de defensa frente a microorganismos patógenos y su relación con la expresión de señales

Defensas comportamentales o de respuesta inmune innata no específicas representan las primeras barreras antibacterianas (Playfair and Bancroft 2004) frente a una amplia gama de microorganismos, incluyendo a aquellos que son capaces de degradar las plumas ornamentales (Horrocks et al. 2012). Por ello, cuando por ejemplo una hembra elige pareja basándose en la expresión exagerada de estos ornamentos sexuales, en la mayoría de los casos, elije a machos menos infectados y con mayor capacidad de resistencia a los patógenos, que heredarían sus descendientes (Hamilton and Zuk 1982, Folstad and Karter 1992).

En el caso de las plumas, existen características y estrategias dirigidas al mantenimiento y reducción del desgaste. Las aves, por ejemplo, pueden mudar y de esta forma restablecer el plumaje periódicamente. La mayoría de las especies de aves renuevan completamente sus plumas al menos una vez al año, lo cual es costoso en términos de recursos consumidos (Dawson et al.

2000), e indica la importancia de la integridad de las plumas para la vida aviar (Swaddle et al. 1996). Además, los patrones de muda son paralelos a algunas de las características del ciclo vital que predicen el deterioro de las plumas, lo que sugiere además que su estado de degradación depende de dichas características del ciclo vital (Guallar and Figuerola 2016). El uso de la secreción oleosa de la glándula uropigial para el acicalamiento y cuidado de las plumas es otra estrategia bien conocida. La glándula uropigial es un complejo holocrino exclusivo de las aves (Jacob and Ziswiler 1982). Su secreción tiene actividad antibacteriana y se utiliza para acicalar y proteger las plumas de los agentes abrasivos, incluidas las bacterias que degradan las plumas (Shawkey et al. 2003, Møller et al. 2009, Ruiz-Rodríguez et al. 2009, Soler et al. 2012a, Fülöp et al. 2015, Moreno-Rueda 2017, Verea et al. 2017). Por estudios previos sabemos que el nivel de respuesta inmune innata (Soler et al. 2011) y el tamaño de la glándula uropigial (Soler et al. 2012a) se asocian con la carga de bacterias en la superficie de los huevos en aves, y que el tamaño de esta glándula está negativamente relacionado con la densidad de las bacterias que degradan las plumas en las golondrinas (*Hirundo rustica*) (Møller et al. 2009). Además, la manipulación experimental del ambiente microbiano conduce a cambios en el tamaño de la glándula y en la composición de la secreción (Jacob et al. 2014).

La estructura y composición química de las plumas también podrían afectar a su susceptibilidad a la degradación bacteriana (Ruiz-de-Castañeda et al. 2012). Aunque el principal componente químico de las plumas es la β-queratina, estas normalmente están coloreadas y, una ligera variación en la composición y tipo de pigmento, puede influir en su susceptibilidad a la degradación. Sabemos, por ejemplo, que la queratina melánica es más difícil de romper que la no melánica (Bonser 1995), lo que explica que las tasas de desgaste (Burtt 1979), rotura (Kose and Møller 1999) y degradación bacteriana (Goldstein et al. 2004, Gunderson et al. 2008, Ruiz-de-Castañeda et al. 2012),

sean menores en las plumas melánicas que en las plumas pálidas no melanizadas. Esta quizás sea la razón de que las aves de plumaje oscuro sean relativamente más abundantes en hábitats húmedos, donde los bacilos degradadores de plumas son también más activos y abundantes (Burtt and Ichida 2004).

Distintas especies explotan hábitats asociados a distintos ambientes bacterianos y, por tanto, asociados a distintos niveles de riesgos de degradación de plumas por microorganismos. Por ello, el estado de degradación de las plumas de distintas especies, así como su resistencia a la degradación bacteriana, debería de ser distinto. Aunque la variación en la resistencia a la degradación de plumas por bacterias ha sido estudiada intra-específicamente en relación, por ejemplo, a la pigmentación de las plumas (Ruiz-de-Castañeda et al. 2012) y las características radioactivas del hábitat (Ruiz-Rodríguez et al. 2016), hasta donde sabemos, la variación inter-específica ha sido cuantificada sólo una vez en varias especies de loros (Burtt et al. 2011)). La variación en la degradación que presentan las plumas de distintas especies en una misma etapa del ciclo vital nunca ha sido cuantificada. Esta tesis intenta llenar ese hueco.

4. Variación intra-específica en plumas ornamentales. El caso del estornino negro

La resistencia al ataque por bacterias queratinolíticas y la degradación de las plumas varía incluso dentro del mismo individuo. Existen plumas más frágiles y más expuestas a microorganismos y a otros agentes degradadores y que, por tanto, son buenos candidatos a señalizar características antimicrobianas de los organismos (Shawkey et al. 2007, Ruiz-Rodríguez et al. 2015). Por ejemplo, las plumas ornamentales de la garganta de los machos de estornino (Ruiz-Rodríguez et al. 2015) presentan la parte apical alargada y estrecha (Fig. 1) (Lezana et al. 2000), y son mucho más largas que en las hembras (Hiraldo and

Herrera 1974, López-Rull et al. 2007a). Sabemos también que la degradación por bacterias queratinolíticas es mayor en estas plumas ornamentales que en otras no ornamentales de la misma zona (Ruiz-Rodríguez et al. 2015). Además, las propiedades antimicrobianas de la secreción uropigial están relacionadas negativamente con el nivel de degradación de las plumas ornamentales, pero no con el de las no ornamentales (Ruiz-Rodríguez et al. 2015). Estas plumas también pueden reflejar otras características de los portadores relacionadas con su calidad fenotípica y genética (Aparicio et al. 2001), por lo que la información que transmiten no se relaciona exclusivamente con capacidades antimicrobianas.

Existen evidencias de que plumas ornamentales de estorninos están sometidas a selección sexual, ya que su longitud se asocia con el éxito de emparejamiento, con los niveles de homocigosidad (Aparicio et al. 2001), y con los efectos inhibidores de la testosterona sobre la respuesta inmunitaria mediada por macrófagos (Gil and Culver 2011). Durante el cortejo, los estorninos machos exhiben sus llamativas plumas de la garganta mientras cantan posados en lugares muy visibles.

Conocidos el dimorfismo sexual que presentan estas plumas y su posible función como señal sexual, en esta tesis nos planteamos la posibilidad de que este ornamento informe sobre la calidad fenotípica y/o genética del individuo. Esta posibilidad la analizamos estudiando la asociación entre la longitud de las plumas y la longitud y acortamiento de los telómeros de los cromosomas, que son indicadores de la calidad genética y del nivel de estrés sufrido durante el crecimiento. También exploramos la relación entre estas plumas ornamentales y otro carácter sexual secundario de los estorninos como es el color del pico.

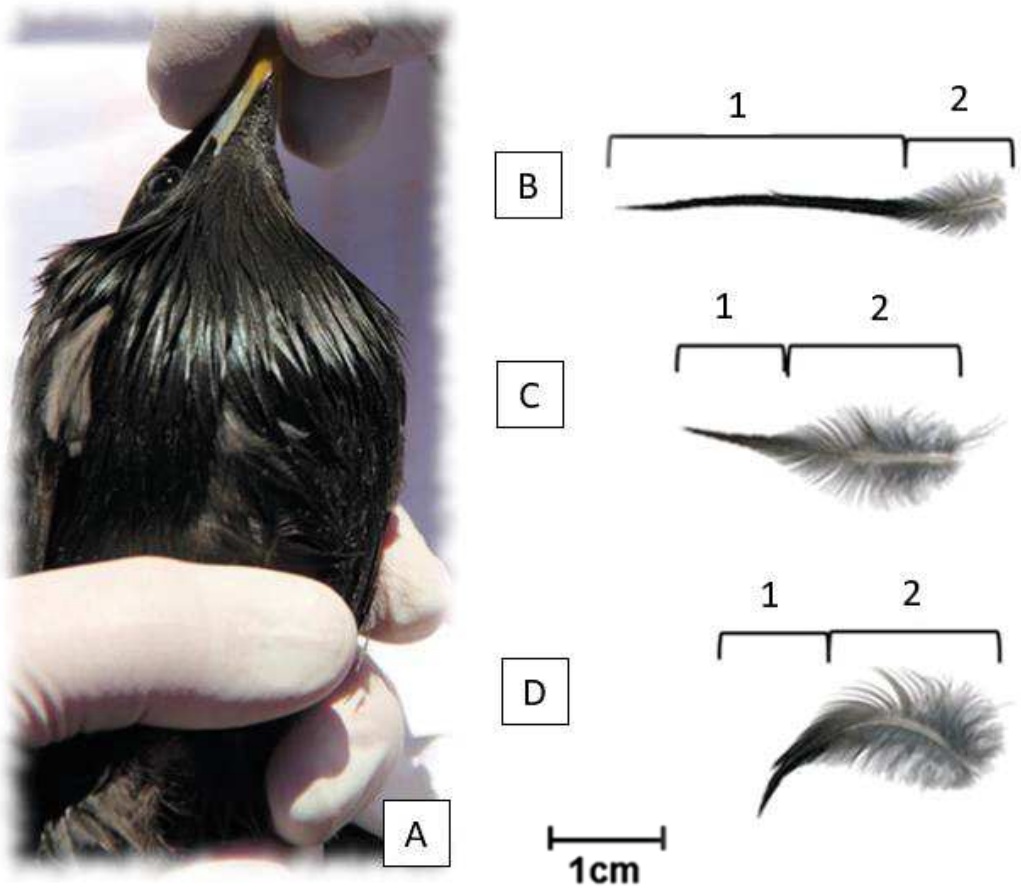

Figura 1: (A) Estornino macho capturado durante el período de emparejamiento con aparentes y llamativas plumas ornamentales en la garganta. (B) Plumas de la garganta ornamentales y (C) no ornamentales del macho. También se muestran (D) plumas de la garganta de la hembra. En la imagen se distingue entre la parte apical (1) y basal (2) de las plumas. (Foto y figura modificadas de: Ruiz-Rodríguez, M. et al. (2015)).

4.1 Relación de las plumas ornamentales con la dinámica y longitud de los telómeros

La longitud y dinámica de los telómeros son cualidades que informan de la calidad fenotípica y genética de los individuos y, también, del nivel de estrés soportado durante períodos de tiempo concretos (Bauch et al. 2013). Los telómeros son regiones de secuencias cortas y repetidas de ADN no codificante (TTAGGGG) que cubren el extremo de los cromosomas eucariotas. Entre otras funciones, protegen la integridad de la información genética durante la división

celular (Blackburn 1991). Debido a que los fallos de replicación ocurren frecuentemente al final de los cromosomas, los telómeros se acortan continuamente como consecuencia de la división celular (Boonekamp et al. 2017). Así, su longitud es un buen indicador de procesos de senescencia, tanto a nivel celular como del organismo (Suram et al. 2012, Campisi 2013), y de las perspectivas de supervivencia (Monaghan and Haussmann 2006, Kotrschal et al. 2007, Soler et al. 2015a, pero ver Van Lieshout et al. 2019). Además, la longitud y el desgaste de los telómeros, tienen componentes genéticos significativos (Dugdale and Richardson 2018), y están relacionados con las capacidades antioxidantes (Beaulieu et al. 2011, Badás et al. 2015, Kim and Velando 2015) y con el nivel de estrés sufrido durante el desarrollo (Boonekamp et al. 2014, Herborn et al. 2014), incluyendo el relacionado con infecciones por microorganismos (Asghar et al. 2015, Eisenberg et al. 2017, 2018). La longitud de los telómeros también se asocia con el éxito reproductor en aves silvestres (Boonekamp et al. 2014, Parolini et al. 2017, Bauer et al. 2018). Por lo tanto, poner de manifiesto relaciones entre ornamentos sexuales y la longitud y/o desgaste de los telómeros, indicaría que el ornamento puede informar sobre distintas capacidades (inmunitaria, antioxidativa, estrés, etc.), todas ellas indicadoras de la calidad fenotípica y/o genética del portador.

La asociación entre caracteres sexuales y longitud y dinámica de telómeros sólo ha sido explorada y detectada en tres especies de aves. En dos de ellas, la golondrina común (Parolini et al. 2017) y el cuelliamarillo común (*Geothlypis trichas*) (Taff and Freeman-Gallant 2017), el carácter estudiado fue la coloración del plumaje. En golondrinas, la longitud de los telómeros de machos y hembras se correlaciona con la coloración del plumaje ventral; un carácter sexualmente dimórfico en esta especie (Parolini et al. 2017). En el caso de los cuelliamarillos, la coloración de las plumas de sus baberos amarillos estaba relacionada con la longitud y tasa de desgaste entre años de sus telómeros, pero sólo en los machos (Taff and Freeman-Gallant 2017). El

tercer ejemplo se refiere a la coloración de los huevos de estorninos, que es un ornamento sexual de las hembras de esta especie (Soler et al. 2008b) y que predice la longitud de los telómeros de los polluelos (Soler et al. 2018). Todas estas evidencias son correlativas y, en esta tesis, damos un paso más y testamos experimentalmente las relaciones esperadas. Por otro lado, todos los estudios mencionados tratan sobre caracteres mediados por pigmentos. Nosotros utilizamos un carácter sexual no mediado por pigmentos; las plumas ornamentales de la garganta de los machos de estornino.

4.2 Relación entre la expresión de distintos ornamentos del estornino en un contexto de señales múltiples

Los machos típicamente poseen múltiples rasgos que pueden transmitir información a las hembras (Møller and Pomiankowski 1993). Aunque la mayor parte de la investigación sobre la evolución y función de las señales se ha centrado en rasgos únicos, en los últimos años se ha destacado la importancia de estudiar estos caracteres dentro de un marco teórico más realista que contemple la comunicación social como el resultado de múltiples señales (Candolin 2003, Hebets and Papaj 2005). Una señal puede proporcionar información sobre distintas características o capacidades del portador, y varias señales pueden informar sobre el mismo tipo de capacidades. Diferentes señales pueden, por ejemplo, implicar mensajes múltiples o redundantes, o pueden ser más eficientes en entornos particulares o en la estimulación de determinados canales sensoriales (Møller and Pomiankowski 1993, Candolin 2003, Hebets and Papaj 2005). Por tanto, en este marco teórico, los caracteres o señales individuales que conforman las señales múltiples o complejas no se consideran independientes, sino que su función sería el resultado de la interacción entre ellos. Hebets y Papaj (2005) desarrollaron un marco teórico sobre el funcionamiento de señales múltiples del que se desprendían hipótesis y predicciones comprobables sobre su evolución y funcionamiento.

Destacaron por ejemplo que, al considerar señales complejas o múltiples, la unidad de selección pasaría a ser el conjunto de señales.

Las investigaciones sobre señalización compleja se han centrado en explorar las asociaciones entre varias señales (Perrier et al. 2002, Bro-Jorgensen and Dabelsteen 2008, Mason et al. 2014, Chaine and Lyon 2015, Girard et al. 2015), o entre señales y diferentes aproximaciones de la calidad fenotípica incluyendo la condición física (Balmford et al. 1992, Martin and Lopez 2009), el éxito de apareamiento (Møller and Pomiankowski 1993) o la eficacia de la transmisión de la señal en diferentes entornos o ambientes (Endler and Houde 1995). La existencia de una asociación positiva entre dos ornamentos sugeriría que la información transmitida es redundante o complementaria, mientras que una asociación negativa podría indicar que, aunque puedan transmitir el mismo tipo de información, existe una compensación o compromiso entre los caracteres de señalización. Finalmente, la ausencia de asociación entre distintas señales sugeriría que transmiten información diferente a los receptores (Candolin 2003, Hebets and Papaj 2005).

El desarrollo o la expresión de una señal puede influir o interaccionar con el desarrollo de otro ornamento. Esto puede ocurrir por ejemplo cuando los rasgos implicados no se producen simultáneamente, y la exhibición del primero que se desarrolla influye en el fenotipo del segundo rasgo en desarrollarse (Johnstone 1996, Candolin 2003). Es posible que los beneficios asociados a la expresión de una señal, reduciendo por ejemplo interacciones sociales agonísticas (Senar and Camerino 1998, Morales and Velando 2018), faciliten el desarrollo de ornamentos que se expresen con posterioridad. Este podría ser el caso de ciertas características del plumaje de las aves que reducen los costos sociales antes de la reproducción (Senar et al. 2000), y que podrían potenciar la expresión de otros rasgos sexualmente seleccionados, como son el canto u otros ornamentos que se expresan exclusivamente durante la

reproducción (Badyaev et al. 2002, Mason et al. 2014). En este escenario, la expresión de los ornamentos desarrollados antes y durante la reproducción podrían estar positivamente relacionados. Aquí testamos esta hipótesis mediante el estudio de la relación entre dos caracteres sexuales del estornino, las plumas de la garganta y el color de la base del pico.

En este escenario complejo de pistas y señales proporcionando información a distintos tipos de receptores, y donde las bacterias juegan un papel clave determinando sus características y evolución, es en el que se encuadra la tesis doctoral. En ella nos planteamos los siguientes objetivos específicos que expongo a continuación.

OBJETIVOS ESPECÍFICOS

1- Explorar experimentalmente **el uso de pistas visuales** de ectoparasitismo en nidos de estornino por hembras parásitas de cría en la elección de nidos hospedadores (capítulo 1).
2- Explorar experimentalmente **el uso de pistas (químicas)** asociadas a la rotura de sacos fecales de pollos de estornino en sus nidos, por depredadores y parásitos (capítulo 2).
3- Comprobar la relación entre coloraciones de caracteres sexuales secundarios (pico, plumas y tarso) y diferentes rasgos relacionados con las **defensas antimicrobianas y éxito reproductor de hembras** (capítulo 3)**.**
4- Poner de manifiesto variaciones inter-específicas en las **características de las plumas** relativas a su **susceptibilidad a la degradación bacteriana** y otros agentes degradadores (capítulo 4).
5- Comprobar experimentalmente la **relación entre un carácter sexual** (la longitud de las plumas de la garganta de estorninos) **e indicadores de calidad fenotípica o genética** (la longitud y dinámica de los telómeros) (capítulo 5).
6- Comprobar experimentalmente la existencia de **asociaciones entre caracteres sexuales** (longitud de las plumas de la garganta y color del pico de machos de estornino) en un marco teórico de señales múltiples (capítulo 6).

Para llevar a cabo la mayoría de estos objetivos específicos, hemos realizado una serie de experimentos y aproximaciones correlativas en una población de estornino negro, una especie en la que, como ya se ha destacado,

sus machos presentan unas plumas alargadas en la garganta con las que podrían mostrar sus características genéticas (Aparicio et al. 2001) y la capacidad de defenderse de bacterias patógenas (Ruiz-Rodríguez et al. 2015). Los seis objetivos planteados los hemos desarrollado en sendos capítulos. A continuación, describo brevemente la importancia de cada uno de ellos en el marco teórico de esta tesis.

Capítulo 1: A la hora de elegir el nido a parasitar, las hembras parásitas de cría pueden tener en cuenta las características de sus hospedadores potenciales que se relacionen con el ambiente en el que crecerá su descendencia, en términos de, por ejemplo, mejor territorio y menor probabilidad de ectoparasitismo (Paasivaara et al. 2010, Tomás et al. 2017). Además, el grado de ectoparasitismo se ha relacionado con modificaciones en la carga bacteriana del nido (Tomás et al. 2018). En este capítulo, abordamos la posibilidad de que las hembras parásitas utilicen las motas de los huevos, producidas por la actividad de los ectoparásitos, como pista para seleccionar a su hospedador en base al ambiente en el que se desarrollarán sus pollos; una hipótesis propuesta recientemente. Para ello, manipulamos las motas de los huevos y exploramos la frecuencia de parasitismo en nidos en los que los huevos estaban sin motas o con motas experimentales de distintos colores.

Capítulo 2: El ambiente microbiano del nido en el que crecen las aves durante los primeros días de vida también puede afectar a su desarrollo (Potti et al. 2002) y, por lo tanto, la eliminación de los sacos fecales por los padres puede ser una estrategia para disminuir la contaminación bacteriana, o evitar las pistas producidas por sustancias químicas presentes en las heces y que pudieran atraer a depredadores y/o ectoparásitos (Herrick 1900, Petit et al. 1989). En este capítulo, comprobamos esta hipótesis mediante la rotura experimental de sacos fecales dentro del nido. Este experimento puede provocar un aumento de la densidad bacteriana en los nidos que podría afectar negativamente al crecimiento de los pollos.

Capítulo 3: En este capítulo exploramos la posibilidad de que ornamentos femeninos se asocien con capacidades inmunitarias. Hemos estudiado (i) la fluctuación de la inversión en defensas antimicrobianas a lo largo de la temporada de cría, (ii) la relación entre la inversión en defensas y el éxito reproductor, y (iii) la posible relación de la capacidad individual para defenderse de infecciones con coloraciones del cuerpo.

Capítulo 4: En este capítulo cuantificamos la variación inter-específica en el deterioro de las plumas y su resistencia a la degradación por bacterias queratinolíticas en 16 especies de aves. También cuantificamos la carga bacteriana de los nidos como indicativo de las presiones selectivas ejercidas en cada especie, debido por ejemplo a los distintos hábitats que ocupan. Detectar la existencia de variaciones inter-específicas en las características de las plumas se podría interpretar como una evidencia del papel de los microorganismos degradadores de plumas en la evolución de estas estructuras aviares. Además, encontrar una asociación entre la carga bacteriana de los nidos y el grado de degradación y de resistencia de las plumas, también apoyaría esta posibilidad.

Capítulo 5: En este capítulo estudiamos la asociación entre la longitud y dinámica de los telómeros (como indicadores de calidad fenotípica/genética y de esperanza de vida) y la longitud de las plumas de los estorninos macho; un carácter sexual de la especie. Además, manipulamos la longitud de estas plumas y exploramos los efectos en la tasa de reducción de telómeros un año más tarde. Nuestras predicciones son que la longitud original de las plumas de la garganta (antes del experimento) de los machos, pero no la de plumas de las hembras, debería relacionarse positivamente con el éxito reproductor, y negativamente con la fecha de apareamiento y la tasa de deterioro de los telómeros. Además, la reducción experimental de la longitud de la pluma de la garganta debería resultar en un menor éxito reproductor, fechas de

reproducción más tardías, y mayores tasas de deterioro de los telómeros en los individuos experimentales.

Capítulo 6: En este capítulo exploramos experimentalmente la asociación entre dos caracteres sexuales, la longitud de las plumas de la garganta y el color azulado de la base del pico de los machos. Si el acortamiento de las plumas aumenta las interacciones agonísticas sociales antes de la reproducción, la energía y los recursos disponibles para desarrollar la coloración sexual del pico durante la reproducción serán menores para estos machos. Sin embargo, si el experimento no aumenta los costos sociales, los machos experimentales podrían compensar la pérdida de una señal sexual aumentando la expresión de la otra.

MATERIAL Y MÉTODOS

1. Especie de estudio: El estornino negro

El estornino negro es un ave semicolonial perteneciente a la familia *Sturnidae*, del orden de los *Passeriformes*. Esta especie de tamaño medio (longitud pico-cola = 21-23 cm, envergadura alar = 38-42 cm) es endémica de la región mediterránea occidental. Los estorninos negros son sexualmente dimórficos. Los machos, por ejemplo, son más pesados que las hembras (media macho = 93.4 g, media hembra = 88.5g) (Cramp 1998, Veiga and Polo 2016), presentan un plumaje más brillante, y además, la coloración de la base del pico en la época reproductiva es azulada, mientras que las hembras presentan un tono rosáceo (Fig. 2) (Veiga and Polo 2016). La coloración amarilla del pico se relaciona con la concentración de carotenoides y vitamina A en el plasma sanguíneo (Navarro et al. 2010). La diferenciación sexual más llamativa la encontramos en la longitud y forma de las plumas ornamentales de la garganta (más largas y estrechas en la zona apical en los machos que en las hembras, ver Fig. 1) (Ruiz-Rodríguez et al. 2015, Veiga and Polo 2016). El macho, durante la época de cortejo, extiende estas plumas y las exhibe parado en lugares visibles mientras canta (Fig. 2C) (Ruiz-Rodríguez et al. 2015). La muda post-reproducción en esta especie comienza a finales de junio y se completa entre mediados de septiembre y mediados de octubre (Veiga and Polo 2016). El huevo típico de la especie es de color azul verdoso inmaculado (Cramp 1998), con un pequeño pico en la longitud de onda ultravioleta (Soler et al. 2008b).

En nuestra población de estudio el cortejo se produce durante los meses de febrero-marzo, y la temporada reproductiva comienza a principios de abril. La mayoría de los individuos hacen una segunda puesta durante el mes

de mayo. El tamaño de puesta más común es de 4-5 huevos. La fase de incubación suele comenzar con el penúltimo huevo, lo que hace que el último polluelo eclosione más tarde que los demás (Veiga and Polo 2016). La incubación es realizada principalmente por hembras con la ayuda esporádica de machos, y dura 14 días. El período de estancia de los pollos en el nido es 21-22 días (Cramp 1998). Tanto el macho como la hembra contribuyen a la alimentación y a la extracción de los sacos fecales de los pollos (Cramp 1998). Durante todas las etapas de reproducción, los adultos llevan plumas y plantas aromáticas al nido, las cuales han demostrado tener funciones antimicrobianas beneficiosas (Ruiz-Castellano et al. 2016, Soler et al. 2017). El parasitismo de cría entre conespecíficos es un fenómeno común en esta especie que ocurre en alrededor del 25 % de los nidos (Calvo et al. 2000).

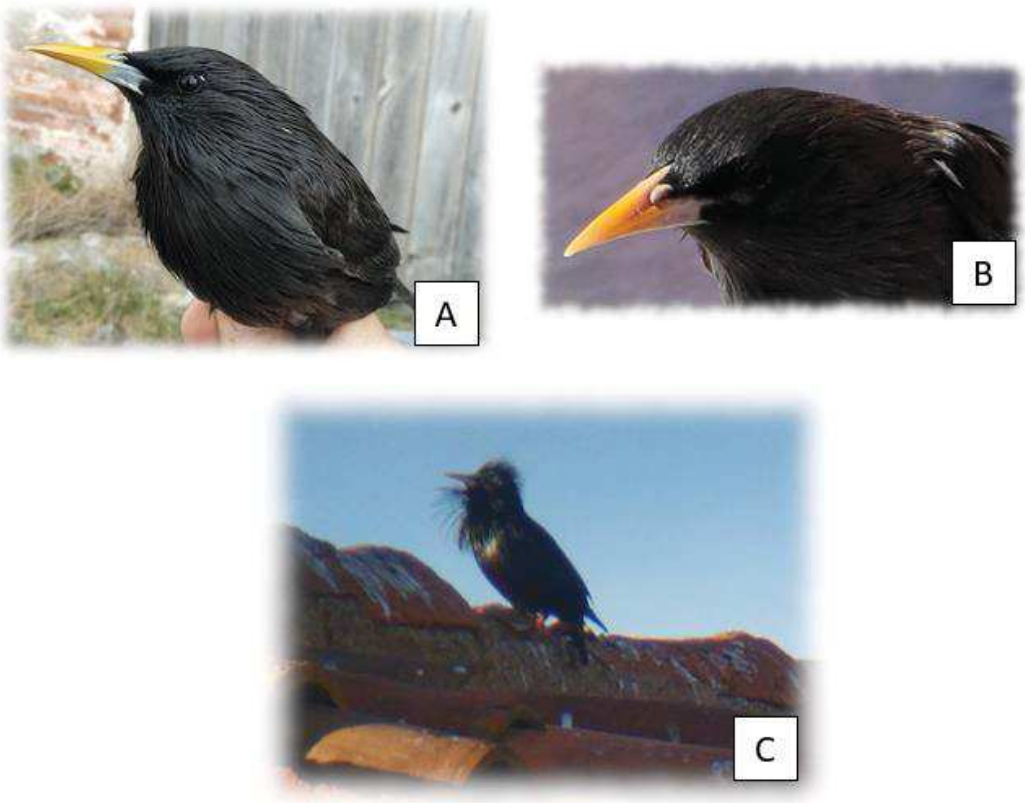

Figura 2: Coloración del pico en la época reproductiva de (A) machos y (B) hembras de estornino. También se muestra a (C) a un macho exhibiendo las plumas de la garganta (fotos realizadas por distintos miembros de nuestro grupo durante el trabajo de campo).

2. *Área de estudio*

El trabajo de campo se realizó entre los años 2015 y 2017 durante la época reproductiva del estornino en la "Hoya de Guadix", al sureste de España; una meseta de aproximadamente 1000 m de altitud con clima semiárido y precipitaciones anuales inferiores a 300 L/m^2 (Pérez-Contreras et al. 2008). Nuestra población de estudio se encuentra en las antiguas estaciones de ferrocarril de La Calahorra (37°15' N, 3°01' W) y de Huéneja (37°13' N, 2°56' W), separadas entre sí 20 km, y donde hay 84 y 24 cajas-nido instaladas respectivamente desde 2005 (altura interna x ancho x profundidad = 350 x 180 x 210 mm, altura desde el fondo al agujero de entrada = 240 mm). Estas cajas-nido se fijan a los troncos de los árboles o paredes de construcciones, a 2-4 m sobre el suelo y, en su mayoría, las ocupan estorninos negros (Fig. 3). El éxito reproductor del estornino en el área de estudio varía típicamente entre años y puestas y, entre otros factores, depende de la probabilidad de depredación. Los depredadores más comunes de los nidos de estorninos en nuestra área de estudio son las ratas (*Rattus rattus*), las serpientes de escalera (*Rhinechis scalaris*), los mochuelos comunes (*Athene noctua*) y los lagartos ocelados (*Timon lepidus*).

3. *Trabajo de campo*

3.1 Captura de adultos y mediciones generales

Durante el cortejo, algunas aves duermen dentro de las cajas-nido, hecho que aprovechamos para realizar sesiones de captura de los adultos entre febrero y mediados de marzo. Una hora antes del amanecer, cerrábamos la entrada de todas las cajas-nido con esferas de espuma densa, e inmediatamente después del amanecer, cogíamos a todos los individuos que se encontraban en el interior. Las aves capturadas se mantuvieron individualmente en bolsas de

algodón limpias. Las bolsas las manteníamos colgadas de un palo para mantener tranquilas a las aves. Al finalizar la toma de datos de cada individuo se le liberaba, de modo que el tiempo máximo que un estornino capturado estuvo en la bolsa nunca excedió las tres horas. También realizábamos intentos de captura de los adultos durante la reproducción (4-5 días después de la fecha de eclosión) colocando trampas en el interior del nido que cerraban la puerta de la caja una vez el ave entraba. El tiempo máximo que manteníamos la trampa en la caja nido era de una hora. Todos los adultos capturados (machos y hembras) fueron anillados con una anilla metálica numerada (si no estaban ya anillados) y con una combinación única de anillas de tres colores. Se pesaron con una balanza (Pesola 0-300 g, precisión 2 g), y la longitud de su tarso, pico y ala se midió con un calibre digital (precisión 0,01 mm) y una regla (precisión 1 mm), respectivamente. Además, tomábamos las medidas de altura, anchura y longitud de la glándula uropigial (capítulo 3) para estimar su volumen (Martín-Vivaldi et al. 2009). La secreción de la glándula uropigial (capítulo 3) se extrajo colocando un micro-capilar estéril (32mm/20µl) en la abertura de la glándula, y presionándola ligeramente para estimular la salida de la secreción. La longitud del capilar con secreción se utilizó como indicador del volumen de secreción producida. Posteriormente, se recogieron muestras de sangre pichando con una aguja estéril la vena braquial y recogiendo la sangre en dos o tres capilares heparinizados (75mm^3/75µl). Los capilares se vaciaron en microtubos de 1.5 cm^3 que se mantuvieron en neveras portátiles a unos 4 °C y, una vez en el laboratorio (en un plazo máximo de 6 horas), se centrifugaron (5 min a 14000 rpm) para separar el plasma de la fracción celular. El plasma se introdujo en microtubos de 1.5 cm^3, y se conservó a -80 °C hasta su análisis (capítulo 3 y 5). En el campo, también medimos la longitud de las plumas de la garganta de machos y hembras con una regla. Estas medidas las realizamos tres veces para estimar la repetibilidad (capítulos 5 y 6). Además, en el campo, también se midió el color de la base (capítulo 6) y la punta del pico, las patas, y las plumas del dorso y el pecho (capítulo 3) con un

espectrómetro Ocean Optics S2000 conectado a una luz halógena de deuterio (D2-W, Mini) (ver a continuación).

Figura 3: Área de estudio en la Hoya de Guadix: (A) Edificio principal y (B) arboles con cajas-nido de la Estación de La Calahorra y (C) Estación de Huéneja (algunos ejemplos de cajas nido señalados con flechas rojas).

3.2 Medidas del color

Para estandarizar las condiciones de luz ambiental, las medidas se tomaban en un edificio cercano, o en una tienda de campaña. Además, el sujeto a medir (huevos o adultos) se colocaba dentro de una bolsa de tela opaca-negra, que también envolvía la punta de la fibra óptica. Antes de la medición de cada individuo, calibramos el espectrómetro utilizando una referencia estándar en blanco y negro. Se obtuvieron espectros de reflectancia a intervalos de 1 nm en el intervalo de longitud de onda 300-700 nm. El color se midió tres veces en cada zona y se obtuvieron valores medios. A continuación, para la base del pico (capítulo 3 y 6), estimamos la proporción de reflectancia total dentro del

rango de la luz azul (λ = 400-475 nm, color al que tiende la base del pico del macho, en adelante intensidad del color azul) e hicimos lo mismo con el rango de croma amarillo-rojo del espectro (λ = 570-700 nm, en adelante intensidad de color amarillo-rojo). Este rango coincide con el típico aumento gradual de la forma espectral del color de las feomelaninas (Navarro et al. 2010), y en el que hay una gran variación en los valores de reflectancia de machos y hembras en la base del pico. Para las demás áreas (capítulo 3), además de estos dos rangos, estimamos la proporción de reflectancia para el ultravioleta (UV) (λ = 300-400 nm), UV-azul (λ = 300-450 nm) y azul-verde (λ = 450-570 nm) de los espectros visuales tetracromáticos de las aves. Obtuvimos estas variables mediante el software Avicol V.6 (Gomez 2006). Antes de realizar todos los análisis, se corrigieron los valores negativos de reflectancia, fijándose a cero, y el ruido de las curvas de reflectancia se corrigió siguiendo el método de alisamiento triangular (Gomez 2006).

3.3 Experimento de acortamiento de plumas ornamentales

Manipulamos la longitud de las plumas de la garganta de algunos machos capturados antes de la reproducción cortándolas, y dejando otros individuos sin cortar como control. El tratamiento se asignó alternativamente según el orden de captura. Se recortó aproximadamente el 50 % (en torno a 1.5 cm) de la porción distal de las plumas de la garganta con unas tijeras (Fig. 4) antes de la reproducción. Posteriormente, exploramos los efectos experimentales sobre el acortamiento de los telómeros (capítulo 5) y el color (croma y brillo) de la base del pico durante la reproducción, un año después del experimento (capítulo 6). Las plumas de la garganta se midieron antes y después del experimento.

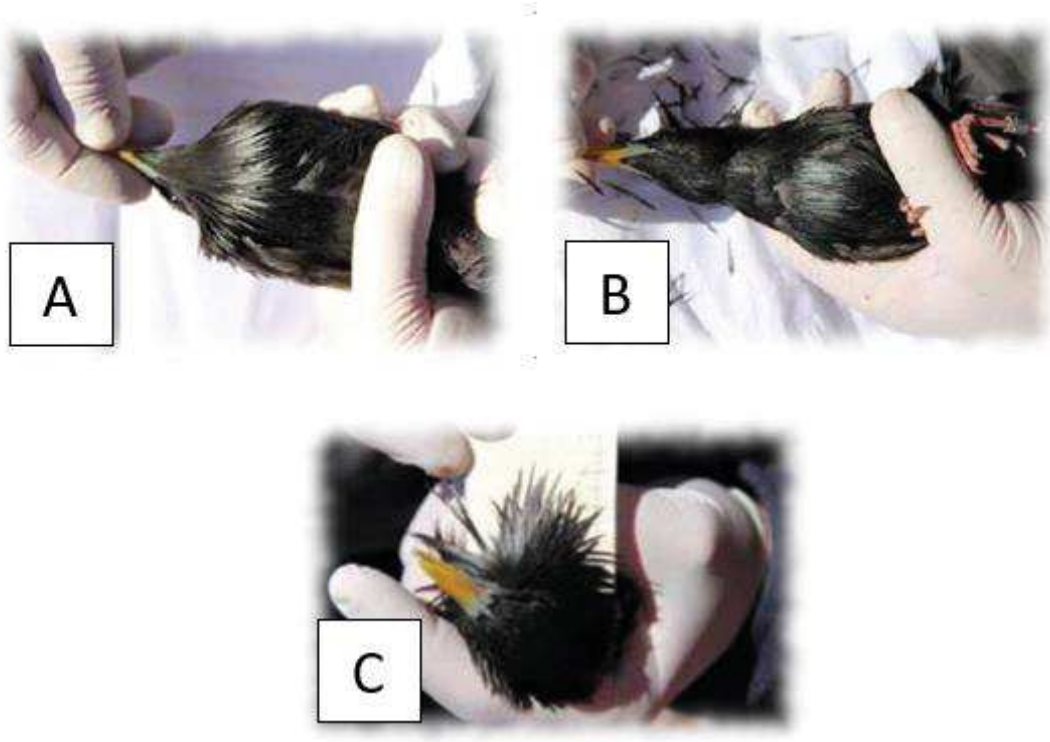

Figura 4: Experimento de acortamiento de plumas de la garganta en machos de estornino negro. Aspecto de un individuo control (A), otro experimental (B), y estimación de la longitud de las plumas de la garganta (C) (fotos realizadas por distintos miembros de nuestro grupo durante el trabajo de campo)

3.4 Seguimiento de los nidos y mediciones de pollos

Durante los tres años de estudio, los nidos se comenzaron a visitar a finales de marzo, cada tres días, hasta que detectamos el primer huevo. Exceptuando el año del experimento de parasitismo (capítulo 1, ver más adelante), el nido se visitaba doce días después y, en adelante, cada día hasta la eclosión del primer huevo (día 1 del período de pollos). Dividiendo el número de pollos eclosionados final entre el número de huevos de la puesta, calculamos el éxito de eclosión del nido. Las siguientes visitas eran los días 5, 8, 11 y 14 del período de pollos. En cada visita se anotaba el número de pollos que había, se les medía el tarso y el ala, y se les pesaba con una balanza electrónica (precisión de 0.01 g) los días 5 y 8, y con un dinamómetro los días 11 y 14. El día 8 todos los pollos fueron anillados con anillas metálicas, y el día 14 (cuando los pollos

estaban cercanos a volar) estimamos la abundancia de dípteros ectoparásitos del género *Carnus* (mayoritariamente *Carnus hemapterus*) (capítulo 2). Utilizamos una escala semicuantitativa en función de la densidad de mordeduras y restos de sangre y heces detectados en las alas y el vientre de cada uno de los pollos del nido (ver capítulo dos para detalles de la escala).

Durante la estación reproductora de los años 2015 y 2016 realizamos grabaciones de 1 hora de las entradas de las cajas-nido, 2 o 3 días después de la eclosión del primer huevo. Estas grabaciones las utilizamos para identificar a los adultos con ceba que entraban a las distintas cajas. Las cámaras se situaban a 3-5 metros de la caja nido a grabar.

3.5 Experimento de selección de hospedador en un contexto de parasitismo de cría intra-especifico

En el año 2016 realizamos un experimento para testar la hipótesis de que las hembras de estornino parásitas de cría utilicen las motas de las cáscaras de los huevos (ver Introducción, Fig. 5A) como pistas sobre el nivel de ectoparasitismo de los nidos. Para ello, manipulamos las motas de los huevos en los nidos, y exploramos el efecto sobre la probabilidad de que el nido fuera parasitado por otra hembra. Al detectar el primer huevo en un nido, se contaban el número de motas procedentes de la actividad parasitaria de *Carnus* (López-Rull et al. 2007b). También se medían la longitud (L) y anchura (A) de los huevos con un calibre para estimar su superficie según la fórmula superficie = (3.155 − 0.0136L + 0.0115A)LA (Narushin 2005). Después, todas las motas se eliminaban de los huevos (Fig. 5B) limpiándolos suavemente con un hisopo estéril (Sterile R, Nuova Aptaca S.R.L.) ligeramente humedecido con tampón fosfato (0,2 M; pH = 7,2). Posteriormente, dependiendo del tratamiento aleatorio asignado al nido, se pintaron 40-50 motas (i) de color marrón-rojizas (similar a la coloración de las motas naturales producidas por *Carnus*) (Fig.

5C) o (ii) turquesa-azuladas (un color similar al de las cáscaras de los huevos de estornino muy pigmentadas) (Fig. 5D). Para pintar las motas utilizamos rotuladores resistentes al agua y de punta fina (Letraset® Promarker, marrón-ámbar R646 y azul C247 respectivamente). En un tercer grupo de nidos, (iii) no se pintaron manchas en las cáscaras de los huevos. Las cajas nido las volvíamos a visitar cada dos días hasta el final de la puesta. En cada visita se contaban las manchas naturales producidas por *Carnus* en todos los huevos y se aplicaban los tratamientos experimentales a los nuevos huevos encontrados, repasando los anteriores si era necesario. Una vez terminada la puesta, los nidos fueron visitados cada 4 días hasta la eclosión. Los nidos se visitaban aproximadamente a la misma hora y, dado que los estorninos ponen un huevo por día (Calvo et al. 2000), al encontrar más de 2 huevos nuevos en visitas sucesivas durante el período de puesta, se pudo inferir la existencia de un evento de parasitismo de cría, lo que en todos los casos se confirmaba visualmente al comparar la apariencia (color y/o tamaño) de los huevos individuales dentro de la nidada (Fig. 6). La combinación de estos dos métodos para detectar eventos de parasitismo de cría de conespecíficos es precisa y fiable en esta especie (Lyon and Eadie 2017, Tomás et al. 2017).

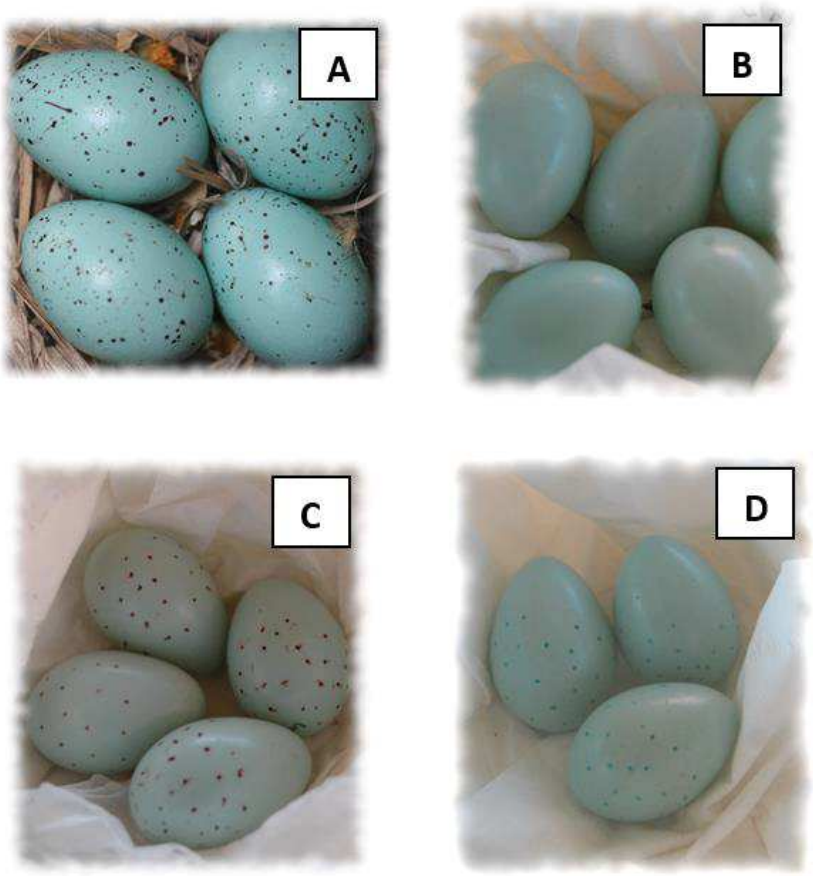

Figura 5: Fotos de (A) huevos de estornino con motas naturales (con un alto nivel de infestación, foto de Gustavo Tomás), y de puestas experimentales bajo los tres tratamientos de pintado de motas: (B) limpios de motas naturales, y limpios y pintados con motas (C) marrones (imitando las manchas de *Carnus*), o (D) azules (semejante a la cascara del huevo).

Figura 6: Ejemplo de nido parasitado donde se puede distinguir visualmente el huevo parásito.

3.6 Experimento de rotura de sacos fecales en los nidos

Con el objetivo de comprobar los efectos que tendría sobre los pollos la no retirada de sacos fecales del nido, realizamos un experimento que simulaba el fallo en la retirada de algunos sacos por parte de los padres (capítulo 2). El experimento se realizó en todos los nidos en el año 2015 y en una pequeña muestra en el año 2016 (ver más adelante). El experimento comenzó el día 2 del período de pollos. Brevemente, utilizando guantes de látex nuevos y lavados con etanol al 70 % para evitar la contaminación bacteriana entre nidos, se introducían todos los pollos en una bolsa de algodón. Después, con un hisopo estéril (Sterile R, Nuova Aptaca S.R.L.) ligeramente humedecido con tampón fosfato de sodio estéril (0,2 M; pH = 7,2), muestreábamos la comunidad bacteriana del cuenco del nido durante 10 segundos. El hisopo se introducía en un tubo Eppendorf con 1,2 ml de tampón, que se conservaba a 4-6 °C hasta su procesamiento en el laboratorio en las siguientes 24 horas. Después, a los pollos se les estimulaba la cloaca para que expulsaran los sacos fecales. Estos se rompían y se mezclaban en un recipiente de plástico y, con la ayuda de una pequeña cuchara, se esparcía la mezcla correspondiente a aproximadamente tres sacos fecales en los cuencos de los nidos experimentales (la mitad de los nidos), asegurándonos de no dejar piezas grandes que pudieran ser fácilmente retiradas por los padres. En la otra mitad de los nidos (grupo control), el material también se tocó de la misma manera con una cucharita limpia. Este proceso se repitió los días 5, 8, 11 y 12. El experimento se realizó tanto en las primeras como en las segundas puestas.

3.7 Estudio de la variabilidad inter-específica en la resistencia de las plumas

En los años 2015 y 2016, se recogieron plumas de pollos de 16 especies de aves para estimar su deterioro y su resistencia a la degradación por bacterias queratinolíticas. También se muestrearon con hisopos los nidos para estimar la

carga bacteriana por métodos tradicionales de cultivo en los siguientes 4 días a la recogida de la muestra (ver metodología en la descripción del experimento anterior y en el capítulo 4). Los muestreos se realizaron en la Hoya de Guadix y en el bosque de Valsaín (Segovia, 40º 54' N, 04º 01' W), en el centro de España, zona descrita en González-Braojos et al. (2017). Con ayuda de tijeras previamente lavadas en etanol, recolectamos dos o tres plumas (coberteras de segundas y terceras secundarias) de al menos dos pollos de cada nido al final de la etapa de crecimiento (en la Tabla 1 se exponen los tamaños de muestras conseguidos). Dependiendo de la especie, recolectamos plumas con partes pigmentadas y no pigmentadas. Las plumas de cada pollo se mantuvieron en bolsas de plástico nuevas con cremallera y se almacenaron en el laboratorio a 4 °C hasta su análisis.

Tabla 1: Especies y tamaños de muestra tomadas para la estimación del deterioro y el grado de resistencia a la degradación bacteriana (degradabilidad) de las plumas, y de carga bacteriana del nido.

	N = Nidos (plumas)		N = Nidos	N = Nidos
	Degradación		Degradabilidad	Carga Bacteriana
	Melanizadas	No Melanizadas		
Athene noctua	13	13	9	13
Clamator glandarius	9	9	15	9
Coracias garrulus	6	6		6
Corvus monedula	6		6	6
Cyanistes caeruleus	5	5	10	5
Ficedula hypoleuca	23	25	10	24
Hirundo rustica	3	0		3
Oenanthe leucura	3	0		3
Otus scops	12	12	5	11
Parus major	19	18	18	17
Passer domesticus	8	8		8
Petronia petronia		3	4	3
Pica pica	17	17	15	17
Pyrrhocorax pyrrhocorax	5		11	5
Sturnus unicolor	5		9	5
Upupa epops	26	26	8	26

4. Trabajo de laboratorio

4.1 Estudio de la carga bacteriana de los nidos

Los tubos con los hisopos de muestras de los nidos (capítulos 2 y 4) se agitaron vigorosamente en un vortex (Boeco V1 Plus) durante 10 segundos y, posteriormente, se sembraron 100 µl en platas de Petri mediante la técnica de *siembra en superficie* en cuatro medios sólidos diferentes (Peralta-Sánchez et al. 2010): Tryptic Soy Agar (TSA), un medio generalista en el que crecen bacterias mesófilas, y tres medios selectivos: Kenner Fecal Agar (KF) para el cultivo de bacterias pertenecientes al género *Enterococcus*; Vogel-Johnsson Agar (VJ) para bacterias del género *Staphylococcus*; y Hecktoen Enteric Agar (HK) para bacterias Gram-negativas de la familia *Enterobacteriaceae*. Se hicieron diluciones seriadas de las muestras originales de hasta 10^{-4} en TSA, y 10^{-2} en el resto. La densidad bacteriana de los nidos se expresó como el número de unidades formadoras de colonias (UFC) por ml (Nº colonias * $10^{\text{factor de dilución}}$) / 100 ml de dispersión).

4.2 Estimación de la capacidad del plasma para inhibir bacterias

Se realizaron pruebas de antagonismo del plasma frente a 12 bacterias indicadoras (capítulo 3): *Bacillus licheniformis, Bacillus megatherium, Bacillus thuringensis, Enterococcus faecalis, Enterococcus faecium, Lactobacilus paracasei, Latobacillus plantarum, Lactococcus lactis, Listeria inocua, Listeria monocytogenes, Proteus sp. y Staphylococcus aureus*. Para preparar las placas de antagonismo, se agregaron 100 µL de un cultivo de 12 horas de cada bacteria indicadora a 15 ml de un medio tamponado estéril (1,8 % de Brain Heart Infusion (BHI) y 0,8 % de agar, en tampón fosfato 0,1 M pH 7). Después, se inocularon 2 ml de cada muestra de plasma en las placas solidificadas con la bacteria indicadora, y se incubaron durante 12 h a 28 °C.

A continuación, se revisaron las placas para detectar halos de inhibición (Fig. 7), es decir, zonas transparentes alrededor del plasma en las que se inhibió el crecimiento de la bacteria indicadora. Los halos se midieron en mm, desde el límite de la gota de plasma hasta el final del halo (es decir, donde comienza el crecimiento de las bacterias indicadoras). Con esas medidas se calculó un índice de actividad antagónica como la media de la actividad (es decir, el diámetro del halo) mostrada frente total de bacterias testadas.

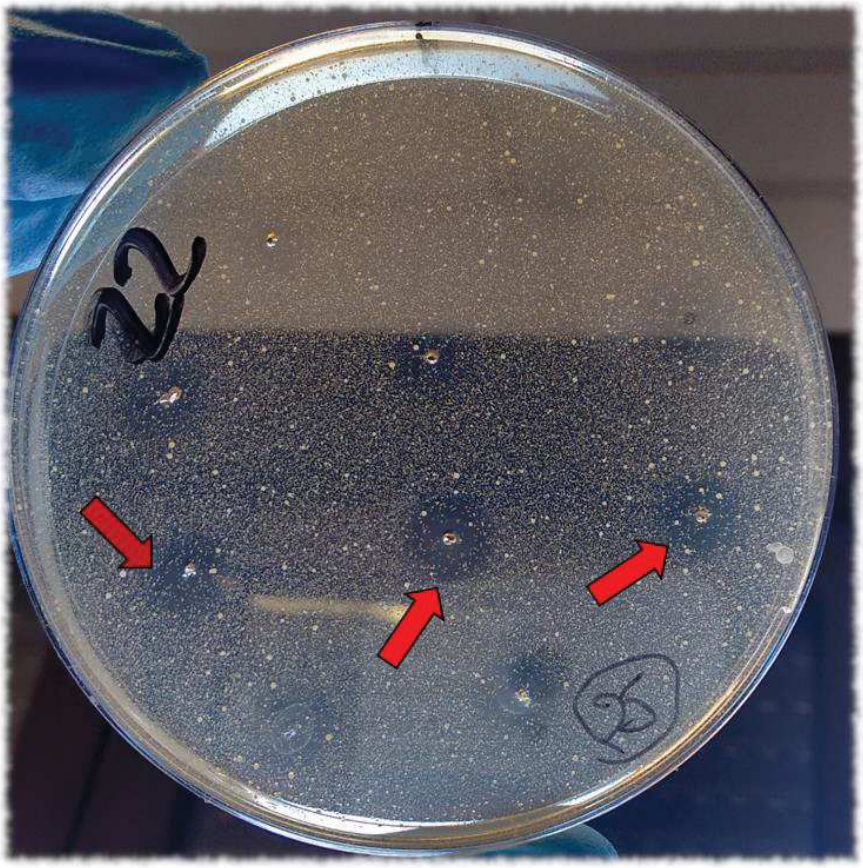

Figura 7: Ejemplo de halos mostrando el grado de antagonismo del plasma frente a un tipo bacteriano. Algunos ejemplos de halos de antagonismo señalados en rojo.

4.3 Evaluación de la capacidad de respuesta inmune innata

La respuesta inmune mediada por anticuerpos naturales (capítulo 3) fue estimada en dos placas de ensayo de poliestireno de 96 pocillos siguiendo el procedimiento descrito en Matson, Ricklefs y Klasing (2005) (Fig. 8). La

cuantificación de la capacidad de lisis y aglutinación se hace considerando el número del pocillo con la última dilución plasmática en la que se observa la reacción de lisis o aglutinación del plasma que estamos testando frente a la sangre de conejo (Matson et al. 2005). La lisis refleja la acción del sistema del complemento por la cantidad de hemoglobina que liberan los eritrocitos de la sangre de conejo, mientras que la aglutinación refleja la interacción entre los anticuerpos naturales y el antígeno, que en este caso es la sangre de conejo (Matson et al. 2005).

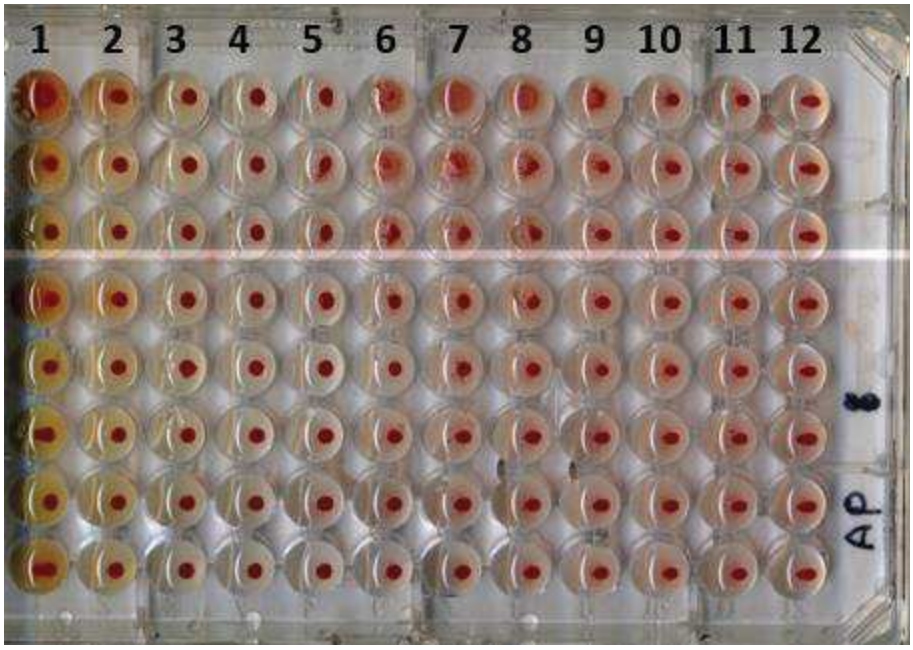

Figura 8: Placa de poliestireno en la que se muestra la reacción de lisis y aglutinación de muestras de plasma. En la primera fila, el pocillo 1 muestra el proceso de lisis, y del 2 al 5 la forma más compacta de la aglutinación. Los pocillos del 6 al 9 muestran una forma menos compacta de aglutinación, en el 10 y 11 no hay lisis ni aglutinación. La última columna es un control. Por lo tanto, en este ejemplo las estimas de respuesta inmune en la fila 1 tendrían valores de 1 para lisis y 9 para aglutinación.

4.4 Susceptibilidad de las plumas a la degradación

La susceptibilidad de las plumas a la degradación por bacterias queratinolíticas se exploró utilizando plumas completas y siguiendo el protocolo descrito en (Gunderson et al. 2008, Ruiz-Rodríguez et al. 2009, Ruiz-de-Castañeda et al.

2012, Ruiz-Rodríguez et al. 2015) (capítulo 4). Las plumas se pesaron en una balanza de precisión (AB135-5/FACT (0.000001 g), Mettler Toledo, España) y se esterilizaron en autoclave antes de comenzar el experimento de laboratorio. Cada pluma se introdujo en un tubo de vidrio esterilizado que contenía 4 mL de tampón (9,34 mM de NH4Cl, 8,55 mM de NaCl, 1,72 mM de K_2HPO_4, 2,92 mM de KH_2PO_4, 0,49 mM de $MgCl_2$-$6H_2O$ y 0,01 % de extracto de levadura en 100 mL de agua destilada). Además, se introdujo una colonia de *Bacillus licheniformis* previamente aislada en placas TSA en cada tubo. Se incubaron en agitación constante a 37 °C y 120 rpm en un agitador orbital (VWR, España). Se recolectó 1 mL de cada tubo antes de comenzar el experimento y al final de la incubación, 21 días después. Estas muestras se centrifugaron para eliminar las células bacterianas, y la absorbancia del sobrenadante se estimó utilizando un espectrofotómetro (Helios Zeta UV-Vis, Thermo Scientific, Reino Unido) a 230 nm (Goldstein et al. 2004). La concentración de oligopéptidos en el sobrenadante proviene principalmente de la degradación de la queratina y, por lo tanto, está directamente relacionada con la cantidad de pluma degradada. Las curvas de calibración de absorbancia y concentración de oligopéptidos (de 0 a 300 μg x mL^1) se obtuvieron mediante el uso de albúmina de suero bovino (BSA) (R^2 = 0,98 en ambas curvas), lo que permitió extrapolar las absorbancias a los valores de concentración de oligopéptidos. También utilizamos tubos controles de tres tipos: (i) solo con tampón, (ii) tampón + bacilo y (iii) tampón + una pluma para estandarizar la concentración de oligopéptidos en tubos experimentales con respecto a la observada en estos tubos controles.

4.5 Estima del nivel de degradación de las plumas

El grado de desgaste que presentaban las plumas se estimó siguiendo el protocolo descrito en Ruiz-Rodríguez et al. (2015) (capítulo 4). Las plumas se

fotografiaron (2x) con una cámara (Nikon Digital Sight DS Fi1) conectada a una lente binocular (Nikon SMZ1500, Melville, NY, USA). Dos técnicos de laboratorio, que desconocían el origen de las plumas (es decir, especie, parte del cuerpo, etc.), evaluaron todas las fotografías, según el nivel de degradación en una escala semicuantitativa (desde 0 (sin degradación) a 3 (más de 2/3 de la pluma dañada)) (Ruiz-Rodríguez et al. 2015). Las estimas de los dos técnicos fueron repetibles. Por lo tanto, se utilizaron los valores promedios de desgaste de plumas del mismo nido en los análisis.

4.6 Estudio de la longitud y dinámica de los telómeros

La longitud y dinámica de los telómeros como indicadores de la calidad individual (capítulo 5) se estimaron con material genético de células sanguíneas, siguiendo el protocolo de Criscuolo et al. (2009). La concentración de ADN se ajustó a 20 ng μL^{-1} con agua destilada y se congeló a -20 ºC hasta los análisis. La longitud relativa de los telómeros fue estimada por q-PCR y el acortamiento se calculó restando el valor de longitud de las muestras de la última captura al de la primera. Estas diferencias se estandarizaron dividiendo el acortamiento de los telómeros por la longitud de los mismos en muestras de las primeras capturas.

5. Análisis estadísticos

Para el análisis de los datos hemos utilizado modelos lineales generales (GLM), asumiendo distribuciones normales, o binomiales (modelos generalizados, GLZ). Hemos tenido en cuenta la estructura y el nivel de réplica estadísticamente independiente de los datos incluyendo el nido o el pollo como factores aleatorios en los modelos, o utilizando ANOVAs de medidas repetidas. Además, en el caso de que existiera un factor fijo dentro del aleatorio

(i.e. pollos experimentales y controles dentro del mismo nido), hemos incluido la interacción entre el factor aleatorio y el fijo como otro factor aleatorio en los modelos. En el caso de estar interesados en los efectos fijos sobre varias variables dependientes, utilizamos MANOVAs con modelos similares a los descritos arriba. En todos los modelos, hemos incluido variables para controlar los efectos explorados por otras variables que pudieran estar afectando tanto a la variable dependiente como a la independiente. El software que hemos utilizado en la mayoría de los análisis fue el Statistica V13 (Dell-Inc. 2015), y en alguna ocasión también utilizamos R software v3.2.5 (R Core Development Team 2016). En los distintos capítulos se puede encontrar una explicación detallada de los modelos utilizados en cada uno de los test estadísticos.

CAPÍTULOS

1. **Spotless starlings prefer spotless eggs: conspecific brood parasites cue on eggshell spottiness to avoid nests infested by ectoparasites.** Azcárate-García M., Díaz-Lora S., Tomás G. & Soler J. J. *Journal of Animal Ecology* (submitted)

2. **Experimentally broken faecal sacs affect nest bacterial environment, development and survival of spotless starling nestlings.** Azcárate-García M., Ruiz-Rodríguez M., Díaz-Lora S., Ruiz-Castellano C. & Soler J. J. *Journal of Avian Biology* 50(3) (2019).
doi: https://doi.org/10.1111/jav.02044

3. **The antimicrobial capacity of spotless starling (*Sturnus unicolor*) females is related to their reproductive success and tegument colouration.** Ruiz-Rodríguez M., Azcárate-García M., Ruiz-Castellano C., Tomás G., Díaz-Lora S. & Soler J. J. *Journal of Avian Biology* (submitted)

4. **Interspecific variation in deterioration and degradability of avian feathers: The evolutionary role of microorganisms.** Azcárate-García M., González-Braojos S., Díaz-Lora S., Ruiz-Rodríguez M., Martin-Vivaldi M., Martínez-Bueno M., Moreno J. & Soler J. J. *Journal of Avian Biology* (second revision)

5. **Length of ornamental throat feathers predicts telomere dynamic and hatching success in spotless starling (*Sturnus unicolor*) males.** Azcárate-García M., Ruiz-Rodríguez M., Díaz-Lora S., Ruiz-Castellano C., Martín-Vivaldi M., Figuerola J., Martínez-de la Puente J., Tomás G., Pérez-Contreras T. & Soler J. J. *Frontiers in Ecology and Evolution* (second revision)

6. **Beak colouration of starling (*Sturnus unicolor*) males depends on the length of their throat feathers.** Azcárate-García M., Ruiz-Rodríguez M., Ruiz-Castellano C., Díaz-Lora S., Tomás G., Martín-Vivaldi M. & Soler J. J. *Behavioral Ecology* (first revision)

CAPÍTULO 1

Spotless starlings prefer spotless eggs: conspecific brood parasites cue on eggshell spottiness to avoid nests infested by ectoparasites

Abstract

Avian brood parasites are expected to select host nests according to characteristics that maximize offspring fitness, such as probability of ectoparasitism. Spotless starlings (*Sturnus unicolor*) lay immaculate blue eggs that sometimes become brownish-spotted due to the activity of the ectoparasitic fly *Carnus hemapterus* on incubating birds. Therefore, parasitic females should adaptively avoid for parasitism nests with spotted eggshells. Here, we manipulated perceived risk of ectoparasitism by painting the eggs with either brown (similar to those due to ectoparasite activity), or blue spots. A third group of nests were maintained with immaculate eggshells. Nests with non-spotted eggshells showed the highest rate of brood parasitism, while nests with brownish or bluish spots on the eggshells were parasitized at a similar lower rate. These results suggest that brood parasitic females use achromatic information of the eggshells in their selection of host nests. This study adds to the scarce evidence showing that brood parasitic birds select host nests with low risk of ectoparasitism, and is pioneering demonstrating that colour patterns of the eggshell (i.e. spottiness) of their potential hosts represent a major cue employed to appraise risk of ectoparasitism.

Keywords: Eggshell spottiness, *Sturnus unicolor*, Intraspecific nest parasitism, Host-selection mechanisms, *Carnus hemapterus*, Ectoparasitism.

Introduction

Avian brood parasitism is a reproductive strategy in which brood parasitic females lay their eggs in the nests of other females of the same (conspecific) or different (interspecific) species (Soler 2017a). Parasitic individuals take advantage of parental effort of their hosts and save most of the reproductive costs, but instead have to overcome antiparasitic defences of their hosts as well as to find and choose suitable host nests (Rothstein 1990, Davies 2000, Lyon and Eadie 2008, Soler 2017b). Because parasites should choose hosts with characteristics that maximize their fitness, brood parasitism is expected to occur non-randomly within the host populations. Therefore, identifying host characteristics that increase reproductive success of parasites is pivotal to understanding the evolution of conspecific (Lyon and Eadie 2008, Tomás et al. 2017) and interspecific brood parasitism (Parejo and Avilés 2007, Soler et al. 2009, Soler et al. 2014).

Conspecific brood parasitism is widespread in birds (Yom-Tov and Geffen 2017). Although it has received much less attention than interspecific brood parasitism (Lyon and Eadie 2008), there is sparse evidence suggesting that it occurs non-randomly within the available host nests (Pöysä et al. 2014). Brood parasitic females might for instance choose host nests located in territories of better quality, which can be inferred by nest-site characteristics, and that will result in higher resource availability for their offspring (Paasivaara et al. 2010). They can also choose nests with reduced risk of predation by cuing on several anti-predatory characteristics of nests (Pöysä and Pesonen 2007, Pöysä et al. 2010, Soler et al. 2014). Moreover, they might also choose nests with smaller number of eggs, which might indicate that incubation has not yet started, favouring a better match with the host egg laying period, and thus a proper incubation of their eggs (Odell and Eadie 2010). Parasitic females might also cue on sexually selected traits indicating parental quality of potential hosts, and choose host nests for parasitism accordingly

(Soler et al. 1995, McLaren and Sealy 2003, Parejo and Avilés 2007). It has also been suggested that brood parasitic females could avoid for parasitism those nests with higher abundance of nest dwelling ectoparasites (Brown and Brown 1991), for which there is recent experimental evidence (Tomás et al. 2017). Most of this evidence is however correlational, and experimental manipulation of host nest features is necessary to further understand the non-random choice of conspecific host nests for parasitism (Pöysä et al. 2014).

Nest dwelling ectoparasites are hematophagous arthropods that cause anaemia in their avian hosts (O'Brien et al. 2001, Hannam 2006, Minias 2015), which might pose drastic negative effects to fledging success (Brown and Brown 1991, Richner et al. 1993). Moreover, ectoparasite activity will also increase nutrient availability that enhance bacterial growth in the avian nests and on the eggshells (Tomás et al. 2018), which will negatively affect egg hatchability (Peralta-Sánchez et al. 2018) and nestling development (Soler et al. 2017, Azcárate-García et al. 2019). Thus, recognising and avoiding nests with high risk of ectoparasitism would be of clear selective advantage for brood parasitic females. In accordance with the existence of this kind of bias, conspecific brood parasitism by cliff swallows (*Petrochelidon pyrrhonota*) was more common in nests with a low probability of infestation by fleas and bugs (Brown and Brown 1991). Furthermore, an experimental increase of *Carnus hemapterus* flies in nests of spotless starlings (*Sturnus unicolor*) reduced probability of conspecific brood parasitism (Tomás et al. 2017). How brood parasitic individuals could manage to infer ectoparasitism status remains however unclear. The first, most direct possibility is that parasitic individuals directly evaluate abundance of nest-dwelling ectoparasites when inside the nest cavity. Another possible mechanism includes the evaluation of physical condition of nest owners, which may reflect their parasitism status. Brood parasitic females could also use other indirect cues of ectoparasite presence, such as conspicuous spots on the eggshells, which are ectoparasite faeces and

blood remains derived from ectoparasite activity on incubating adults, and appear frequently in nests parasitized by different ectoparasite taxa (Tomás et al. 2017). Interestingly, these cues will inform brood parasitic females not only on the abundance of ectoparasites (López-Rull et al. 2007, Avilés et al. 2009, Tomás et al. 2017), but also on the incubation stage of the host nests, because these cues of blood sucking activity do not appear until incubation has started (Tomás et al. 2017). Since avoiding nests where incubation has already started is of prime importance for brood parasites (Wiley 1988, Kattan 1997, Soler et al. 2015, Tomás et al. 2017), usage of these cues of ectoparasitism would imply a two-fold advantage and, thus, should be rapidly selected within the brood parasitic strategy. Here, we tested this possibility by experimentally painting conspicuous spots on the eggshells of spotless starlings, simulating those derived by *Carnus hemapterus* (hereafter *Carnus*) activity (López-Rull et al. 2007), and exploring its effects on probability of brood parasitism.

Carnus is a blood-sucking ectoparasite of nestling (and incubating adult) birds (Grimaldi 1997), commonly found in nests of spotless starlings in their adult form a few days before full incubation starts (López-Rull et al. 2007). The abundance of flies in a nest at the nestling stage can be anticipated few days after the onset of incubation by the density of spots on the eggshells (López-Rull et al. 2007). Interestingly, we know that nests of spotless starlings where eggshells were cleaned from *Carnus*' spots deserved more paternal investment (in terms of feeding visits) during the nestling phase (Avilés et al. 2009). This experimental result suggests that spotless starlings are able to evaluate such parasitic cue and, thus, open the possibility that brood parasitic individuals use eggshell spottiness in their selection of host nests. In accordance, Tomás and coauthors (Tomás et al. 2017) showed that experimental parasitism with *Carnus* flies, which increased the abundance of spots on the eggshells, decreased the probability of brood parasitism. However, these results could also be explained if brood-parasitic females followed other

related cues such as the direct detection of abundant ectoparasites or associated changes in the nest environment or in the behaviour of potential host birds (Tomás et al. 2017, 2018). Thus, to further demonstrating that brood parasitic females use eggshell colour patterns (i.e. *Carnus*-provoked spots on the eggshells) to choosing host nests for parasitism, we experimentally manipulated eggshell spottiness and explored its effects on probability of brood parasitism. Briefly, we cleaned spots from all the eggs in the nests and painted their eggshells with spots of two different colours in different groups of nests: (i) reddish-brown (hereafter brown), simulating colouration of *Carnus*-spots, and (ii) bluish-turquoise (hereafter blue), a colour similar to that of highly chromatic spotless starling eggshells (see Fig 1). In a third group of nests, (iii) we cleaned the eggs from *Carnus*-spots, but did not paint spots on the eggshells. If brood parasitic females use *Carnus*-spots on the eggshells as cues to decide where to lay their eggs, we expected that they avoid the nests with experimental brown spots on their eggs simulating a high rate of ectoparasitism.

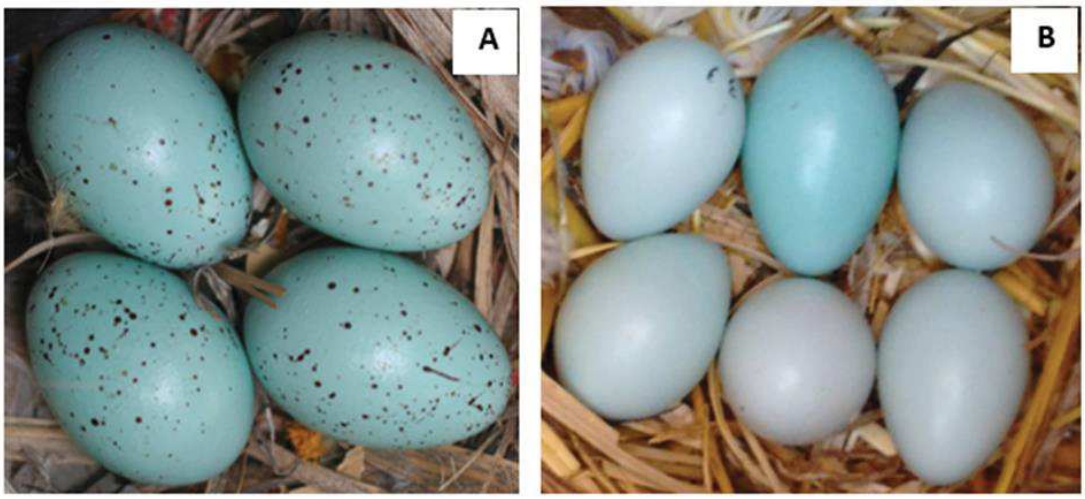

Fig. 1: Appearance of spotless starling clutches with natural spots due to *Carnus* ectoparasitism (A), and with a larger and more intensely coloured parasitic egg (B).

Material and Methods

Study area and study species

The study was conducted during the year 2016 in a south-eastern region of Spain (Hoya de Guadix, 37º15'N, 3º01'W). Our two study areas are located at La Calahorra (37º15' N, 3º01' W) and Huéneja (37º13' N, 2º56' W) railway stations, where nest-boxes attached to tree trunks or walls at 3-4 m above ground are available for starlings to breed in (for further information on the study area see previous work by Soler and coauthors (Soler et al. 2017)).

The spotless starling is a medium-sized, hole-nesting passerine that mostly breed in colonies (Cramp 1998, Veiga and Polo 2016). It lays immaculate blue-greenish eggs (Cramp 1998) with a small peak at the ultraviolet wavelength (Soler et al. 2008). In this species, conspecific brood parasitism is a common phenomenon occurring in around 25% of nests (Calvo et al. 2000). Overall, 17% of nests are parasitized during the host's egg-laying stage and 9% during incubation, with the latter representing 32–53% of total parasitism depending on the year (Calvo et al. 2000).

In the studied populations, the reproductive season starts in early April, and most individuals lay a second clutch during May-June. The most common clutch size is 4-5 eggs. The incubation phase usually begins with the penultimate egg, which promotes that the last egg hatches one day after the others (Soler et al. 2008, Veiga and Polo 2016). Incubation is mostly carried out by females with sporadic help from males, and extends for around 14 days, while the nestling period lasts 18-25 days (Cramp 1998). Soon after the onset of incubation, immaculate blue eggs sometimes become brownish spotted as a result of the viscous faeces (and blood remains) derived from *Carnus* adults feeding on incubating females (López-Rull et al. 2007, Avilés et al. 2009; Fig. 1, Tomás et al. 2017). *Carnus* is a 2 mm blood-sucking fly found in nests of an extremely wide diversity of birds. It has been found parasitizing 64 host species

from 24 avian families (Grimaldi 1997, Brake 2011, Tomás et al. 2017). After emergence from overwintering pupae inside nests, winged adults may disperse, and lose their wings once a suitable nest is found (Grimaldi 1997). *Carnus* flies are hardly visible within the nest materials but easily detected on the nestling skin (Tomás et al. 2017, Azcárate-García personal observation). Infestation at the incubation stage can therefore be appraised by prospecting parasitic birds almost exclusively from egg spottiness (López-Rull et al. 2007).

Fieldwork and experimental procedure

The experimental design aims at detecting if starlings use eggshell colour patterns, including cues of *Carnus* parasitism, to select the nests where to lay their parasitic eggs. With this purpose, from the end of March, we visited the nest-boxes every 3-4 days until starling eggs were detected. Once a nest was detected with eggs (in all cases it was between first and third egg laying day), it was sequentially assigned to one of the three experimental treatments We first counted the number of natural spots of each egg and, then, measured egg length and breadth with a digital calliper (to the nearest 0.01 mm) to estimate eggshell surface following Narushin's formula (Narushin 2005, $(3.155 - 0.0136L + 0.0115B)LB$). Afterwards, we cleaned all eggs by gently swabbing their shells with a sterile swab (Sterile R, Nuova Aptaca S.R.L.) slightly wet with phosphate buffer (0.2 M; pH = 7.2). Then, we painted 40-50 (i) brown spots in each egg simulating a high *Carnus* infestation level (López-Rull et al. 2007), or (ii) blue spots, with waterproof markers (Letraset® Promarker, brown-amber R646 and blue C247 respectively). Blue is a colouration close to that of heavily pigmented starling eggshells (Soler et al. 2008) and, thus, spots of this colour will contrast with the colouration of the eggshells, but at a lower degree than the brown spots. In a third group of nests, (iii) eggs were not painted after cleaning. All the eggs were numbered on the thick pole, which

allowed identification in subsequent visits. After the first visit, nests were checked every second day until the end of egg laying, i.e., five or six days after the first egg was laid. During each visit, we counted the number of natural Carnus-spots to calculate natural eggshell spottiness as the number of spots / cm2 of eggshell surface, and within-nest mean eggshell spottiness per egg was used in the analyses. Experimental treatments were applied to any new eggs found (and to previous eggs if necessary). From then on, nests were visited every 4 days until egg hatching and natural eggshell spottiness recorded on each visit. Data on natural eggshell spottiness from the different visits after experimental manipulation were averaged for each nest. Natural eggshell spottiness before, as well as after experimental manipulation were reduced (mean (SE) values of density of spots per cm^2; before: 0.15 (0.009); after: 0.31 (0.013)), and similar in nests under the different experimental treatments, either in first and in second breeding attempts (all $P > 0.12$). Each nest was visited at a similar time of the day and, as starlings lay one egg per day (Calvo et al. 2000), the occurrence of brood parasitism events was detected when more than two new eggs were found between successive visits. Events of brood parasitism were also visually confirmed by comparing the appearance (colour and/or size) of individual eggs within the clutch (see Fig. 1). Combination of these two methods for detecting events of conspecific brood parasitism is accurate and reliable in this species (Lyon and Eadie 2017, Tomás et al. 2017).

Sample size and statistical analyses

We followed 142 starling nests (74 first and 68 second clutches). One hundred and thirty of those nests were located at La Calahorra and 12 nests at Huéneja area. Moreover experimental treatments were balanced within first and second clutches (first clutches: 26 brown / 25 blue / 23 unspotted; second clutches: 23

/ 22 / 23) and within study areas (La Calahorra: 44 / 44 / 42; Huéneja: 5 / 3 / 4) (Pearson $\chi^2 < 0.56$, df = 5, $P > 0.98$).

We were interested in detecting possible bias in host selection and, thus, we considered each parasitic egg as an independent event of parasitism. As nests available for parasitism, we considered those nests that were in their laying or early incubation stage (i.e. 1 – 8 days after the first egg was laid) at the time of parasitism in the area where a target parasitic egg was detected.. With this data set, possible bias in host nest choice for brood parasitism in relation to experimental treatment was analysed by means of Generalized Linear Models (GLZ) with binomial distribution and logit link function. Information on whether or not a parasitic egg was laid in each of the available nests was used as the dependent binomial variable, and experimental treatment as the independent factor. Study area, breeding attempt (i.e. first or second clutch), and whether parasitism occurred in nests when incubation had (i.e. from the fifth to the eighth day after the first egg was laid) or had not (from the first to the fourth day of laying) started (hereafter incubation stage), were considered additional discrete independent factors. Natural eggshell spottiness, both before, and after experimental manipulation, were considered additional continuous independent variables. Because of the relatively low number of brood parasitism events detected, and the impossibility of testing interactions between the several additional independent factors considered, the effects of each of these factors on probability of parasitism were tested in separate models that also included experimental treatments. The effects of the interactions between independent factors were also explored to test the possibility that expected effects of experimental treatment depended on the study area, incubation stage, natural eggshell spottiness or breeding attempt. The main effects were estimated in models that did not include the interaction, while the effect of the interactions was estimated in models that also included the main effects. Probabilities were estimated by means of type 3 likelihood

ratio tests after correcting for overdispersion. All analyses were performed in Statistica V13 (Dell-Inc. 2015).

Results

We detected 23 parasitic eggs in 18 out of the 142 (12.38 %) monitored starling nests. Most (88.9%, N = 18) of the parasitized clutches received a single parasitic egg, while the other two parasitized nests received three and four parasitic eggs.

In accordance with the hypothesis tested, experimental treatment affected probability of parasitism (Table 1). Brood parasitic females preferentially selected nests with eggs that were experimentally cleaned but were not painted (unspotted vs blue spots, $\chi^2_1 = 4.87$, P = 0.027; unspotted vs brown spots, $\chi^2_1 = 5.93$, P = 0.015), while nests containing eggs with painted spots, either blue or brown, were parasitized at similar lower rates ($\chi^2_1 = 0.07$, P = 0.786) (Fig. 2). The experimental effect was detected even after controlling for statistically significant effects of study area and incubation stage (higher parasitism rate in nests when incubation had started), and the non-significant effects of natural eggshell spottiness and breeding attempt (Table 1). Moreover, the interactions between experimental treatment with incubation stage, natural eggshell spottiness, and study area did not reach statistical significance (Table 1), suggesting that the detected experimental effects do not depend on these additional independent factors. However, the interaction between experimental treatment and breeding attempt reached statistical significance (Table 1), suggesting that the experimental effects were more evident in first than in second clutches.

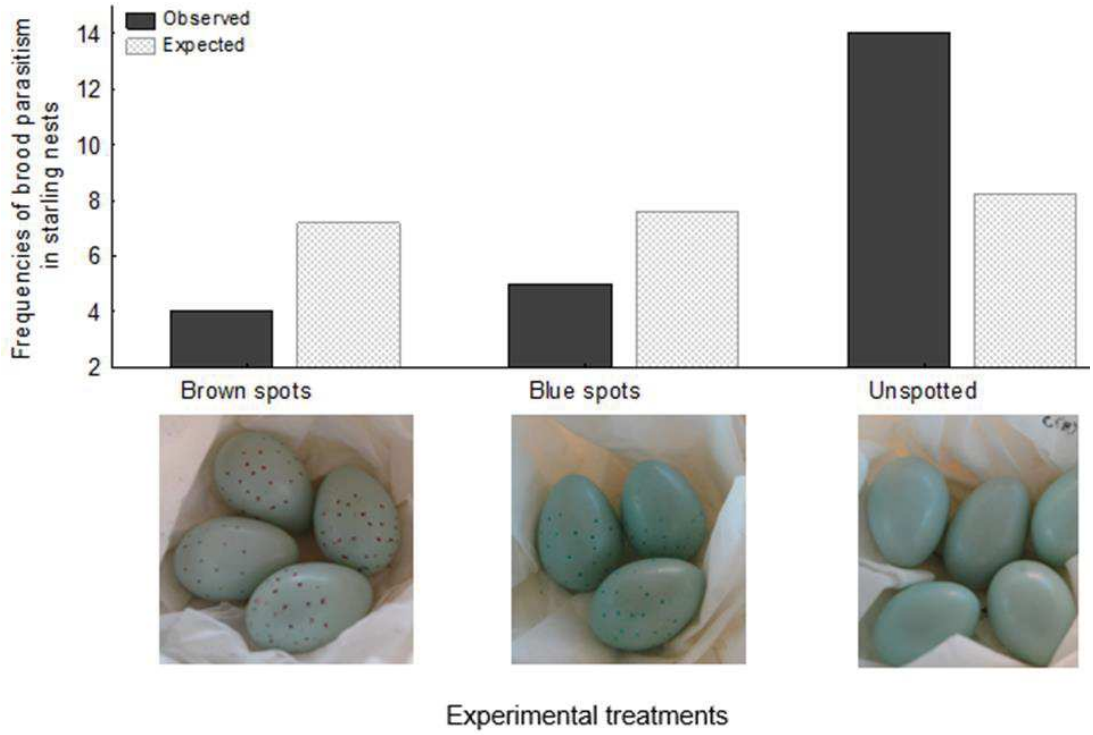

Fig. 2: Expected and observed frequencies of brood parasitism in nests of spotless starlings under different experimental treatments (with painted brown or bluie spots, or unspotted on the eggshells). Expected frequencies of brood parasitism were estimated considering nests available for parasitism at the date when each of the 23 events of brood parasitism occurred, and assuming no preference for any experimental treatment. Representative clutches for each experimental treatment are shown.

Discussion

By painting spots on spotless starling eggshells, simulating cues of ectoparasitism by *Carnus* flies, we explored the effects of the presence of such cues on the probability of conspecific brood parasitism. Our study revealed that nests without spotted eggshells showed the highest rate of brood parasitism, even after controlling for the effects of study area, natural eggshell spottiness, incubation stage, and breeding attempt. These results therefore confirm that brood parasitic females select host nests with low risk of ectoparasitism, and convincingly show that risk of ectoparasitism is appraised through an indirect cue of ectoparasite infestation such as eggshell spottiness.

Table 1: Rates (%) of brood parasitism in nests of spotless starlings under different experimental treatments, as number of parasitism events in relation to the sum of the number of nests (in parenthesis) that were available at the date of each event of parasitism. Results from Generalized Linear Models testing the effect of experimental treatment and of a second additional independent factor (study area, incubation stage, breeding attempt, or pre- and post-experimental natural eggshell spottiness), as well as the interactions between the two factors included in each model, are also shown.P-values lower than 0.05 are in bold.

Additional independent factors	Frequencies of parasitism (%)				Statistical tests					
	Experimental treatments			Parasitism events	Experimental treatment		Additional independent factor		Interaction	
	Brown spots	Blue spots	Unspotted	(N)	χ^2_2	P	χ^2_1	P	χ^2_1	P
Study area					6.94	**0.031**	4.11	**0.043**	2.09	0.148
La Calahorra	3.85 (N = 104)	3.60 (N = 111)	10.37 (N = 106)	19						
Huéneja	0.00 (N = 6)	25.0 (N = 4)	37.5 (N = 8)	4						
Incubation stage					6.36	**0.042**	6.52	**0.011**	0.27	0.873
Before full incubation	1.61 (N = 62)	2.99 (N = 67)	5.56 (N = 54)	6						
After full incubation	6.25 (N = 48)	6.25 (N = 48)	18.3 (N = 60)	17						
Breeding attempt					7.56	**0.023**	0.07	0.789	5.22	**0.022**
First	0.00 (N = 39)	4.76 (N = 42)	16.67 (N = 42)	9						
Second	5.63 (N = 71)	4.11 (N = 73)	9.72 (N = 72)	14						
Pre-experimental natural spottiness				23	8.11	**0.017**	2.07	0.151	0.91	0.635
Post-experimental natural spottiness				23	8.54	**0.014**	2.69	0.101	1.46	0.480
N_{Total}	110	115	114	23						

Ectoparasites produce severe negative effects in developing nestlings (Soler et al. 1999, Martín-Vivaldi et al. 2006, e.g. Avilés et al. 2009, Hoi et al. 2010, Hoi et al. 2018). Thus, interpreting cues that reliably anticipate intensity of parasitism before the nestling phase would be of selective advantage. This would be the case, not only for brood parasitic females (Tomás et al. 2017), but also for adoptive parents, which have the opportunity to adjust reproductive investment to perceived risk of ectoparasitism (O'Brien and Dawson 2005, Avilés et al. 2009). It might also be advantageous for eavesdropping females in general that visit nests of neighbours to collect public information about nest environmental conditions (Parejo et al. 2008). Consequently, recognizing and assessing eggshell spottiness as a cue of ectoparasitism in decision taking by starlings will evolve more rapidly than expected in the case of advantages exclusively related to brood parasitism.

Our experiment included two treatments consisting on painting spots of two different colours on eggshells: one of brown colouration, simulating spots derived from the parasitic activity of *Carnus* flies, and the other of blue colour. Given that blue is close to the colouration of highly chromatic starling eggs (Soler et al. 2008), this latter treatment was conceived to explore the importance of spot colouration. However, parasitism rates in nests under brown or bluish-turquoise experimental treatments did not differ significantly, while parasitism rates in nests under these two treatments were significantly lower than parasitism rate in nests with eggshells experimentally cleaned. These results allow to infer that chromatic colouration of eggshell spots is not very important for brood parasitic females. Examples of the importance of achromatic colouration determining functionality of coloured signals are not rare in the literature (e.g. Soler et al. 2005, Avilés et al. 2008, Soler and Avilés 2010) and, thus, it is possible that starling females mainly used achromatic information of eggshell spots when selecting conspecific nests for brood parasitism. Further experimental work is necessary to confirm this inference.

The detected experimental effects were significantly larger in first than in second clutches. This interaction mainly arose because none of the nests with brown spots on the eggshells was parasitized in first clutches, when brood parasitism concentrated in nests with cleaned eggshells. This result cannot be explained by differences in availability of nests for brood parasitism since it was higher by the time of second clutches, neither by differences in the rate of parasitism between first and second clutches, which were quite similar. Availability of nest-boxes for building nests can neither be a reason explaining the detected interaction since vacant nest-boxes were available in the study area during the entire breeding season. The reasons we envisage as responsible for the detected interaction between experimental treatment and breeding attempt are related to possible variations in antiparasitic defences of starlings (nest defences) or in the genotype (individual identity) of brood parasitic individuals during first and second clutches. Exploring such interesting possibilities deserves future investigations.

One of the advantages that brood parasitic females would enjoy by avoiding nests with traces of *Carnus* flies activity is that they would elude those nests where incubation has already started (López-Rull et al. 2007, Tomás et al. 2017), and where hatching and fledging success for parasitic offspring may consequently be reduced (Wiley 1988, Kattan 1997, Soler et al. 2015). In fact, brood parasitism in spotless starlings occurs more frequently in nests before the start of incubation (Calvo et al. 2000). However, we found that brood parasitic females preferentially parasitized experimental nests after the start of incubation. These apparently contradictory results may be related to the fact that we manipulated all available nests in our study area. For every studied nest, we cleaned all eggshells at the time of egg laying from stains and spots due to parasitic activity by *Carnus* flies and, thus, brood parasitic females might have considered experimental nests with no painted spots as those where incubation had not started. However, this *a posteriori* explanation would

predict no differences in parasitism rate of nests that had or had not started incubation and, thus, cannot fully explain the detected preference. To properly discussing this result, we would need an additional group of nests with non-manipulated eggshells (i.e. not cleaned) that at present is lacking. We here focused on detecting whether brood parasitic females assess eggshell spotiness to choose nests for brood parasitism and, accordingly, designed an experiment to disentangle the effect of eggshell spottiness from that of ectoparasite infestation. We knew that brood parasitism rate is relatively low in starling nests (Calvo et al. 2000, Tomás et al. 2017) and, trying to obtain enough number of parasitized nests that allowed statistical comparisons between experimental treatments, we did not consider natural nests with and without spots derived from *Carnus* activity. Preferential parasitism of nests after the onset of incubation may arise as a consequence of time constraints for parasite females to detect nests where egg laying has started (parasite eggs laid before host egg laying are ejected from nests in this (Eens and Pinxten 1999) and related starling species (Stouffer et al. 1987, Pinxten et al. 1991, Yamaguchi 1997)). Moreover, it may represent the outcome of a trade-off between matching host egg laying stage and waiting to gather more information on host nest quality (in terms of, for instance, ectoparasitism risk (Tomás et al. 2017)). Thus, additional research is necessary to explore the causes underlying the interesting, but somehow counterintuitive, detected preferential brood parasitism of starling nests that have started incubation.

To conclude, we have confirmed that parasitic starlings avoid host nests infested by ectoparasites (Tomás et al. 2017). Moreover, we have demonstrated experimentally, and independently of the presence of ectoparasites, that the cue used to infer ectoparasitism risk is the presence of spots on the eggshells of potential host nests. Interestingly, results suggest that they mainly use achromatic information of eggshell spottiness. To our knowledge, this is the first experimental demonstration supporting the

hypothesis that brood parasitic females use colour patterns of the eggshell (i.e. spottiness) of their potential hosts to choose the nests where to lay their parasitic eggs. The great and diverse advantages of choosing nests without spots due to activity of ectoparasites, and the widespread parasitism by *Carnus* flies in dozens species of birds as well as by other ectoparasite taxa that deliver similar cues (Tomás et al. 2017), suggest that the biased brood parasitism we detected in spotless starling nests without spots is widespread in nature.

Acknowledgments

We thank Magdalena Ruiz-Rodríguez and Manuel Martín-Vivaldi for their helpful comments on a previous version of the manuscript. The research group benefits from facilities, including an apartment, provided by the city hall of Guadix where a small lab to quickly proceed the samples was installed.

References

Avilés, J. M., Perez-Contreras, T., Navarro, C. and Soler, J. J. 2008. Dark nests and conspicuousness in color patterns of nestlings of altricial birds. -*The American Naturalist,* 171: 327-338.

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

Azcárate-García, M., Ruiz-Rodriguez, M., Diaz-Lora, S., Ruiz-Castellano, C. and Soler, J. J. 2019. Experimentally broken faecal sacs affect nest bacterial environment, development and survival of spotless starling nestlings. -*Journal of Avian Biology,* 50: 10.

Brake, I. 2011. World Catalog of the Family Carnidae (*Diptera, Schizophora*). -*Myia,* 12: 113-169.

Brown, C. R. and Brown, M. B. 1991. Selection of high-quality host nests by parasitic cliff swallows. -*Animal Behaviour,* 41: 457-465.

Calvo, J. M., Pascual, J. A., Deceuninck, B. and Peris, S. J. 2000. Intraspecific nest parasitism in the spotless starling *Sturnus unicolor*. -*Bird Study,* 47: 285-294.

Cramp, S. 1998. The complete birds of the Western Palearctic. *Optimedia.* Oxford University Press.

Davies, N. B. 2000. *Cuckoos, cowbirds, and others cheats,* London, T & A D Poyser.

Eens, M. and Pinxten, R. 1999. Responses of male Spotless Sterlings *Sturnus unicolor* to experimental intraspecific nest parasitism vary in relation to the nesting stage. -*Journal of Avian Biology,* 30: 122-125.

Grimaldi, D. A. 1997. *The bird flies, genus Carnus : species revision, generic relationships, and a fossil Meoneura in amber (Diptera, Carnidae),* New York, NY., American Museum of Natural History.

Hannam, K. 2006. Ectoparasitic blow flies (*Protocalliphora sp.*) and nestling Eastern Bluebirds (*Sialia sialis*): direct effects and compensatory strategies. -*Canadian Journal of Zoology-Revue Canadienne De Zoologie,* 84: 921-930.

Hoi, H., Darolova, A., Kristofik, J. and Hoi, C. 2018. The effect of the ectoparasite Carnus hemapterus on immune defence, condition, and health of nestling European Bee-eaters. -*Journal of Ornithology,* 159: 291-302.

Hoi, H., Kristofik, J., Darolova, A. and Hoi, C. 2010. Are parasite intensity and related costs of the milichiid fly Carnus hemapterus related to host sociality? -*Journal of Ornithology,* 151: 907-913.

Kattan, G. H. 1997. Shiny Cowbirds Follow the Shotgun Strategy of Brood Parasitism. -*Animal Behaviour,* 53: 647-654.

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

Lyon, B. E. and Eadie, J. M. 2008. Conspecific Brood Parasitism in Birds: A Life-History Perspective. -*Annual Review of Ecology, Evolution, and Systematics,* 39: 343-363.

Lyon, B. E. and Eadie, J. M. 2017. Why Do Birds Lay Eggs in Conspecifics' Nests? *Avian Brood Parasitism.* Springer, pp. 105-123.

Martín-Vivaldi, M., Ruiz-Rodríguez, M., Mendez, M. and Soler, J. J. 2006. Relative importance of factors affecting nestling immune response differs between junior and senior nestlings within broods of hoopoes *Upupa epops*. -*Journal of Avian Biology,* 37: 467-476.

Mclaren, C. M. and Sealy, S. G. 2003. Factors influencing susceptibility of host nests to brood parasitism. -*Ethology Ecology & Evolution,* 15: 343-353.

Minias, P. 2015. The use of haemoglobin concentrations to assess physiological condition in birds: a review. -*Conservation Physiology,* 3: 15.

Narushin, V. G. 2005. Production, modeling, and education: Egg geometry calculation using the measurements of length and breadth. -*Poultry Science,* 84: 482-484.

O'brien, E. L. and Dawson, R. D. 2005. Perceived risk of ectoparasitism reduces primary reproductive investment in tree swallows Tachycineta bicolor. -*Journal of Avian Biology,* 36: 269-275.

O'brien, E. L., Morrison, B. L. and Johnson, L. S. 2001. Assessing the effects of haematophagous ectoparasites on the health of nestling birds: haematocrit vs haemoglobin levels in House Wrens parasitized by blow fly larvae. -*Journal of Avian Biology,* 32: 73-76.

Odell, N. S. and Eadie, J. M. 2010. Do wood ducks use the quantity of eggs in a nest as a cue to the nest's value? -*Behavioral Ecology,* 21: 794-801.

Paasivaara, A., Rutila, J., Pöysä, H. and Runko, P. 2010. Do parasitic common goldeneye *Bucephala clangula* females choose nests on the basis of host traits or nest site traits? -*Journal of Avian Biology,* 41: 662-671.

Parejo, D. and Avilés, J. M. 2007. Do avian brood parasites eavesdrop on heterospecific sexual signals revealing host quality? A review of the evidence. -*Animal Cognition,* 10: 81-88.

Parejo, D., Perez-Contreras, T., Navarro, C., Soler, J. J. and Avilés, J. M. 2008. Spotless starlings rely on public information while visiting conspecific nests: an experiment. -*Animal Behaviour,* 75: 483-488.

Peralta-Sánchez, J. M., Martín-Platero, A. M., Wegener-Parfrey, L., Martínez-Bueno, M., Rodríguez-Ruano, S., Navas-Molina, J. A., Vazquez-Baeza, Y., Martín-Galvez, D., Martín-Vivaldi, M., Ibánez-Álamo, J. D., Knight, R. and Soler, J. J. 2018. Bacterial density rather than diversity correlates with hatching success across different avian species. -*Fems Microbiology Ecology,* 94: 13.

Pinxten, R., Eens, M. and Verheyen, R. F. 1991. Responses of male starlings to experimental intraspecific brood parasitism. -*Animal Behaviour,* 42: 1028-1030.

Pöysä, H., Eadie, J. M. and Lyon, B. E. 2014. Conspecific brood parasitism in waterfowl and cues parasites use. -*Wildfowl*: 192–219.

Pöysä, H., Lindblom, K., Rutila, J. and Sorjonen, J. 2010. Response of parasitically laying goldeneyes to experimental nest predation. -*Animal Behaviour,* 80: 881-886.

Pöysä, H. and Pesonen, M. 2007. Nest Predation and the Evolution of Conspecific Brood Parasitism: From Risk Spreading to Risk Assessment. -*The American Naturalist,* 169: 94-104.

Richner, H., Oppliger, A. and Christe, P. 1993. Effect of An Ectoparasite on Reproduction in Great Tits. -*Journal of Animal Ecology,* 62: 703-710.

Rothstein, S. I. 1990. A model system for coevolution: avian brood parasitism. -*Annual Review of Ecology and Systematics,* 21: 481-508.

Soler, J. J. and Avilés, J. M. 2010. Sibling competition and conspicuousness of nestling gapes in altricial birds: a comparative study. -*PLoS ONE,* 5: e10509.

Soler, J. J., Aviles, J. M., Martin-Galvez, D., De Neve, L. and Soler, M. 2014. Eavesdropping cuckoos: further insights on great spotted cuckoo preference by magpie nests and egg colour. -*Oecologia,* 175: 105-115.

Soler, J. J., Martín-Vivaldi, M. and Møller, A. P. 2009. Geographic distribution of suitable hosts explains the evolution of specialized gentes in the European cuckoo *Cuculus canorus*. -*BMC Evolutionary Biology,* 9: 88.

Soler, J. J., Møller, A. P., Soler, M. and Martínez, J. G. 1999. Interactions between a brood parasite and its host in relation to parasitism and immune defence. -*Evolutionary Ecology Research,* 1: 189-210.

Soler, J. J., Moreno, J., Avilés, J. M. and Møller, A. P. 2005. Blue and green egg-color intensity is associated with parental effort and mating system in passerines: Support for the sexual selection hypothesis. -*Evolution,* 59: 636-644.

Soler, J. J., Navarro, C., Pérez-Contreras, T., Avilés, J. M. and Cuervo, J. J. 2008. Sexually selected egg coloration in spotless starlings. -*American Naturalist,* 171: 183-194.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martín-Vivaldi, M., Martínez-De La Puente, J., Ruiz-Rodríguez, M. and Tomás, G. 2017. Telomere length and dynamics of spotless starling nestlings depend on nest-building materials used by parents. -*Animal Behaviour,* 126: 89-100.

Soler, J. J., Soler, M., Møller, A. P. and Martínez, J. G. 1995. Does the great spotted cuckoo choose magpie hosts according to their parenting ability? -*Behav. Ecol. Sociobiol,* 36: 201-206.

Soler, M. 2017a. Avian Brood Parasitism: Behaviour, Ecology, Evolution and Coevolution. In: Soler, M. (ed.) Avian Brood Parasitism: Behaviour, Ecology, Evolution and Coevolution. Springer International Publishing Ag, Cham, pp. 1-574.

Soler, M. 2017b. Brood Parasitism in Birds: A Coevolutionary Point of View. In: Soler, M. (ed.) Avian Brood Parasitism: Behaviour, Ecology, Evolution and Coevolution. Springer International Publishing Ag, Cham, pp. 1-19.

Soler, M., Peréz-Contreras, T. and Soler, J. J. 2015. Synchronization of laying by great spotted cuckoos and recognition ability of magpies. -*Journal of Avian Biology*, 46: 608-615.

Stouffer, P. C., Kennedy, E. D. and Power, H. W. 1987. Recognition and removal of intraspecific parasite eggs by starlings. -*Animal Behaviour,* 35: 1583-1584.

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

Tomás, G., Martín-Gálvez, D., Ruiz-Rodríguez, M. and Soler, J. 2017. Intraspecific avian brood parasites avoid host nests infested by ectoparasites. -*Journal of Ornithology,* 158: 561-567.

Veiga, J. and Polo, P. 2016. Estornino Negro–Sturnus unicolor. En: Enciclopedia Virtual de los Vertebrados Españoles. Museo Nacional de Ciencias Naturales, Madrid, Salvador, A., Morales, M. B. (Eds.).

Wiley, J. W. 1988. Host selection by the shiny cowbird. -*Condor,* 90: 289-303.

Yamaguchi, Y. 1997. Intraspecific nest parasitism and anti-parasite behavior in the grey starling *Sturnus cineraceus*. -*Journal of Ethology,* 15: 61-68.

Yom-Tov, Y. and Geffen, E. 2017. Conspecific brood parasitism among birds: The effects of phylogeny, mode of reproduction and geographic distribution. In: Soler, M. (ed.) *Avian Brood Parasitism: Behaviour, Ecology, Evolution and Coevolution.* Springer International Publishing Ag, Cham, pp. 95-103.

CAPÍTULO 2

Experimentally broken faecal sacs affect nest bacterial environment, development and survival of spotless starling nestlings.

Abstract

Nestlings of most avian species produce faecal sacs, which facilitate the removal of nestlings' excrements by parents, thereby reducing proliferation of potentially pathogenic microorganisms and/or detectability by predators and parasites. The nest microbial environment that birds experience during early life might also affect their development and thus, faecal sacs facilitating parental removal may be a strategy to decrease bacterial contamination of nests that could harm developing nestlings. Here, we tested this hypothesis by experimentally broken faecal sacs and spreading them in nests of spotless starlings (*Sturnus unicolor*), thereby avoiding their removal by adults. In accordance with the hypothesis, experimental nests harboured higher bacterial density than control nests. Nestlings in experimental nests were of smaller size (tarsus length) and experienced lower probability of survival (predation) than those in control nests. Moreover, nestlings in experimental nests tended to suffer more from ectoparasites than those in control nests. We discuss the possible pivotal role of bacteria producing chemical volatiles that ectoparasites and predators might use to find avian nests, and that could explain our experimental results in starlings.

Keywords: bacteria, ectoparasites, faecal sacs, nestling growth, nest predation, nest sanitation, *Sturnus unicolor*

Introduction

Recent works have stressed the importance of considering the role of bacteria and other microorganisms in evolutionary and ecological frameworks (Archie and Theis 2011, Ezenwa et al. 2012, McFall-Ngai et al. 2013). For instance, it was demonstrated that chemicals from bacterial metabolism play important roles in animal communication (Leclaire et al. 2017), and that some parasites cue on such volatiles to find and select hosts (Verhulst et al. 2011, Busula et al. 2017). Bacteria may also have positive (Møller et al. 2012) or negative (Polin et al. 2015) effects on the probability of being captured by predators, or even deter parasitoids from using infected hosts (Costopoulos et al. 2014). Pathogenicity is perhaps the best-known characteristic of bacteria, not only because it directly or indirectly affects survival prospect of organisms, but also because it would constrain the evolution of characteristics that enhance the probability of bacterial infections, as it is probably the case of cognitive skills (Soler et al. 2012b). In birds, pathogenic effects of bacteria have mainly been explored in poultry and, more recently, also in wild birds (Benskin et al. 2009). Negative effects in terms of survival or viability have been detected for embryos (Soler et al. 2012a, Peralta-Sánchez et al. 2018), nestlings, and adult wild birds (Benskin et al. 2009). All these negative effects strongly select for defensive strategies and barriers against bacterial infections, such as particular immune responses (Soler et al. 2011), antimicrobial secretions (Soler et al. 2008, Møller et al. 2009, Ruiz-Rodríguez et al. 2009, Soler et al. 2010, Soler et al. 2012a, Martín-Vivaldi et al. 2014), the use of aromatic plants with antimicrobial capabilities as nest materials (Gwinner and Berger 2005, Mennerat et al. 2009a, Ruiz-Castellano et al. 2016), or nest sanitation behaviours to prevent or limit bacterial growth (Ibáñez-Álamo et al. 2017).

Removal of nestling faeces is the most common form of nest sanitation in birds (Guigueno and Sealy 2012). Nestlings of most avian species recover

the excrements with small mucous sacs that facilitate removal from nests by parents (Herrick 1900, Guigueno and Sealy 2012, Ibáñez-Álamo et al. 2017). These two traits, nestling faeces removal and covering excrements with faecal sacs, are quite spread in birds and correlate to each other along the avian phylogeny (Ibáñez-Álamo et al. 2017), which suggest the existence of important advantages associated to them. Since enteric bacteria include potential pathogens for birds (Brittingham et al. 1988, Lombardo et al. 1996, Westneat and Rambo 2000) that could easily infect growing nestlings, removing nestling faeces from nests would reduce risk of infection and, thus, development and probability of survival of nestlings (Ibáñez-Álamo et al. 2014a). Moreover, the presence of nestling faeces within or close to the nests might for instance result in visual or chemical cues for predators (Petit et al. 1989, Lang et al. 2002) , and/or ectoparasites (Hart 1997, Quan et al. 2015, Ibáñez-Álamo et al. 2016). Thus, removing droppings from nests should reduce probability of parasitism and/or of predation of nestlings. Enteric bacteria in nestling faeces include taxa that, as Clostridium and *Enterococcus*, produce abundant malodourous volatiles (Lemfack et al. 2014) that predators and parasites might follow to localize avian nests. Thus, it is likely that bacteria mediated the expected negative effects of excrements in avian nests (pathogenicity, parasitism and predation) and, consequently the evolution of nest sanitation behaviour of adults, and of faecal sacs by nestlings, as protection against them (Ibánez-Álamo et al. 2014a, 2017). The above scenario therefore suggests that, although removal of nestling faeces by parents is a costly activity (Weatherhead 1984), the associated reduction in probability of predation, parasitism, and/or infection would result in hypotheticals net benefits for parents.

We here tested the predicted negative effects of faecal sacs contents on the nest bacterial environment and development and survival of nestlings. Parents failing to remove nestling faeces from nests might occur in natural

conditions, as a consequence of nestlings excreting in their absence, or because weak faecal sacs. Thus, detecting such negative effects would confirm the hypothesized advantages of removal of nestling faeces from avian nests by parents. We did so through experimentally broken of faecal sacs contents in nests of spotless starlings (*Sturnus unicolor*), a cavity nesting species that readily use artificial nest-boxes. The general expectation is that the experiment should negatively affect nestling growth and/or survival. These effects might be determined either by predators or parasites more easily detecting experimental nests, or by pathogenic bacteria inducing nestlings' death or suboptimal growth.

Our hypothesis proposes a central role for bacterial environment determining the expected effects and, thus, we assumed that the experimentally broken of nestlings' faeces would enhance bacterial colonization and growth in starling nests. Thus, we expect that nest-cups with experimentally broken faecal sacs harboured bacteria at a higher density than control nests (Prediction 1). As this prediction was fulfilled, detected experimental effects could be interpreted as the consequence of differences in bacterial environment. Expected experimental effects include reduced nestling survival, either due to pathogenic bacteria or to predators more easily detecting nests (Prediction 2). We also expect higher ectoparasite loads in experimental nests due to an increase of nest detectability (Prediction 3). Moreover, because growing in nest environments with high bacterial density may have consequences for nestlings' growth, our experiment should affect phenotypes of fledglings. Particularly, we expect that fledglings from experimental nests have lower body mass and shorter tarsus length than those from control nests (Prediction 4). Finding support to any of the predicted negative experimental effects would support our main hypothesis of beneficial effects of nestling-faeces removal by parents.

Materials and Methods

Study area and species

The study was carried out during the 2015-16 breeding seasons in the "Hoya de Guadix", southeast Spain, a high-altitude plateau 1000 m a.s.l. with a semi-arid climate. Our study population are located at La Calahorra (37°15' N, 3°01' W) and Huéneja (37°13' N, 2°56' W) railway stations, where there are 131 nest-boxes installed since 2005 (internal height*width*depth: 350*180*210 mm, bottom-to-hole height: 240 mm). These nest-boxes are attached to tree trunks or walls at 3-4 m above ground and are mostly occupied by spotless starlings.

The spotless starling (hereafter starling) is a medium-sized and hole-nesting passerine (Cramp 1998). In our study area, the reproductive season starts in early April and, depending on the study year, most individuals in the population lay a second clutch during May. The most common clutch size is 4-5 eggs. The incubation phase usually begins with the penultimate egg, which promotes that the last nestling hatches later than the others (Veiga and Polo 2016). Incubation is mostly carried out by females with sporadic help from males and extend for around 14 days, while the nesting period lasts 21-22 days (Cramp 1998). Both male and female parents contribute to feeding the offspring and remove faecal sacs produced by nestlings after feeding (Cramp 1998). In addition, during the whole nesting process, adults renew feathers and aromatic plants deposited in the nest, which have been shown to have antimicrobial-beneficial functions (Ruiz-Castellano et al. 2016).

Reproductive success in the study area typically varies among years and breeding attempt and, among other factors, it depends on probability of predation. The most common predators of starling nests in our study area are rats (*Rattus rattus*), ladder snakes (*Rhinechis scalaris*), little owls (*Athene noctua*) and ocellated lizards (*Timon lepidus*) (pers. obs.).

Fieldwork and experimental protocol

At the end of March, we started to visit the nest-boxes every third day until starling eggs were detected. Nest-boxes were again visited 12 days after the 4th egg was laid and, afterward, every single day until hatching. Visited nest-boxes with hatchlings were sequentially assigned to control or experimental treatments (both in first and second breeding attempts; implying that the same nest-box might have been assigned to different experimental treatments in first and second breeding attempts). The day after the first egg hatched, we sampled nest-cups to assess bacterial density of nest lining material before the experiment started, while keeping the nestlings in a cotton bag (the process lasted around 1-2 minutes). Briefly, we gently rubbed nest-cup material with a sterile swab (Sterile R, Nuova Aptaca S.R.L.) slightly wet with sterile sodium phosphate buffer (0,2 M; pH = 7.2) during 10 seconds. Afterwards, we kept the swab in an Eppendorf tube with 1.2 ml of phosphate buffer at 4-6 °C, in a portable refrigerator until being processed in the lab within the next 24 hours. Moreover, we stimulated nestling cloaca and collected faecal sacs in experimental and control nests. Faecal sacs were mixed in a plastic container and used in experimental nests. Briefly, before nestlings were returned to their nests, with the help of a small teaspoon, we spread the mixture corresponding to approximately three faecal sacs on the nest-cup lining materials of experimental nests. We ensured that we never left large pieces that could be easily removed by parents. Nest-cup material of control nests was also touched in the same way with a clean teaspoon. We repeatedly performed this experimental treatment on days 5, 8 and 11 of the nestling period. In 2015, samples for estimating bacterial density of nest-cups in experimental and control nests were collected on day 14 after hatching. However, we realized that nestlings at this age were very active within nest-boxes, and parents feed them mostly from the nest entrance without removing faecal sacs after feeding (pers. obs.). Thus, control and experimental nests with 14 days old starlings

are typically full of excrements and with similar bacterial loads of nest lining material (see Results). Consequently, nest bacterial loads at this stage is too high (Devaynes et al. 2018) and, thus, are not a good proxy of nest bacterial environment experience by developing nestlings in experimental and control nests. Looking for a better proxy of bacterial environment experienced by nestlings during growing, in the 2016 breeding season, we also sampled nest material on day 12, before parents stopped removing nestling faeces from nest-boxes, in a reduced number of nests (2015: N = 128; 2016: N = 21).

In addition, 8 days after hatching, all nestlings were ringed with aluminium numbered rings. Finally, when nestlings were close to fledging (i.e., 14 days after hatching), they were weighed with a hanging scale (Pesola 0-100 g, accuracy 1 g), and their tarsus and wing length measured with a digital calliper (accuracy 0.01 mm) and a metallic ruler (accuracy 1 mm), respectively. All nestlings were also checked for evidences of parasitism by the diptera *Carnus hemapterus*, the main ectoparasite in our study area. Adults of the genus *Carnus* are blood-sucking ectoparasites of nestling birds (Valera et al. 2004, Brake 2011) and they are highly abundant in Sturnus colonies (Lopez-Rull et al. 2007, Avilés et al. 2009). As a proxy of ectoparasitism loads, we used a semi-quantitative scale depending on density of bites and blood remains detected in wings and belly of each nestling in the nest. Briefly, 0 value was assigned when no bite was detected, 1 when only few (1-10) bites were detected, 2 when more than 10 bites were detected, but skin surface covered by bites or blood remains was less than 50 %. Nestlings with more than 50 % of the skin covered by bites or blood remains of flies were assigned the value 3. Since bites and blood remains of flies accumulate over several days, these estimates provide a measure of intensity of parasitism for a wider time window.

For each nest visit and sampling, to prevent bacterial contamination between nests, we wore new latex gloves washed with ethanol 70 % before nestling manipulation.

Laboratory work

In the lab, tubes containing the bacterial samples were vigorously shaken in a vortex (Boeco V1 Plus) to remove bacteria from swabs. Afterward, we spread homogeneously 100 µl of serially diluted samples until 10^{-4} in the general media (see below) and 10^{-2} in the three used selective media (see below). Plates were incubated at 37 °C during 72 hours, and afterward the number of colonies on each plate was counted. Following the method of Peralta-Sánchez et al. (2010), we used four different solid media (Scharlau Chemie S.A., Barcelona) to grow bacterial samples. We used Tryptic Soy Agar (TSA), a broadly used general medium to grow mesophilic bacteria, and three specific media: Kenner Fecal Agar (KF) for growing bacteria belonging to the genus *Enterococcus*; Vogel-Johnsson Agar (VJ) for bacteria of the genus *Staphylococcus*; and Hecktoen Enteric Agar (HK) for Gram-negative bacteria of the family *Enterobacteriaceae*. Bacterial load of starling nest-cups was expressed as the number of colony forming units (CFU) per ml (Nº colonies * 10 $^{\text{dilution factor}}$) / 100 ml spread).

Statistical Methods

After logarithmical transformation, nest bacterial loads, and intensity of ectoparasitism (average of estimates of belly and wing) did approximately follow a Gaussian distribution. Fledging success was transformed to rank values by using the Statistica 7.1 software. For nests with identical fledging success, we used the mean values of rank positions. The ranked values varied

from 15.5 (= 0 % of fledging success) to 147 (= 100 % fledging success). Variables of predation, nestlings' body mass and tarsus length were not transformed before the analyses. Residuals of all statistical models followed Gaussian distributions.

The effects of experimental treatment on nest bacterial loads were tested by mean of a Repeated-Measures ANOVAs with sampling day (1 and 14 in 2015; 1, 12 and 14 in 2016; see above) as the within factor (i.e., dependent variables) and experimental treatment as the categorical predictor (i.e., between effect). Fisher-LSD tests were used for post-hoc comparisons. The effects of experimental treatment on fledging success (i.e., proportion of hatchlings that reached the fledging stage) were explored in General Linear Models (GLM) with experimental treatment, breeding attempt (first or second clutch), study year and brood size (i.e. number of hatchlings) as fixed factors.

The experimental effects on intensity of ectoparasitism, tarsus length and body mass of nestlings were explored in General Linear Mixed Models (GLMM), with experimental treatment, breeding attempt and year as fixed factors, and nest-box identity nested within the three-level interaction treatment x breeding attempt x year as the random effects. Interactions between fixed effects were tested in separate models that also included main effects. Tarsus length ($\beta \pm SE = -0.14 \pm 0.05$, $F_{1,376} = 7.01$, $P = 0.008$) and body mass marginally ($\beta \pm SE = -0.09 \pm 0.05$, $F_{1,376} = 3.15$, $P = 0.077$), but not parasitism intensity ($\beta \pm SE = 0.06 \pm 0.05$, $F_{1,365} = 1.23$, $P = 0.268$) of starling nestlings did depend on brood size. Thus, to control for this effect we used residuals of body mass and tarsus length after controlling for brood size as dependent variables in the GLMMs.

Identity of the study sub-area (La Calahorra or Huéneja) did not explain significant proportion of variance of any of the considered dependent variable (results not shown), and thus was not included in the models.

Moreover, the experimental treatment received by nest-boxes during the first breeding attempt did not influences values of considered dependent variables (bacterial loads, parasitism loads, probability of predation or nestling growth) measured during the second breeding attempt (Annex 1). Thus, previous experimental treatment was no further considered in our analyses.

The experimental effects on fledging success were tested twice, first, by considering all nests with information (i.e., including both the depredated and not depredated nests), and second, by excluding nests that suffered predation from the analyses. Detecting experimental effects in nests that did not suffer predation will indicate that causes other than predation affected survival of experimental nestlings. The experimental effect on the probability of nest depredation was explored in a Generalized Linear Model (GLZ) with a binomial distribution and logit link function. For these analyses, we considered as predated nests those that suffered predation of the complete brood. Similar to previous models, we included experimental treatment, breeding attempt (first or second clutch), brood size (i.e. number of hatchlings) and year as fixed factors.

All analyses were performed with Statistica 7.1 (Statsoft Inc. 1984-2005).

Results

Before the experiment, nests assigned to experimental and control treatments did not differ in bacterial density (see post-hoc in Table 1). Experimentally broken nestlings' faeces did affect the bacterial load of starling nests 12 days after hatching (Table 1, Fig. 1). These effects disappeared two days later, i.e., in samples collected at the end of the nestling period (see post-hoc in Table 1).

Table 1: Results from Repeated Measures ANOVAs exploring the effects of experimentally broken nestling faecal sacs (treatments: Control and Experimental (Exp.)) on density (log-transformed CFU / ml) of Mesophilic bacteria, *Enterobacteriaceae, Staphylococcus* and *Enterococcus* at three different stages of the nestling period. Analyses were separately performed for samples collected in 2015 (N-control = 69, N-Experimental = 59) and in 2016 (N-control = 10, N-Experimental = 11). Post-hoc LSD tests comparing the effect of the experiment within the same stage are also shown. P-values lower than 0.1 (α-values of one-tailed statistical tests) are in bold.

Media	1 day after hatching			12 days after hatching			14 days after hatching				
	Control Mean (SE)	Exp. Mean (SE)	Post-Hoc P =	Control Mean (SE)	Exp. Mean (SE)	Post-Hoc P =	Control Mean (SE)	Exp. Mean (SE)	Post-Hoc P =		
Year 2015										$F_{1,126}$	P
TSA	0.70 (0.01)	0.71 (0.01)	0.573	-	-	-	0.79 (0.01)	0.79 (0.01)	0.904	0.09	0.765
HK	0.30 (0.03)	0.28 (0.04)	0.751	-	-	-	0.60 (0.02)	0.63 (0.03)	0.413	0.12	0.726
VJ	0.13 (0.03)	0.20 (0.03)	0.102	-	-	-	0.37 (0.03)	0.37 (0.03)	0.952	1.55	0.215
KF	0.61 (0.02)	0.65 (0.02)	**0.095**	-	-	-	0.75 (0.01)	0.73 (0.01)	0.280	0.17	0.679
Year 2016										$F_{1,19}$	P
TSA	0.66 (0.02)	0.66 (0.02)	0.916	0.68 (0.02)	0.75 (0.02)	**0.012**	0.70 (0.02)	0.74 (0.02)	0.167	3.08	**0.095**
HK	0.17 (0.06)	0.27 (0.06)	0.267	0.38 (0.06)	0.60 (0.06)	**0.020**	0.46 (0.07)	0.51 (0.07)	0.621	3.87	**0.064**
VJ	0.37 (0.07)	0.37 (0.06)	0.925	0.23 (0.06)	0.48 (0.05)	**0.005**	0.44 (0.07)	0.48 (0.06)	0.686	4.45	**0.048**
KF	0.59 (0.02)	0.56 (0.02)	0.393	0.65 (0.03)	0.71 (0.03)	**0.057**	0.69 (0.02)	0.72 (0.02)	0.324	0.85	0.368

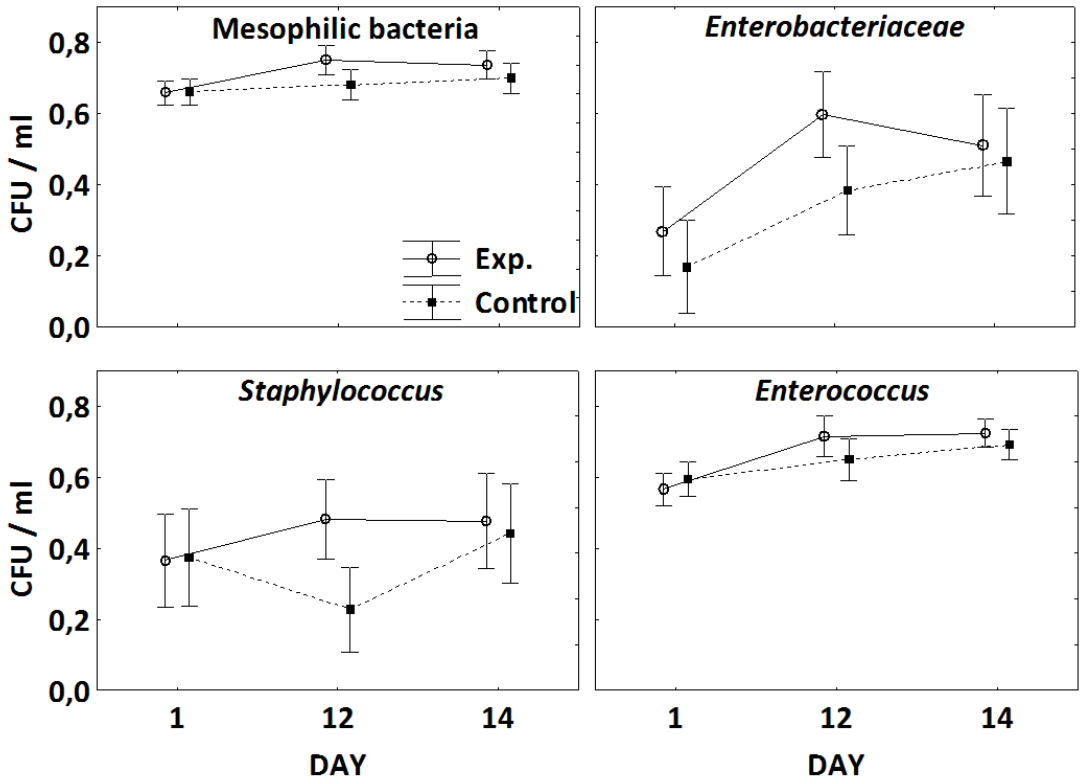

Fig. 1: Nest bacterial loads (log-transformed CFU / ml ± 95 % of Confidence Interval) of experimental (N = 11) and control (N = 10) nests sampled three times along the nestling period in the year 2016.

The fledging success of nestlings in experimental nests was lower than that of control nests (GLM: $F_{1,155} = 3.93$, P = 0.049, Fig. 2), after controlling for the negative effects of breeding attempt (GLM: $F_{1,155} = 7.91$, P = 0.006, $\bar{x}_{\text{first clutch}} \pm SE = 102.98 \pm 7.22$, $\bar{x}_{\text{second clutch}} \pm SE = 81.01 \pm 6.22$) and brood size (GLM: $F_{1,155} = 6.94$, P = 0.009, $\beta \pm SE = -0.20 \pm 0.08$). Interestingly, this effect disappeared when considering only the nests that were not depredated (GLM: $F_{1,129} = 0.63$, P = 0.428), which suggest that the detected effects in fledging success were mainly due to predation mainly affecting experimental nests. In accordance with this inference, we found that experimental nests experienced higher probability of predation (22.47 %, N = 89) than control nests (11.49 %, N = 87; GLZ: $\chi^2_1 = 5.02$, P = 0.025, Fig. 2). That was the case when controlling for the significant effect of breeding attempt (GLZ: $\chi^2_1 = 10.75$, P = 0.001).

Fledglings that developed in nests with experimentally broken faecal sacs had shorter tarsus than those in control nests (GLMM: $F_{1,148} = 5.01$, P = 0.027, Fig. 3) after controlling for the effects of the study year (GLMM: $F_{1,138} = 5.26$, P = 0.048). Control and experimental fledglings did not differ in body mass (GLMM: $F_{1,142} = 1.55$, P = 0.214, Fig. 3)). Finally, intensity of ectoparasitism resulted non-significantly higher in nests with experimentally broken faecal sacs than in control nests (GLMM: $F_{1,133} = 2.98$, P = 0.087, Fig. 3), after controlling for the significant effects of breeding attempt (GLMM: $F_{1,134} = 8.11$, P = 0.005).

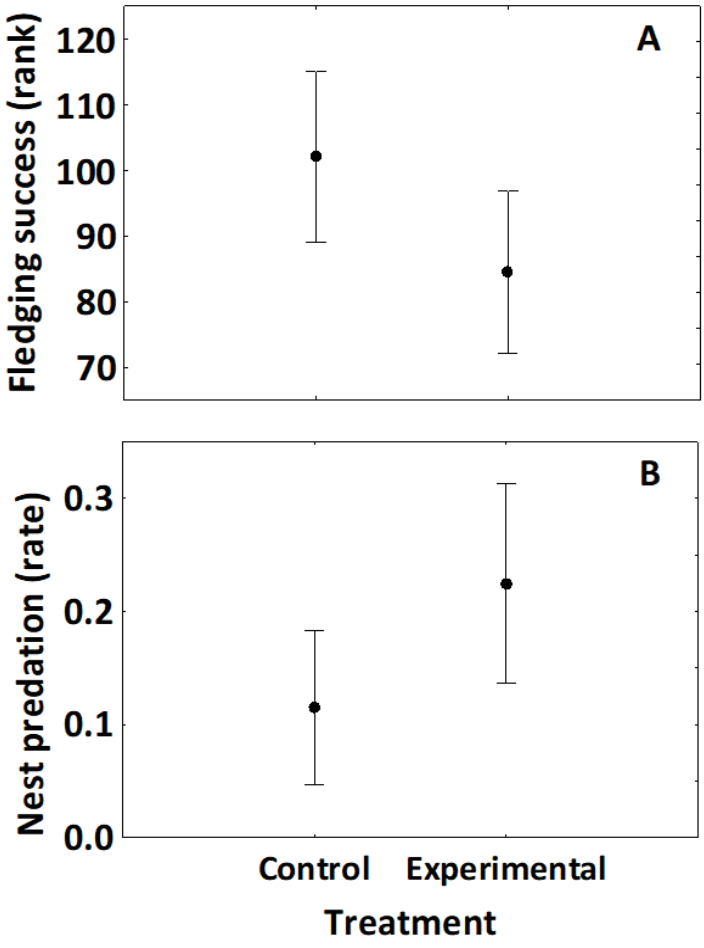

Fig. 2: Differences in (A) fledgling success ($F_{1,155} = 3.93$, P = 0.049) and (B) nest predation rates ($\chi^2_1 = 5.02$, P = 0.025) of starling nests with (Experimental, N = 89) and without (Control, N = 87) experimentally broken faecal sacs. Represented values are means ± CI 95 %

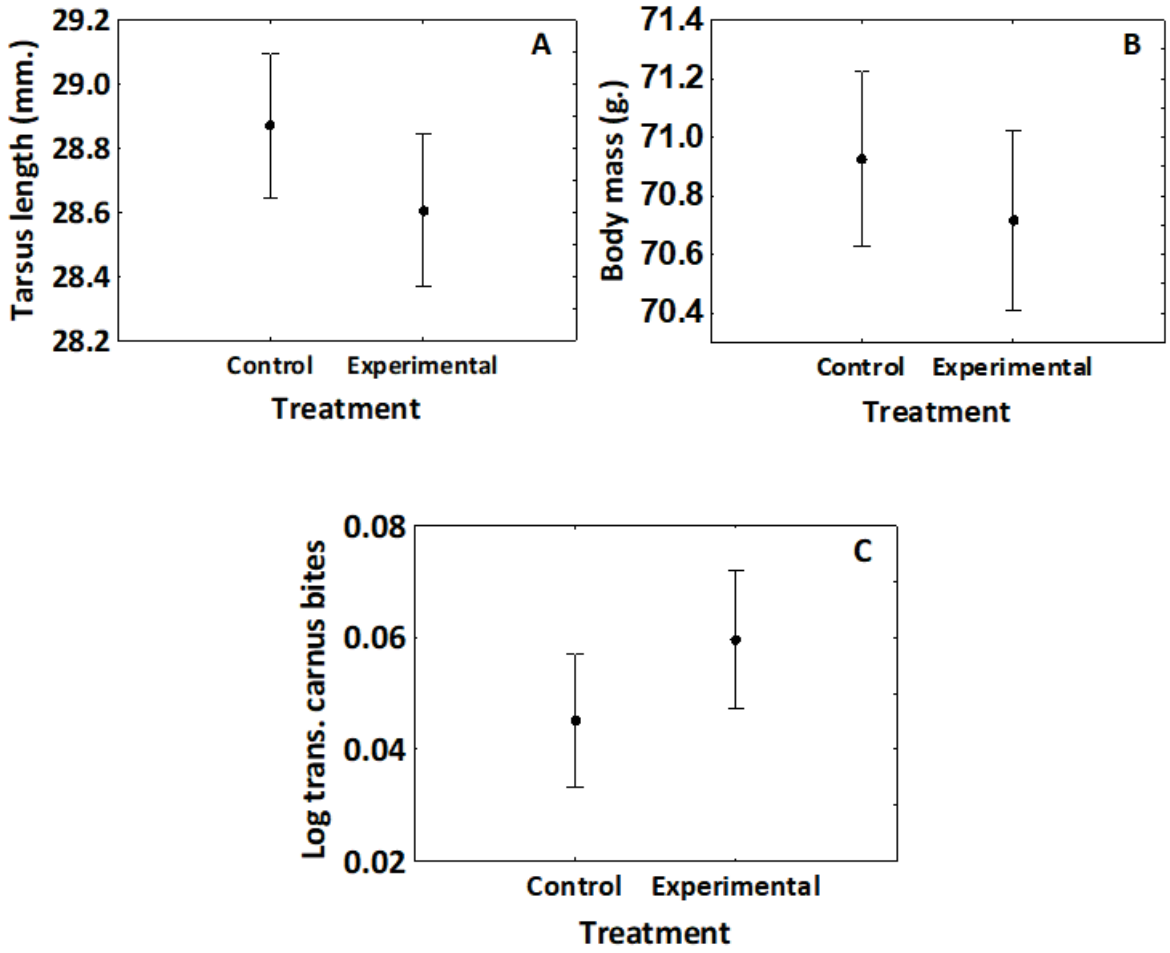

Fig. 3: Differences in (A) tarsus length ($F_{1,148} = 5.01$, $P = 0.027$), (B) body mass ($F_{1,142} = 1.55$, $P = 0.214$) and (C) parasitism intensity (i.e., *Carnus* bites, $F_{1,133} = 2.98$, $P = 0.087$) of spotless starling fledglings that developed in nests with (Experimental, N = 173) or without (Control, N = 198) experimentally broken faecal sacs in their nests. Represented values are least square means ± CI 95 %.

Discussion

By adding experimentally broken faecal sacs of spotless starling nestlings, we successfully modified the bacterial environment of nest-cups in terms of increasing bacterial loads. Those effects were detected before parents stopped removing faecal sacs from nests (only analysed in one of the study years), but not two days later when parental duties related to nest sanitation were greatly

reduced. Several strains of the considered bacterial groups (mesophilic bacteria, *Enterobacteriaceae*, *Staphylococcus* and *Enterococcus*) are potentially pathogenic for adult birds, nestlings or embryos (Devriese et al. 1991, Bruce and Drysdale 1994, Benskin et al. 2009), or produce volatiles (Lemfack et al. 2014) that predators and ectoparasites might use to detect starling nests. Thus, we predicted negative experimental effects on fitness related variables. As expected, the addition of experimentally broken of faecal sacs in nests of spotless starlings increased probability of nest predation, negatively affected nestling growth in terms or tarsus length, and tended to increase intensity of parasitism experienced by nestlings developing in experimental nests. Below we discuss the possibility that changes in the bacterial community of experimental nests mediated these effects.

Our results imply that predators and, at a lower degree, ectoparasites, detected experimental nests more easily. Predators, as well as ectoparasites, follow visual, auditory, or chemical cues to detect avian nests (Tomás and Soler 2016), and our experiment should have influenced one or more of these cues. Petit et al. (1989) deposited experimental faeces that predators could visualize near focal artificial nests and attracted predators. Rubio et al. (2018) performed a similar experiment in inactive nests of a grown nester, but in this case, the presence of faecal sacs did not attract predators. Moreover, Ibáñez-Álamo et al. (2014b) hided experimental faecal sacs to terrestrial predators in common blackbird (*Turdus merula*) active nests, which did not increase the probability of nest predation, suggesting that Petit et al. (1989)'s results were due to visual cues of faecal sacs. Our experiments were performed within nest-boxes and, thus, no visual cues that attract the attention of predators towards experimental nests existed. Thus, most of the detected effects in predation and parasitism should be mediated by olfactory cues related to the experiment.

Changes in olfactory cues due to higher density of volatiles produced by bacteria in experimental nests are a likely explanation of our results. The

role of volatiles-producing bacteria in different ecological scenarios is well known. Recently, the importance of volatiles produced by symbiotic bacteria have been stressed in scenarios of social communication (e.g., Theis et al. 2013, Ezenwa and Williams 2014, Leclaire et al. 2017), predator-prey, or parasite-host interactions (Bucher 1988, Gow et al. 2015, Schulte et al. 2016). Prey might for instance use predator odours in antipredator behaviour (Heise-Pavlov 2016), or predators might use volatiles from symbiotic bacteria of prey as clues to detect their victims (Fogarty et al. 2017, Mihailova et al. 2018). Previous studies have explored the possibility that faecal sacs attract potential predators and/or parasites, but did not find support for the hypothesis. The presence of faeces of common blackbirds nestlings in experimental nests attracted flies but not ectoparasites or predators (Ibáñez-Álamo et al. 2014b, 2016), which a priori suggest a minor role of olfactory cues for predators or ectoparasites detecting active nests of blackbirds. In these experiments, however, faecal sacs were not broken, which might prevent the escape of volatiles and, thus, explain the absence of experimental effects. The mucous covering excrements may prevent dispersion of volatiles produced by enteric bacteria that predator and ectoparasites might use to detect prey and hosts. Moreover, the mucus of faecal sacs has antimicrobial activity (Ibáñez-Álamo et al. 2014a) that would prevent proliferation of enteric bacteria producing volatiles. Thus, it is possible that the experimental addition of broken faecal sacs was necessary for further testing the hypothetical effect of associated volatiles attracting predators and ectoparasites to avian nests. We did so and, in the absence of visual cues, detected the expected increase in predation rates and, at a non-significant level, in ectoparasitism of experimental nests, which is the first experimental demonstration of non-visual cues associated with nestling faeces influencing nest detectability by predators or parasites.

In addition to chemical cues, our experiment could also have indirectly influenced the strength of auditory cues that predators and ectoparasites may

use to detect experimental nests more often than control nests. It is possible for instance that the elevated bacterial loads of experimental nests provoke bacterial infection of nestlings, which would increase their physiological needs and, thus, their begging effort (Martín-Gálvez et al. 2011, Martín-Gálvez and Soler 2016). Detectability by predators and parasites is one of the main costs of begging auditory traits (see Tomás and Soler (2016)) and, therefore, the detected effects on predation rates and ectoparasitism of experimentally broken faecal sacs in starling nests might be partially mediated by the effect on bacterial environment determining health status and/or physiological needs of nestlings. Unfortunately, we did not quantify begging behaviour of nestlings and, thus, we are unable to test the influence of the experimental manipulation on nestlings needs. However, since we found detrimental effects of the experiment in nestlings' growth (see above), we speculate with the possibility that needs, and thus begging effort, was higher for nestlings growing in nests with experimentally broken excrements. Further research is in any case necessary for testing the associations between nest bacterial environment, begging effort, and risk of parasitism and predation. Whatever the mechanism (i.e., chemical or acoustic cues) explaining the detected effects of faecal sacs experimentally broken, our results are likely mediated by the detected differences in bacterial environments of experimental and control nests.

We also found evidence of detrimental effects of the experimentally broken faecal sacs on nestling growth, which are likely explained by the elevated bacterial loads of experimental nests (see Jacob et al. (2015)), but also because the negative effect of ectoparasites that tended to be more abundant in experimental nests. Nestlings that grew in experimental nests developed shorter tarsus, although no effect was detected in nestlings' survival or body mass. Supporting the hypothesis that this effect was mediated by bacterial environments of experimental and control nests, previous studies have shown that antimicrobial materials (Mennerat et al. 2009b, Soler et al. 2017) and

density of bacteria ((Jacob et al. 2015), but see (Mills et al. 1999, Berger et al. 2003)) in avian nests affect nestlings growth. In addition, we also know that increasing nestlings' exposure to microorganisms might affect their immune system development (Hooper et al. 2012), which trade-off with nestlings growth (Szép and Møller 1999, Soler et al. 2003, Brommer 2004). Parents reducing nest attendance when risk of bacterial contamination is high might also mediate the associations between nest bacteria environment and nestling growth. We know that adult starlings reduce feeding effort in nests with high risk of ectoparasitism (Avilés et al. 2009) and that they have an extraordinary olfactory sense (Amo et al. 2012). Therefore, it is possible that parents discern probability of bacterial infection in experimental and control nests and adjust parental effort to detected volatiles from bacterial metabolism. Whatever the mechanism, and in accordance with previous papers, our results suggest that nest bacterial environment determines nestling growth. Thus, since immunocompetence and growth are good predictors of probability of nestling recruitment to reproductive populations (Cichon and Dubiec 2005, Moreno et al. 2005), our experimental results might suggest a reduced probability of recruitment of nestlings that grew in experimental nests.

Taken together, our experimental results suggest fitness effects of experimentally broken faecal sacs in terms of probability of predation, and phenotypic conditions of nestlings, which confirm the adaptive function of faecal sacs facilitating removal by parents of nestlings' faeces. These effects are likely mediated by the elevated bacterial density of experimental nests, which highlights the role that bacteria play as a significant selective pressure in birds' life history characteristics, stimulating the development of nest sanitation behaviours. Future research should focus on directly determining the effects of nest bacterial environment on volatiles and begging behaviour of nestlings explaining the detected effects of predation and the expected effects on ectoparasitism.

Acknowledgments

We thank Manuel Martín-Vivaldi for comments on a previous version of the manuscript that greatly improve its understanding, and Estefanía López Hernández for assistance in laboratory work. This work was supported by the currently named Ministerio de Ciencia, Innovación y Universidades and European (FEDER) funds (CGL2013-48193-C3-1-P, CGL2017-83103-P). MAG was financed by a predoctoral contract (BES-2014-068661) from the Spanish Ministerio de Economía y Competitividad (CGL2013-48193-C3-1-P). The authorization for carrying out the experimental work on animals in the field was granted by the Dirección General de Gestión del Medio Natural of the Consejería de Medio Ambiente of Junta de Andalucía.

References

Amo, L., Avilés, J. M., Parejo, D., Peña, A., Rodríguez, J. and Tomás, G. 2012. Sex recognition by odour and variation in the uropygial gland secretion in starlings. -*Journal of Animal Ecology*, 81: 605-613.

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

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

Benskin, C. M., Wilson, K., Jones, K. and Hartley, I. R. 2009. Bacterial pathogens in wild birds: a review of the frequency and effects of infection. -*Biological Reviews*, 84: 349-73.

Berger, S., Disko, R. and Gwinner, H. 2003. Bacteria in starling nests. -*Journal für Ornithologie*, 144: 317-322.

Brake, I. 2011. World Catalog of the Family Carnidae (Diptera, Schizophora). -*Myia*, 12: 113-169.

Brittingham, M. C., Temple, S. A. and Duncan, R. M. 1988. A survey of the prevalence of selected bacteria in wild birds. -*Journal of Wildlife Diseases*, 24: 299-307.

Brommer, J. E. 2004. Immunocompetence and its costs during development: an experimental study in blue tit nestlings. -*Proceedings of the Royal Society of London. Series B: Biological Sciences*,271: S110-S113.

Bruce, J. and Drysdale, E. 1994. Trans-shell transmission. Microbiology of the avian egg. Springer, pp. 63-91.

Bucher, E. H. 1988. Do birds use biological-control against nest parasites. -*Parasitology Today*, 4: 1-3.

Busula, A. O., Takken, W., De Boer, J. G., Mukabana, W. R. and Verhulst, N.O. 2017. Variation in host preferences of malaria mosquitoes is mediated by skin bacterial volatiles. -*Medical and veterinary entomology*, 31: 320-326.

Cichon, M. and Dubiec, A. 2005. Cell-mediated immunity predicts the probability of local recruitment in nestling blue tits. -*Journal of evolutionary biology*, 18: 962-6.

Costopoulos, K., Kovacs, J. L., Kamins, A. and Gerardo, N. M. 2014. Aphid facultative symbionts reduce survival of the predatory lady beetle *Hippodamia convergens*. -*BMC ecology*, 14.

Cramp, S. 1998. The complete birds of the Western Palearctic. Optimedia. Oxford University Press.

Devaynes, A., Antunes, A., Bedford, A. and Ashton, P. 2018. Bacterial species richness at three stages of the breeding season in *Cyanistes caeruleus* (blue tit). -Acta Oecologica-*International journal of ecology*, 92: 123-130.

Devriese, L. A., Ducatelle, R., Uyttebroek, E. and Haesebrouck, F. 1991. *Enterococcus*-hirae infection and focal necrosis of the brain of chicks. -*Veterinary Record*, 129: 316-316.

Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M. and Xavier, J. B. 2012. Animal behavior and the microbiome. -*Science*, 338: 198-199.

Ezenwa, V. O. and Williams, A. E. 2014. Microbes and animal olfactory communication: Where do we go from here? -*Bioessays*, 36: 847-854.

Fogarty, D. T., Elmore, R. D., Fuhlendorf, S. D. and Loss, S. R. 2017. Influence of olfactory and visual cover on nest site selection and nest success for grassland-nesting birds. -*Ecology and evolution*, 7: 6247-6258.

Gow, E. A., Wiebe, K. L. and Musgrove, A. 2015. Nest sanitation in response to short- and long-term changes of brood size: males clean more in a sex-role-reversed species. -*Animal Behaviour*, 104: 137-143.

Guigueno, M. F. and Sealy, S. G. 2012. Nest sanitation in passerine birds: implications for egg rejection in hosts of brood parasites. -*Journal für Ornithologie*, 153: 35-52.

Gwinner, H. and Berger, S. 2005. European starlings: nestling condition, parasites and green nest material during the breeding season. -*Journal of Ornithology*, 146: 365-371.

Hart, B. 1997. Behavioural defence. -Host-parasite evolution: general principles and avian models. Oxford University Press, Oxford: 59-77.

Heise-Pavlov, S. R. 2016. Evolutionary Aspects of the Use of Predator Odors in Antipredator Behaviors of Lumholtz's Tree-Kangaroos

(*Dendrolagus lumholtzi*). Chemical Signals in Vertebrates 13. Springer, pp. 261-280.
Herrick, F. H. 1900. Care of Nest and Young. -*Auk*, 17: 100-103.
Hooper, L. V., Littman, D. R. and Macpherson, A. J. 2012. Interactions between the microbiota and the immune system. -*Science*, 336: 1268-73.
Ibáñez-Álamo, J. D., Ruiz-Rodríguez, M. and Soler, J. J. 2014a. The mucous covering of fecal sacs prevents birds from infection with enteric bacteria. -*Journal of avian biology*, 45: 354-358.
Ibánez-Álamo, J. D., Ruiz-Raya, F., Roncalli, G. and Soler, M. 2014b. Is nest predation an important selective pressure determining fecal sac removal? The effect of olfactory cues. -*Journal für Ornithologie*, 155: 491-496.
Ibáñez-Álamo, J. D., Ruiz-Raya, F., Rodríguez, L. and Soler, M. 2016. Fecal sacs attract insects to the nest and provoke an activation of the immune system of nestlings. -*Frontiers in zoology*, 13: 9.
Ibáñez-Álamo, J. D., Rubio, E. and Soler, J. J. 2017. Evolution of nestling faeces removal in avian phylogeny. -*Animal Behaviour*, 124: 1-5.
Jacob, S., Parthuisot, N., Vallat, A., Ramon-Portugal, F., Helfenstein, F., Heeb, P. and Martin, L. 2015. Microbiome affects egg carotenoid investment, nestling development and adult oxidative costs of reproduction in Great tits. -*Functional Ecology*, 29: 1048-1058.
Lang, J. D., Straight, C. A. and Gowaty, P. A. 2002. Observations of fecal sac disposal by Eastern Bluebirds. -*Condor*, 104: 205-207.
Leclaire, S., Jacob, S., Greene, L. K., Dubay, G. R. and Drea, C. M. 2017. Social odours covary with bacterial community in the anal secretions of wild meerkats. -*Scientific reports*, 7: 13.
Lemfack, M. C., Nickel, J., Dunkel, M., Preissner, R. and Piechulla, B. 2014. mVOC: a database of microbial volatiles. -Nucleic Acids Res., 42: D744-D748.
Lombardo, M. P., Thorpe, P. A., Cichewicz, R., Henshaw, M., Millard, C., Steen, C. and Zeller, T. K. 1996. Communities of cloacal bacteria in Tree Swallow families. -*Condor*, 98: 167-172.
Lopez-Rull, I., Gil, M. and Gil, D. 2007. Spots in starling *Sturnus unicolor* eggs are good indicators of ectoparasite load by *Carnus hemapterus* (Diptera : Carnidae). -*Ardeola*, 54: 131-134.
Martín-Gálvez, D., Pérez-Contreras, T., Soler, M. and Soler, J. J. 2011. Benefits associated with escalated begging behaviour of black-billed magpie nestlings overcompensate the associated energetic costs. -*Journal of Experimental Biology*, 214: 1463-1472.
Martín-Gálvez, D. and Soler, J. J. 2016. Decoding colouration of begging traits by the experimental addition of the appetite enhancer cyproheptadine hydrochloride in magpie (*Pica pica*) nestlings. -*Journal of avian biology*, 48: 353-361.

Martín-Vivaldi, M., Soler, J. J., Peralta-Sánchez, J. M., Arco, L., Martín-Platero, A. M., Martínez-Bueno, M., Ruiz-Rodríguez, M. and Valdivia, E. 2014. Special structures of hoopoe eggshells enhance the adhesion of symbiont-carrying uropygial secretion that increase hatching success. -*Journal of Animal Ecology*, 83: 1289-301.

Mcfall-Ngai, M., Hadfield, M. G., Bosch, T. C., Carey, H. V., Domazet-Loso, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E., Rawls, J. F., Reid, A., Ruby, E. G., Rumpho, M., Sanders, J. G., Tautz, D. and Wernegreen, J. J. 2013. Animals in a bacterial world, a new imperative for the life sciences. -*Proceedings of the National Academy of Sciences of the United States of America*, 110: 3229-36.

Mennerat, A., Mirleau, P., Blondel, J., Perret, P., Lambrechts, M. M. and Heeb, P. 2009a. Aromatic plants in nests of the blue tit *Cyanistes caeruleus* protect chicks from bacteria. -*Oecologia*, 161: 849-55.

Mennerat, A., Perret, P., Bourgault, P., Blondel, J., Gimenez, O., Thomas, D. W., Heeb, P. and Lambrechts, M. M. 2009b. Aromatic plants in nests of blue tits: positive effects on nestlings. -*Animal Behaviour*, 77: 569-574.

Mihailova, M., Berg, M. L., Buchanan, K. L. and Bennett, A. T. D. 2018. Olfactory eavesdropping: The odor of feathers is detectable to mammalian predators and competitors. -*Ethology,* 124: 14-24.

Mills, T. K., Lombardo, M. P. and Thorpe, P. A. 1999. Microbial colonization of the cloacae of nestling tree swallows. -*Auk*, 116: 947-956.

Møller, A. P., Czirjak, G. A. and Heeb, P. 2009. Feather micro-organisms and uropygial antimicrobial defences in a colonial passerine bird. -*Functional Ecology*, 23: 1097-1102.

Møller, A. P., Peralta-Sánchez, J. M., Nielsen, J. T., López-Hernández, E. and Soler, J. J. 2012. Goshawk prey have more bacteria than non-prey. -*Journal of Animal Ecology*, 81: 403-410.

Moreno, J., Merino, S., J Sanz, J., Arriero, E., Morales, J. and Tomás, G. 2005. Nestling cell-mediated immune response, body mass and hatching date as predictors of local recruitment in the pied flycatcher *Ficedula* . -*Journal of avian biology*, 36: 251-260.

Peralta-Sánchez, J. M., Møller, A. P., Martín-Platero, A. M. and Soler, J. J. 2010. Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: an experimental study. -*Functional Ecology*, 24: 426-433.

Peralta-Sánchez, J. M., Martín-Platero, A. M., Wegener-Parfrey, L., Martínez-Bueno, M., Rodríguez-Ruano, S., Navas-Molina, J. A., Vazquez-Baeza, Y., Martín-Galvez, D., Martín- Vivaldi, M., Ibánez-Álamo, J. D., Knight, R. and Soler, J. J. 2018. Bacterial density rather than diversity

correlates with hatching success across different avian species. -*FEMS microbiology ecology*, 94: 13.

Petit, K. E., Petit, L. J. and Petit, D. R. 1989. Fecal sac removal: Do the pattern and distance of dispersal affect the chance of nest predation? -*Condor*, 91: 479-482.

Polin, S., Le Gallic, J. F., Simon, J. C., Tsuchida, T. and Outreman, Y. 2015. Conditional Reduction of Predation Risk Associated with a Facultative Symbiont in an Insect. -*PLoS One*, 10: e0143728.

Quan, R. C., Li, H., Wang, B. and Goodale, E. 2015. The relationship between defecation and feeding in nestling birds: observational and experimental evidence. -*Frontiers in zoology*, 12: 7.

Rubio, E., Sanllorente, O., Tieleman, B. I. and Ibáñez-Álamo, J. D. 2018. Fecal sacs do not increase nest predation in a ground nester. -*Journal für Ornithologie*, 1-6.

Ruiz-Castellano, C., Tomás, G., Ruiz-Rodríguez, M., Martín-Galvez, D. and Soler, J. J. 2016. Nest Material Shapes Eggs Bacterial Environment. -*PLoS One*, 11: e0148894.

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

Schulte, B. A., Goodwin, T. E. and Ferkin, M. H. 2016. Chemical Signals in Vertebrates 13, Springer.

Soler, J. J., De Neve, L., Perez-Contreras, T., Soler, M. and Sorci, G. 2003. Trade-off between immunocompetence and growth in magpies: an experimental study. -*Proceedings of the Royal Society of London. Series B: Biological Sciences*, 270: 241-8.

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

Soler, J. J., Martín-Vivaldi, M., Peralta-Sánchez, J. M. and Ruiz-Rodríguez, M. 2010. Antibiotic-producing bacteria as a possible defence of birds against pathogenic microorganisms. -*The open ornithology journal*, 3: 93-100.

Soler, J. J., Peralta-Sánchez, J. M., Flensted-Jensen, E., Martín-Platero, A. M. and Møller, A. P. 2011. Innate humoural immunity is related to eggshell bacterial load of European birds: a comparative analysis. -*Naturwissenschaften*, 98: 807-813.

Soler, J. J., Peralta-Sánchez, J. M., Martín-Platero, A. M., Martín-Vivaldi, M., Martínez-Bueno, M. and Møller, A. P. 2012a. The evolution of size of the uropygial gland: mutualistic feather mites and uropygial secretion

reduce bacterial loads of eggshells and hatching failures of European birds. -*Journal of Evolutionary Biology*, 25: 1779-1791.

Soler, J. J., Peralta-Sánchez, J. M., Martín-Vivaldi, M., Martín-Platero, A. M., Flensted-Jensen, E. and Møller, A. P. 2012b. Cognitive skills and bacterial load: comparative evidence of costs of cognitive proficiency in birds. -*Naturwissenschaften*, 99: 111-122.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martín-Vivaldi, M., Martínez-De La Puente, J., Ruiz-Rodríguez, M. and Tomás, G. 2017. Telomere length and dynamics of spotless starling nestlings depend on nest-building materials used by parents. -*Animal Behaviour*, 126: 89-100.

Szép, T. and Møller, A. P. 1999. Cost of parasitism and host immune defence in the sand martin *Riparia riparia*: a role for parent-offspring conflict? -*Oecologia*, 119: 9-15.

Theis, K. R., Venkataraman, A., Dycus, J. A., Koonter, K. D., Schmitt-Matzen, E. N., Wagner, A. P., Holekamp, K. E. and Schmidt, T. M. 2013. Symbiotic bacteria appear to mediate hyena social odors. -*Proceedings of the National Academy of Sciences of the United States of America* , 110: 19832-19837.

Tomás, G. and Soler, J. J. 2016. Begging and ectoparasite attraction. -*Animal Behaviour*, 113: 93-98.

Valera, F., Hoi, H., Darolova, A. and Kristofik, J. 2004. Size versus health as a cue for host choice: a test of the tasty chick hypothesis. -*Parasitology*, 129: 59-68.

Veiga, J. P. and Polo, V. 2016. Estornino Negro - *Sturnus unicolor*. In: A., M., M.B. (EDS.) (ed.) Enciclopedia Virtual de los Vertebrados Españoles. Museo de Ciencias Naturales, Madrid, España., En: Salvador.

Verhulst, N. O., Qiu, Y. T., Beijleveld, H., Maliepaard, C., Knights, D., Schulz, S., Berg-Lyons, D., Lauber, C. L., Verduijn, W., Haasnoot, G. W., Mumm, R., Bouwmeester, H. J., Claas, F. H. J., Dicke, M., Van Loon, J. J. A., Takken, W., Knight, R. and Smallegange, R. C. 2011. Composition of Human Skin Microbiota Affects Attractiveness to Malaria Mosquitoes. -*Plos One*, 6.

Weatherhead, P. J. 1984. Fecal sac removal by tree swallows - the cost of cleanliness. -*Condor*, 86: 187-191.

Westneat, D. F. and Rambo, T. B. 2000. Copulation exposes female Red-winged Blackbirds to bacteria in male semen. -*Journal of avian biology*, 31: 1-7.

Annex: Influence of the experimental treatment that nest-boxes received during the first breeding attempt on reproductive outcomes of the second breeding attempt.

We found that none of the considered measures of second breeding attempt was affected by the treatment that the next-box received during the first breeding attempt **(**Table A1A, Table A1B). Neither, the experimental treatment during the first breeding attempt influenced probability of predation (GLZ, $\chi^2 = 0.91$, P = 0.341).

Table A1A. Means and standard deviation of the log-transformed bacterial loads (mesophilic bacteria (TSA), enterobacteria (HK), staphylococci (VJ) and enterococci (KF)) in Control (C) and Experimental (E) nest-boxes the day after hatching in first (C_1 and E_1) and second (C_2 and E_2) breeding attempts.

$Treatment_{Brood}$	N	$Mean(SD)_{TSA}$	$Mean(SD)_{HK}$	$Mean(SD)_{VJ}$	$Mean(SD)_{KF}$
C_1	24	0.66(0.10)	0.26(0.24)	0.07(0.15)	0.61(0.10)
E_1	31	0.67(0.11)	0.23(0.28)	0.12(0.17)	0.63(0.06)
C_2	24	0.73(0.10)	0.35(0.29)	0.31(0.24)	0.63(0.15)
E_2	30	0.77(0.11)	0.38(0.28)	0.28(0.22)	0.64(0.11)

Table A1B. Results from GLM models testing whether the experimental treatment of nests during the first breeding attempt influenced detected effects during the second attempt. Models include experimental treatment of the first breeding attempt as the independent fixed factor, and tarsus length, body mass and *Carnus* bites of nestlings as dependent variables. Average nest values were used in the analyses. In the case of the bacterial load, we analysed estimations for day 1 of the nestling periods, before the start of the experiment.

Variable	Beta(SE)	DF	F	P
TSA Media	-0.22(0.13)	1,52	2.77	0.102
HK Media	-0.06(0.14)	1,52	0.22	0.644
VJ Media	0.08(0.14)	1,52	0.33	0.570
KF Media	-0.04(0.14)	1,52	0.10	0.760
Fledging success	0.16(0.14)	1,53	1.40	0.241
Tarsus	0.01(0.17)	1,35	0.00	0.957
Body mass	0.13(0.17)	1,35	0.61	0.439
Carnus Bites	0.03(0.17)	1,35	0.04	0.838

CAPÍTULO 3

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The antimicrobial capacity of spotless starling (*Sturnus unicolor*) females is related to their reproductive success and tegument colouration.

Abstract

Pathogenic microorganisms select for a plethora of defensive mechanisms on their hosts. Antimicrobial capacity of individuals typically predicts reproductive success, and dynamically adapts to changing environments. Moreover, flashy traits might signal antimicrobial capacity and, thus, be favoured in scenarios of sexual selection. These associations are well known for males, but evidences are scarce for females. We here explore those predictions on spotless starling (*Sturnus unicolor*) females.

We studied the antimicrobial properties of blood plasma through direct tests of inhibition of 12 bacterial strains, and quantified the non-specific first barrier of protection of hosts against microbial parasites, the constitutive innate immunity. In addition, we measured the uropygial gland and quantified the volume of secretion as a proxy to defence against feather degrading bacteria. On the other hand, we measured colourations of throat and back feathers, legs, and beak. All these information were collected, first, during the mating period, and later during the nestling stage.

We found an increase in the potential of bacterial inhibition of the plasma from the mating to the nestling period, and a decrease on the plasma capacity of antigens agglutination. During the mating period, the plasma antimicrobial potential, as well as the immune response, are positively related to the reproductive success of females. In addition, the level of antimicrobial defences were related to coloration of different teguments (legs, beak and back feathers). Therefore, similar to males, females may show their capacity of

fighting against microbial infections through their physical appearance, information that could be evaluated by their potential partners.

Keywords: Animal communication, Antimicrobial defences, Sexual selection, *Spotless starlings*

Introduction

The exponential increase of studies on animal-microorganisms interactions during the last years, has become us aware of the importance of bacteria in shaping hosts' life histories (e.g. McFall-Ngai et al. 2013, Rosenberg and Zilber-Rosenberg 2016, Douglas 2018). Among microorganisms, there are plenty of pathogenic strains that may cause serious problems and even death (Gyles et al. 2010). Therefore, animals has developed a wide array of defensive mechanisms to prevent bacterial infections, or reduce their negative effects. The complex immune system is the main antimicrobial barrier (Schmid-Hempel 2003). Specifically, the constitutive innate immunity provides the first-line of protection against invading microbes, through different mechanisms such as natural antibodies and complements (Thornton et al. 1994, Carroll and Prodeus 1998, Ochsenbein and Zinkernagel 2000, Matson et al. 2005).

Another defensive line that contribute to prevent bacterial infections in animals are exocrine glands, as it is the case of the uropygial gland of birds (Jacob and Ziswiler 1982, Shawkey et al. 2003). The uropygium is a holocrine complex situated at the base of the tail. Its relative size greatly varies among species and is associated with their life history characteristics, probability of infection, and production of natural antibodies (Haribal et al. 2005, Vincze et al. 2013). This gland produces a sebaceous and hydrophobic secretion with antibiotic activity mainly composed of aliphatic alcohols, fatty acids and glycerides (Jacob and Ziswiler 1982, Martín-Vivaldi et al. 2010, Moreno-Rueda 2017). Because of the presence of antibiotic compounds in the secretion (Martín-Platero et al. 2006; Martín-Vivaldi et al. 2010; M. Ruiz-Rodríguez et al. 2009; Ruiz-Rodríguez et al. 2013), the gland is directly implicated in bacterial load regulation of the eggshell (Soler et al. 2012) and feathers (Shawkey et al. 2003, Møller et al. 2009). Actually, the amount of secretion produced is negatively related to the plumage bacterial load in spotless

starlings (*Sturnus unicolor*) (Ruiz-Rodríguez et al. 2015). Therefore, by considering together estimates on immune response and uropygial gland measurements, we may properly characterize the antimicrobial capacity of individual birds.

These antimicrobial defences are costly to produce (Habig and Archie 2015) and, thus, its expression is mostly parasite context dependent. Indeed, animals modulate their investment on antiparasitic defences according to environmental factors that might reflect probability of infections (Capilla-Lasheras et al. 2017, Ruiz-Rodriguez et al. 2017). Abiotic conditions, such as temperature and humidity, typically influence microbial communities of their avian hosts (Cook et al. 2005a, Peralta-Sánchez et al. 2012) and, thus, are partially responsible of the phenological variation of microbial communities even within the same locality (Bisson et al. 2007). Therefore, individuals should adjust their potential defensive capacities to abiotic and biotic changes during the reproductive period that influence probability of bacterial infection. However, immunological responses and reproduction are costly activities and might be involved in a trade-off (Harshman and Zera 2007). In accordance with a seasonal variation in immunocompetence, we for instance know that magpie (*Pica pica*) nestlings of delayed clutch showed lower immune response (Sorci et al. 1997), while those of barn swallows showed the opposite pattern, likely as a result of adjusting immune response to probability of pathogenic infections (Merino et al. 2000). Moreover, because only animal in prime condition would be able of mounting strong immune responses, those with higher antimicrobial capacities should be those of higher phenotypic quality and stronger condition (Schmid-Hempel 2003). In the nests, high microbial loads may negatively influence eggs (Cook et al. 2005b) and nestlings (Azcárate-García et al. 2019) development. Adults might transmit pathogenic microorganisms to their offspring while incubating, brooding or feeding their offspring in the nests. Therefore, those with higher level of antimicrobial

defences will experience lower probability of pathogens' transmission to their offspring thereby increasing the reproductive success.

Communicating abilities and phenotypic conditions to conspecifics is important in different social contexts. It for instance reduces the strength of agonistic interactions among conspecifics, influences mate choice, or selects for differential investment in reproduction of partners in scenarios of intra- and inter-sexual selection (Andersson 1994, Westneat 2012). The study of evolution of traits indicating individuals' quality (including antiparasitic defences) through sexual selection has been mostly focussed on males (Andersson and Simmons 2006). That was also the case in birds, where males typically are the extravagant and singing sex (Dale et al. 2015, Romano et al. 2017). Although conspicuous female ornamentation was first explained as a consequence of genetic correlation with males (Bonduriansky and Chenoweth 2009, Dale et al. 2015), nowadays, it is accepted that elaborate female traits might evolve independently of those of males (de Neve *et al.* 2007; Soler and Moreno 2012; Price 2015; Webb *et al.* 2016). Selection pressures acting on females may differ from those acting on males, mainly during mating and reproduction, because of sexual differences in hormones' profiles and reproductive duties. Consequently, information provided by equivalent sexual characters in males and females might also differ sexually (Tobias et al. 2012). Therefore, studies on female signalization are needed to complete the whole view of social and sexual selection, conventionally focused only in male ornamentation.

Female plumage coloration have been shown to indicate their status in social interactions (Murphy et al. 2009a, b, Tobias et al. 2012), and to play a role in sexual selection (Amundsen et al. 1997, Soler et al. 2019). Females' ornaments may for instance reflect fecundity, and males prefer ornamented partners for reproduction (Clutton-Brock 2007). In accordance, the carotenoid-based coloration of blue tit (*Cyanistes caeruleus*) females is positively

associated to reproductive success (clutch size, fledgling success and recruitment), while their UV-blue coloration is positively correlated to survival and laying date (Doutrelant et al. 2008). Moreover, ornamental plumage of females is also related to immunocompetence and capacity of defending themselves from parasites (Amundsen 2000, Roulin et al. 2001, Morales et al. 2007). Similarly, some other ornamental structures, as flamboyantly coloured naked parts of the body (i.e., without feathers), also transmit information about females quality. It occurs for instance with the foot blue colouration of the blue-footed boobies (*Sula nebouxii*) (Torres and Velando 2005), or with the carotenoids-based colouration of the beaks of mallard ducks (*Anas platyrhynchos*) (Butler and McGraw 2011) and zebra finches (*Taeniopygia guttata*) (Alonso-Alvarez et al. 2004).

In the present work, we aimed to study different characteristics related to antimicrobial defences in spotless starling females in relation to traits that might function as signals of these capacities. We paid special attention to possible seasonal changes in the strength of the expected associations. Particularly, we compared (i) antimicrobial defences of females during mating and reproduction, and (ii) explored the relationships between antimicrobial defences before and during reproduction and reproductive success. We also explored the association (iii) between level of antimicrobial defences before and during reproduction, and females' characteristics (different body colourations) possibly reflecting their immune capacities.

Spotless starling females wear a dark, almost black, plumage. During the non-reproductive period, the beak and legs of starlings are black but, some weeks before reproduction, it turns to flamboyant colourations. The legs turn to conspicuous red colouration in both sexes, the beak base became blue in males and pink in females, and the beak tip turns from black to yellow in both sexes (Cramp 1998). Interestingly, during the mating period, the beak tip colour intensity reflects the plasma concentration of carotenoids and vitamin

A in both sexes (Navarro et al. 2010) and, thus, it could also reflect immune capacity. As a proxy of antimicrobial defences, we measured the direct capacity of blood plasma to inhibit bacterial growth by confronting the plasma with different standard bacterial strains in antagonistic plates. In addition, we measured two humoral components of the constitutive innate immunity: natural antibodies and complements. Furthermore, we measured the uropygial gland volume and quantified the amount of preen secretion produced. As traits that could reflect the quality of females, we measured the colours of different body parts: throat and back feathers, legs, and beak (tip and base).

Material and Methods

Study area and species

The field work was carried out during the years 2015 and 2016, in the Hoya de Guadix (Granada, South-eastern Spain, 37º15'N, 3º01'W), where there are placed more than 100 nest-boxes (internal height * width * depth: 350 * 180 * 210 mm; hole height: 240 mm.) attached to tree trunks or walls at 3-4 m above-ground, which are frequently used by spotted starlings to breed in (see Soler *et al.* (2017) for further information). There, starlings start the reproduction in early April, and most individuals lay a second clutch during May-June. The incubation is mainly performed by females with sporadic help from males, and extends for around 14 days. Nestlings stay in the nests around 21-22 days (Cramp 1998).

Starlings' captures and sampling

During March, and with time gap of two weeks, we carried out two journeys of capture in the study area. Briefly, one hour before dawn, we closed the entrance of all nest boxes in the study area, and immediately after dawn, we captured all individuals roosting inside. Captured birds were kept in clean

cotton bags separately, hanging from a stick to keep birds quiet, and after sampling, they were immediately released (Ruiz-Rodríguez et al. 2015). During the nesting period, 4-5 days after hatching, we did another capture attempt by using nest-box traps for a maximum time of one hour, both in the first (at the end of April) and the second (around mid-June) clutches. Apparently, those procedures does not result in negative effects on breeding performance of captured birds (Soler et al. 2008).

Captured individuals were ringed and their uropygial gland measured (height, width, and length) to estimate its volume by following Martín-Vivaldi *et al.* (2009). The uropygial gland secretion was extracted by putting a sterile micro-capillary (32mm/20µl) at the gland opening and slightly pressing the gland to completely remove its secretion. The length of the capillary with secretion was used as proxy of secretion volume. The uropygial gland measures were taken only in individual captured during the mating period, i.e., before breeding. Afterwards, we collected blood samples from the brachial vein by filling two-three heparinized capillary (75mm/75µl) that were emptied into microfuge tubes and kept in portable fridges at 4 ºC. Later, within 6 hours, tubes were centrifuged to separate the plasma from cellular blood fraction, which was kept at -80 ºC until analyses.

Finally, we measured colouration of the beak tip and base, legs and body feathers (back and breast) of females by means of an Ocean Optics S2000 spectrometer connected to a deuterium-halogen light (D2-W, Mini). To standardize ambient light conditions, we used a black bag that wrapped the tip of the probe containing optical fibres and the female body part measured. Before the measurement of each individual, we calibrated the spectrometer using a standard white and black reference. We obtained reflectance spectra at 1 nm intervals from 300-700 nm for all measurements. The colour was measured three times on each part, and then the average calculated. We estimated chromatic colouration as the proportion of total reflectance within

the ultraviolet (UV) (λ = 300-400 nm), UV-blue (λ = 300-450), blue (λ = 400-475 nm), blue-green (λ = 450-570 nm) and yellow-red (λ = 570-700nm), ranges of the tetrachromatic avian visual spectra. Achromatic colouration (i.e., brightness) was estimated as average reflectance value across the entire spectrum (λ = 300-700 nm). Prior to analysis, negative values were setting to zero and reflectance curves were corrected for noise by using triangular smoothing (Gómez 2006). Since the six colour variables might be related to each other within each measured body part (i.e., back, leg, breast, beak base and beak tip), we ran a Principal Component Analysis (PCA) to reduce the number of dependent variables and assure statistical independence among them. PCA factors were rotated (varimax normalized), and their significance established by cross-validation (McGarigal et al. 2000). Only those PCA factors with Eigenvalues higher than 1 were considered as summarising colour variance of each measured body parts (Table 1).

Finally, to estimate variables related to reproductive success, all the nests were visited every third day from the end of March to detect the beginning of the egg-laying, and then they were periodically visited to estimate the total number of eggs until the end of laying. Nest-boxes were again visited 12 days after the 4th egg was laid and, afterward, every single day until hatching, to estimate the number of hatched nestlings and, thus, hatching success, calculated as the number of hatched eggs divided by the clutch size. Then, nests were again visited the day 14 after hatching, to assess the number of nestlings, and thus, the fledging success, calculated as number of nestlings that fledge divided by the number of hatched eggs.

The identification of females during breeding was performed by capturing them at the nest boxes during the nesting phase (see above), or by visualizing the unique combinations of colour rings in 1h video recordings of nest-box entrances during the second-third day after hatching.

Table 1. Results of Principal Component Analysis (PCA) summarizing all the colour measurements. For each PCA factor, the loading coefficients, the power and relative importance of each colour, are represented. Also, the explained variance and Eigenvalues.

				Loadings	
		Power	**Importance**	**PCA1**	**PCA2**
Back	**Brightness**	0.30	6	0.55	0.09
	Yellow-Red	0.96	3	-0.98	-0.08
	UV Chroma	0.98	2	0.68	-0.72
	UV Blue	0.99	1	0.86	-0.50
	Blue-Green	0.93	4	0.63	0.73
	Blue	0.93	5	0.89	0.37
Variance explained	**%**			0.61	0.24
	Total			0.85	
Eigenvalues				3.65	1.46
Legs	**Brightness**	0.88	6	0.67	
	Yellow-Red	0.99	1	-0.99	
	UV Chroma	0.83	4	0.91	
	UV Blue	0.92	3	0.96	
	Blue-Green	0.84	5	0.83	
	Blue	0.97	2	0.98	
Variance explained	**%**			0.80	
	Total			0.80	
Eigenvalues				4.80	
Throat	**Brightness**	0.43	6	0.66	-0.03
	Yellow-Red	0.94	3	-0.96	0.12
	UV Chroma	0.97	2	0.55	0.82
	UV Blue	0.98	1	0.81	0.58
	Blue-Green	0.92	4	0.58	-0.76
	Blue	0.90	5	0.87	-0.39
Variance explained	**%**			0.57	0.29
	Total			0.86	
Eigenvalues				3.41	1.76

				Loadings	
		Power	**Importance**	**PCA1**	**PCA2**
Beak base	**Brightness**	0.36	6	0.50	-0.34
	Yellow-Red	0.99	1	-0.97	-0.22
	UV Chroma	0.91	5	0.93	-0.24
	UV Blue	0.97	2	0.98	-0.11
	Blue-Green	0.95	3	0.07	0.97
	Blue	0.92	4	0.93	0.23
Variance explained	**%**			0.65	0.20
	Total			0.85	
Eigenvalues				3.89	1.23
Beak tip	**Brightness**	0.76	6	0.13	0.86
	Yellow-Red	0.98	1	-0.97	0.19
	UV Chroma	0.78	5	0.84	0.28
	UV Blue	0.94	2	0.95	0.21
	Blue-Green	0.79	4	0.69	-0.56
	Blue	0.82	3	0.90	0.02
Variance explained	**%**			0.64	0.20
	Total			0.85	
Eigenvalues				3.86	1.22

Laboratory procedures

To estimate the antimicrobial defences in the blood plasma, we performed antagonistic and immunological tests. Antagonistic tests were performed against twelve indicator bacteria belonging to different taxonomic groups: *Bacillus licheniformis*, *Bacillus megatherium*, *Bacillus thuringensis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Lactobacilus paracasei*, *Latobacillus plantarum*, *Lactococcus lactis*, *Listeria inocua*, *Listeria monocytogenes*, *Proteus sp*. and *Staphylococcus aureus*. Antagonistic plates were prepared as follows: 15 mL of a culture medium previously prepared and sterilized (1.8 % of brain–heart infusion (BHI) and 0.8 % agar in 0.1 M pH 7 phosphate buffer) was melted and then maintained at 50 °C for 10 min. Then, 100 µL of a 12-h culture of each indicator bacteria was added to the medium,

vigorously vortexed and spread onto a Petri dish. After solidifying about 30 min later, 2 mL of each plasma sample was placed on the plates and later incubated for 12 h at 28 °C. After incubation, plates were checked for inhibition halos, that is, transparent zones around the plasma in which the growth of the indicator bacterium was inhibited (Fig. 1). Halos were measured (in mm) from the limit of the plasma drop to the end of the halo (i.e., where the indicator bacteria growth begins). Antagonistic tests against each bacterium species were made to all the samples in the same randomly selected order. Then, an index of antagonistic activity was calculated as the average activity intensity (i.e., halo diameter) against the twelve indicator bacteria tested. For some of the samples, there was insufficient plasma for testing all indicator bacteria, and in those cases, the index was calculated with the number of tested bacteria. We performed antagonistic tests to 124 plasma samples from 77 females from both study years.

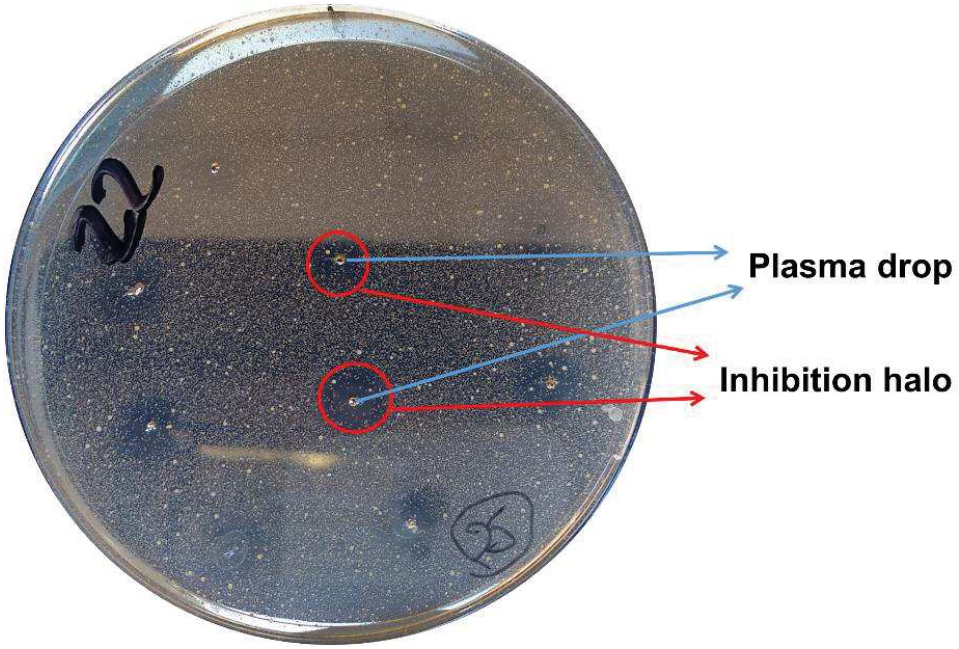


Fig. 1. Antagonistic plate in which an indicator bacteria strain was inoculated, and then a drop (2 µl) of plasma was deposited. After incubation, the clear halo around the plasma indicate its inhibition capacity.

The immune responses mediated by natural antibodies (NAb) and complement were estimated by following the procedure described in Matson, Ricklefs and Klasing (2005). The NAbs serve as a recognition molecules capable of opsonizing invading microorganisms and initiating the complement enzyme cascade, which ends in cell lysis. On the other hand, levels of complement are positively correlated with NAb diversity (Matson et al. 2005).

Briefly, 50 µL of plasma were serially diluted in sodium phosphate buffer (PBS) in two consecutive polystyrene 96-well assay plates where 25 µL of 1% rabbit blood cell suspension (Hemostat laboratories, Dixon, CA 95620, USA) in PBS was added. Quantification of lysis and agglutination titers was assessed as the number of titers with the last plasma dilution at which the lysis or agglutination reaction of rabbit blood was observed (Matson et al. 2005). In the case of samples that did not reach 50 µL, we used 25 or 12.5 µL in the second or third titer depending on availability. Since the lysis reaction occurred in the first titers and we did not have plasma in some of them, we counted until the last titer in which the plasma had any activity (i.e., at the end of the agglutination) to avoid bias due to the absence of plasma in the first titers. These values were used as indicators of immunocompetence (Fig. 2). The agglutination and lysis variables were not correlated in our samples ($F_{1,76}$ = 0.06, R^2 = 0.0009, P = 0.79, estimate (SE) = 1.61 (0.18)). We analysed 78 plasma samples belonging to 54 females in 2015.

Statistical analyses

To explore variation in the plasma antimicrobial defences along the breeding season, we used only the information of females that were captured twice during the same breeding season (Table 2). All considered females were first captured before reproduction, during the mating period, while the second capture of the same females occurred, either during reproduction (few days after hatching the eggs of first or second breeding events) or before

reproduction (two or three weeks after the first capture). Thus, to explore between captures differences in immune response, we used Repeated Measures ANOVAs (RMA) with the two subsequent measures of antimicrobial capacity and immune response (agglutination and lysis) as the within factor, and breeding status of females when the second captures (i.e., before breeding (N = 4), first (N = 7) or second (N = 9) clutch) as the between factor.

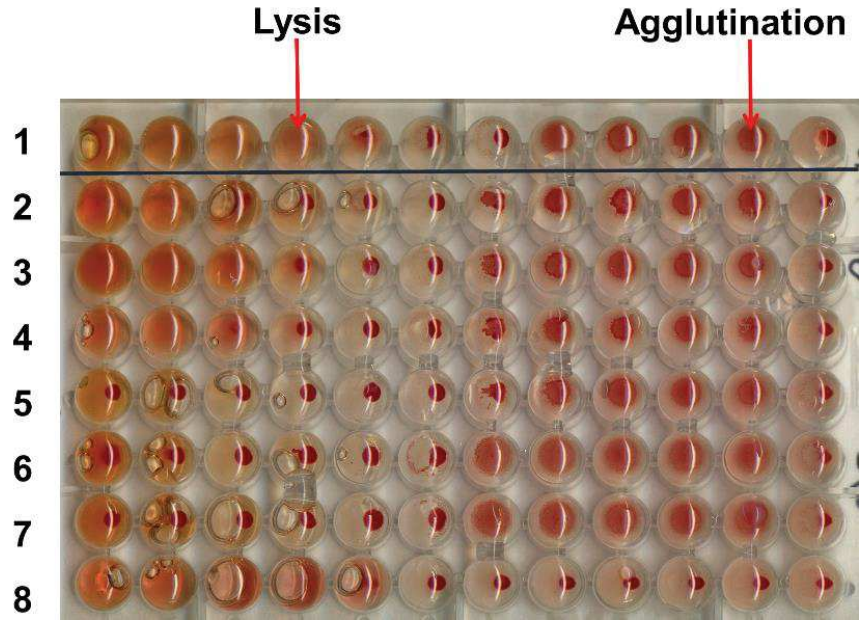


Fig. 2. Result of a plate with an immune essay. For example in line 1, the lysis value would be 4, and the agglutination 11.

Table 2. Number of females captured every year, before breeding started (BB), and during the first (C1) or second (C2) clutch. Also, the number of females captured before reproduction and first / second clutch.

		2015	**2016**	**Both years**	**Total**
Before Reproduction		44	31	14	61
First clutch		15	14	1	28
Second clutch		20	6	2	24
Total females		55	47	22	**80**
BB and	**C1**	5	1		6
	C2	10	3		13

To explore the association between antimicrobial defences and reproductive success, we performed General Linear Mixed Models (GLM) in which the dependent variable was each proxy of reproductive success: clutch size, brood size, hatching, and fledging success. Values of antimicrobial defences were introduced as continuous predictors (plasma antimicrobial activity, agglutination and lysis capacity, uropygial gland volume and preen secretion quantity), and the study year as the fixed factor (except for the analyses of the immune response which was sampled for a single study year). The individual identity was introduced as the random factor. All analyses were separately performed for first and second clutches (except in the uropygial gland measures, that were taken just before breeding).

Finally, to know if the antimicrobial capacity of females is reflected by their colouration, we performed GLMs in which the dependent variables were the PCA factors of colour measurements. As explanatory variables we used (i) the antagonistic index, (ii) the plasma immune variables (lysis and agglutination), and (iii) uropygial size and secretion as the continuous predictors. The effects of each of these continuous predictors on different colour variables were tested in separate models that also included study year and breeding status (i.e. before breeding, first or second clutch) as fixed categorical factors, and individual identity as the random factor.

Since different GLMs were implemented for each dependent variable, the false-discovery-rate (FDR) correction were applied to establish the appropriate Q values (Pike 2011). All the analyses were performed with Statistica 12 software (Dell-Inc. 2015).

Results

Fluctuation of immune capacities along the breeding season

The antimicrobial capacity of the females' blood plasma increases from first to second captures of the same individual during the breeding season (RMA, $F_{1,17}$ = 11.75, p = 0.003, Fig. 3A). Interestingly these between-captures differences did not depend of the breeding status of the second capture (i.e., the interaction between repeat measures and the breeding status of second time captured females; RMA, $F_{2,17}$ = 1.80, p = 0.19). Post-hoc analyses revealed that the detected increase in antimicrobial capacity from first to second clutch came from comparison of females that were first captured before breeding and again during first (Post-hocs, LSD Tests, p = 0.04) or second (Post-hocs, LSD Tests, p = 0.001) clutches. However, no differences were detected in those females captured twice before reprodcution (Post-hocs, LSD Tests, p = 0.65) (Fig. 3A). Therefore, the antimicrobial capacity of females' plasma is significantly higher during the nestling period than before breeding, independently of being the first or second clutch.

In the case of the natural antibodies, the lysis capacity of females' plasma from first and second captures did not differ (RMA, $F_{1,16}$ = 0.005, p = 0.94, Fig. 3B), independently of whether second time captured females were reproducing or mating (RMA, $F_{2,16}$ = 3.20, p = 0.06). Finally, the agglutination capacity of plasma of females decreased from first to second captures (RMA, $F_{1,16}$ = 5.47, p = 0.03, Fig. 3C), independently of the reproductive event at second captures (RMA, $F_{2,16}$ = 0.66, p = 0.52).

Relationship among immune capacities and reproductive success

The antimicrobial capacity of the females' blood plasma during the mating period resulted positively related to breeding and fledging success of second clutches even after statistically correcting for the significant effect of the study

year (Table 3). Clutch size of second broods marginally associated with the plasma lysis capacity of females during the mating period, while the plasma agglutination significantly explained the hatching success of the first clutch (Table 3). All the significant relationships were positive, suggesting that females with higher capacity of antimicrobial defence at the time of mating also experienced higher breeding success. However, antimicrobial capacity of females captured during the nestling period of first or second broods did not predict any of the variables related to breeding success (all $Q > 0.28$). Finally, after applying the FDR correction, none of used variables of reproductive success did associate with the uropygial gland size or the amount of preen secretion (all $Q > 0.16$).

Relationship among antimicrobial defences and colouration

We found that some of the variables describing level of antimicrobial defences of starling females were related to colorations of different body parts (see Table 4). Preen oil volume was positively associated to the back colouration PCA1, and the uropygial gland size negatively to the PCA1 of the base of the beak colouration. In this case, there was also a significant negative effect of the study year (Table 4 and A1), which means that in the second year (2016) the yellow-red intensity of the beak base in the population was higher than in 2015.

Moreover, the plasma agglutination capacity was positively associated with the legs colouration, i.e., negatively related to the yellow-red colouration but positively to the rest of the colours, including brightness (Table 1, 4 and A2). In addition, legs colouration was also negatively explained by the breeding status of the captured females (Table 4 and A2), which means that when the season progress, the legs yellow-red colouration increase, although the opposite occurs with the rest of the chroma.

Finally, we failed in detecting a relationship between the plasma antagonistic index and measured colour variables (Table A3).

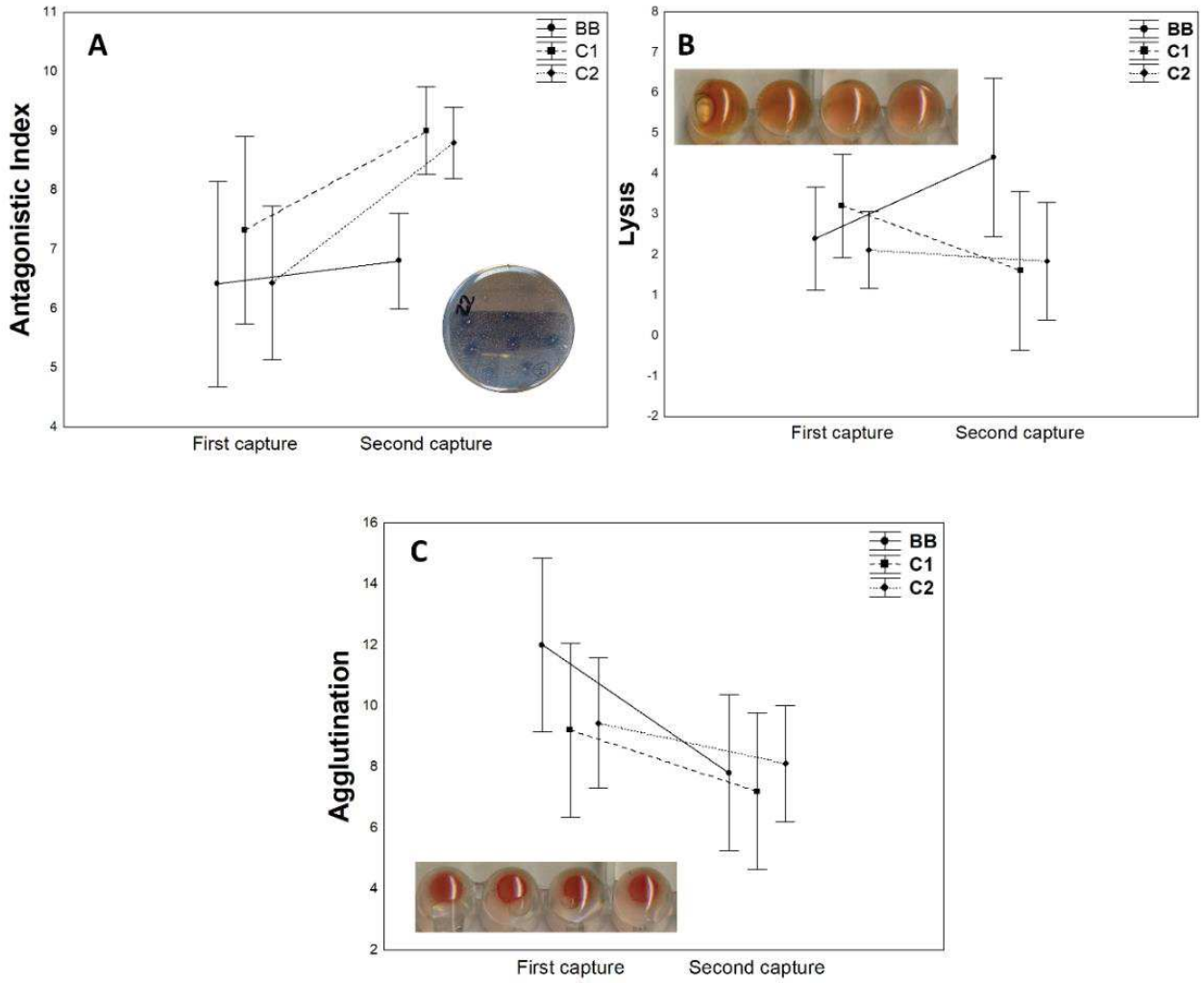


Fig. 3. Differences in the plasma capacity of (A) inhibition of bacterial pathogens, (B) lysis and (C) agglutination, in females captured twice, when the second capture was performed before breeding (BB), or with nestlings from the first (C1) or second clutch (C2). Vertical bars denote 0.95 confidence intervals.

Table 3. Results of GLM analysis on the association between the antimicrobial defences variables and the indicators of reproductive success. Q corresponds to p-values after the FDR correction. Values in bold are statistically significant, and in italics marginally significant.

Dependent variable	**Explaning factor**	**First Clutch**						**Second Clutch**					
		F	df	p	β	SE(β)	Q	F	df	p	β	SE(β)	Q
Clutch size	Antagonistic index	0.11	1,18	0.74	-0.16	0.5	0.74	0.33	1,10	0.57	0.55	0.96	0.66
	Year	0.16	1,18	0.69	-0.2	0.5	0.75	0.33	1,10	0.57	0.56	0.96	0.57
Hatching success	Antagonistic index	0.33	1,17	0.57	-0.29	0.5	0.74	0.21	1,9	0.66	0.46	1.02	0.66
	Year	0.09	1,17	0.75	-0.16	0.5	0.75	0.34	1,9	0.57	0.6	1.02	0.57
Fledgings number	Antagonistic index	4.06	1,18	0.05	-0.89	0.44	0.2	6.15	1,10	0.03	1.63	0.65	*0.06*
	Year	5.54	1.18	0.03	-1.04	0.44	0.12	9.85	1,10	0.01	2.06	0.65	**0.02**
Fledging success	Antagonistic index	1.09	1,15	0.31	-0.56	0.53	0.62	14.5	1,9	0.004	2.11	0.55	**0.016**
	Year	2.15	1,15	0.16	-0.73	0.53	0.32	20.59	1,9	0.001	2.52	0.55	**0.004**
Clutch size	Lysis	0.89	1,11	0.36	0.28	0.29	0.48	10.33	1,5	0.02	0.82	0.25	*0.08*
Hatching success		1.22	1,10	0.29	-0.33	0.29	0.48	1.02	1.5	0.35	-0.41	0.4	0.46
Fledgings number		2.39	1,11	0.15	-0.42	0.27	0.48	3.8	1.5	0.11	0.65	0.33	0.22
Fledging success		0.04	1,8	0.84	-0.07	0.35	0.84	0.33	1,5	0.58	-0.25	0.43	0.58
Clutch size	Agglutination	1.57	1,11	0.23	-0.35	0.28	0.36	0.1	1,5	0.76	-0.14	0.44	1
Hatching success		24.97	1,10	0.0005	0.84	0.17	**0.002**	1.46	1,5	0.28	0.47	0.39	1
Fledgings number		0.77	1,11	0.39	0.25	0.29	0.39	<0.001	1,5	1	<0.001	0.44	1
Fledging success		1.41	1,8	0.27	-0.38	0.32	0.36	0.31	1,5	0.6	-0.24	0.43	1

Table 4. Significant results of GLMs on the associations between the antimicrobial defences and the body coloration of starling females. Values in bold are statistically significant. The rest of the results are in Table S1, S2 and S3.

Explaining variable	Dependent variable	F	df	p	β	SE (β)	Q
Secretion volume	Back colouration (PCA1)	11.23	1,20	0.00	1.19	0.36	**0.04**
Uropygial gland size		5.87	1,20	0.03	0.37	0.15	0.13
Year		7.82	1,20	0.01	0.56	0.20	0.09
Individual		1.59	74,20	0.12	-0.08	0.14	0.31
Secretion volume	Beak base colouration (PCA1)	0.93	1,20	0.35	-0.25	0.26	0.62
Uropygial gland size		30.83	1,20	<0.001	-0.63	0.11	**<0.001**
Year		18.13	1,20	<0.001	-0.63	0.15	**<0.001**
Individual		2.48	74,20	0.01	0.09	0.10	0.07
Lysis	Leg colouration (PCA1)	3.25	1,15	0.09	0.22	0.12	0.30
Agglutination		11.45	1,15	<0.001	0.52	0.15	**0.04**
Breeding status		19.40	2,15	<0.001	-0.78	0.13	**<0.001**
Individual		3.08	50,15	0.01	-0.29	0.11	0.09

Discussion

Our main results indicate that antimicrobial defence of spotless starling females (i) vary depending on reproductive stages (i.e., mating vs breeding), and that (ii) level of antimicrobial defences during mating, but not that during reproduction, is related to reproductive success and (iii) to colouration of body feathers, beak base or legs. These results therefore confirm that antimicrobial capacities of females predict reproductive success, and that males would be able to infer such capacities from females' colourations. Below we discuss possible explanations of such results, as well as the possible role of females' colouration signalling their antimicrobial capacity in scenarios of sexual selection.

Females were captured first before breeding, and most of the recaptures took place during reproduction, when nestlings were 4-5 days old, either in the first or second broods. We detected significant differences in immune capacity of plasma from first and second time captured females. It is likely that abiotic (e.g. temperature, humidity) and biotic (e.g. females condition, microbial environment) factors that determine bacterial environment for females varied between captures. As the season progresses in our study population, temperature typically increases while relative humidity decrease, which should affect bacterial environment in general and that of avian nests in particular (Cook et al. 2005b, Peralta-Sánchez et al. 2012, Soler et al. 2015). Moreover, breeding activity such as incubation or brooding (Cook et al. 2005b, Peralta-Sánchez et al. 2012, Soler et al. 2015), ectoparasites (Tomás et al. 2018), egg breakage (Soler et al. 2015), or nestlings faeces that parent failed to remove (Ibáñez-Álamo et al. 2014), will also affect bacterial environment of nests. All those evidences together suggest that females experience higher probability of microbial infection during the nestling period than during the pre-breeding (i.e., mating) period. Thus, because immune responses are typically positively associated to the risk of infection (e.g. Moller

and Erritzoe 1996), immune response of starling females should also be higher during reproduction, when the risk of infection is the highest; prediction that our results on antimicrobial potential of plasma fulfilled. Contrary to predictions claiming that immune response should increase as the season progress, when considering costs of reproduction, the sign of the association might change because immunity is also costly (Sorci et al. 1997). In agreement with this prediction, immunocompetence of females tree swallows (*Tachycineta bicolor*) tended to decrease along the breeding season (Ardia et al. 2003), as well as occurred with the antigens agglutination capacity of our starling females, which were lower when reproducing than when mating. Thus, depending on the study species and the kind of immune response considered, the expected association between immunity and phenology me be positive, negative, or masked by costs associated to reproduction, or to different lines of immunity (Schmid-Hempel 2003, Soler et al. 2003, Martin et al. 2008). Whatever the reasons explaining the sign of the detected association between immunity and phenology, factors associated to reproduction, rather than those associated with climatic conditions, are likely the cause of the detected patterns. In agreement with this possibility, between-captures differences in plasma antimicrobial capacity of females that were recaptured during their first or second reproduction were similar. Likewise, antimicrobial capacity of plasma of females that were captured twice during the mating period (i.e., with two weeks of delay) did not change.

An alternative explanation that do not imply and adjustment of immunity to environmental conditions is that the immune capacity is the result of historical contacts with antigenic microorganisms. The plasma antibacterial activity integrates cytological (Keusch et al. 1975) and serological (Merchant et al. 2003) immune components. The previous exposure to microorganisms closely related to the indicator strains assayed may affect results on bacterial inhibition capacity (Matson et al. 2006), directly, by the production of specific

antibodies of IgY (Roitt 1997), or indirectly by increasing nonspecific antibodies (IgM, Reid *et al.* 1997). Infection status of bleeding birds might also determine antimicrobial capacity of animals (Millet et al. 2007). To overcome this potential problem, it is advisable to perform the assays with a range of different microorganisms to have a more complete picture of the whole response (Matson et al. 2006), as we did in our experiments. Our inference comes from interpreting results that integrates multiple components of innate immunity and assume that higher diversity of *in vitro* bacteria-killing capacity of indicator strains reflects a higher immune capacity of individuals (Matson et al. 2006). Thus, even though some of the detected bacterial inhibition might be due to historical contexts, it will hardly explain the detected patterns.

We have also found that the studied immunological variables of females at the time of mating predicted reproductive success. Thus, if females were able to show their immune capacity to conspecifics throughout some other characters, males that choose females with high immunocompetent values will enjoy direct and indirect fitness benefits (Horváthová et al. 2011, Tobias et al. 2012). Meanwhile, females with exaggerated traits would enjoy direct benefits from the expected differential investment in reproduction of their mates (Burley 1986, Sheldon 2000). Here we explored and found support to the possibility that coloured traits of females associated with immune responses. Our correlative results, however, do not allow us to reach to further conclusions on signalization of those characteristics, but urge for further studies that test the signalling roles of these traits.

Starling males show their capacity of fighting against bacterial infections through their ornamental throat feathers, which is not present in females (Ruiz-Rodríguez et al. 2015). Therefore, different selective pressures on both sexes may lead to a diversification on their way to reflect the same characteristics (Tobias et al. 2012). Previous works also showed that during the mating period, starlings (both females and males) reflect their plasma

concentration of carotenoids and vitamin A in the colouration intensity of the beak tip (Navarro et al. 2010). The uropygial gland size resulted related to the colouration of the beak base, which is a sexual dimorphic character in this species, and the agglutination capacity was related to legs colouration. Hence, starling females may be reflecting different capabilities through the colouration of different parts of the body. Thus, similarly to females, it is possible that males use multiple signalling characters in mate choice (Møller and Pomiankowski 1993, e.g. Candolin 2003, Loyau et al. 2005, Robson et al. 2005); a possibility that deserve further investigation.

To summarize, we found that starling females' antimicrobial related traits may change along the breeding season, and those females that are able to defend themselves from bacterial infections at a higher extent during the mating period, are those with higher reproductive success. In addition, their antimicrobial capacity is related to different body colours, which may open the possibility that sexual selection processes in females explain those colourations. In general, all those results support the crucial role of microorganisms in shaping hosts life-histories.

Acknowledgments

Estefanía López helped with the laboratory analysis, and Natalia Juárez and Carmen Soler Zamora participated in the starlings captures.

References

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Gaillard, M., Prost, J., Faivre, B. and Sorci, G. 2004. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. - *The American Naturalist*. 164: 651–659.

Amundsen, T. 2000. Why are female birds ornamented? - *Trends in Ecology*

& Evolution. 15: 149–155.
Amundsen, T., Forsgren, E. and Hansen, L. T. T. 1997. On the function of female ornaments: Male bluethroats prefer colourful females. - *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 264: 1579–1586.
Andersson, M. 1994. Sexual selection. - Princeton University Press.
Andersson, M. and Simmons, L. W. 2006. Sexual selection and mate choice. - *Trends in Ecology & Evolution* 21: 296–302.
Ardia, D. R., Schat, K. A. and Winkler, D. W. 2003. Reproductive effort reduces long-term immune function in breeding tree swallows (*Tachycineta bicolor*). - *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 270: 1679–1683.
Azcárate-García, M., Ruiz-Rodríguez, M., Díaz-Lora, S., Ruiz-Castellano, C. and Soler, J. J. 2019. Experimentally broken faecal sacs affect nest bacterial environment, development and survival of spotless starling nestlings. - *Journal of avian biology*. 50: 1–11.
Bisson, I. A., Marra, P. P., Burtt, E. H., Sikaroodi, M. and Gillevet, P. M. 2007. A molecular comparison of plumage and soil bacteria across biogeographic, ecological, and taxonomic scales. - *Microbial Ecology*. 54: 65–81.
Bonduriansky, R. and Chenoweth, F. 2009. Intralocus sexual conflict. - *Trends in Ecology & Evolution* 24: 280–288.
Burley, N. 1986. Sexual Selection for Aesthetic Traits in Species with Biparental Care. - *The American Naturalist* 127: 415–445.
Butler, M. W. and McGraw, K. J. 2011. Past or present? Relative contributions of developmental and adult conditions to adult immune function and coloration in mallard ducks (*Anas platyrhynchos*). - *Journal of Comparative Physiology B*. 181: 551–563.
Candolin, U. 2003. The use of multiple cues in mate choice. - *Biological reviews*. 78: 575–595.
Capilla-Lasheras, P., Dominoni, D. M., Babayan, S. A., O'Shaughnessy, P. J., Mladenova, M., Woodford, L., Pollock, C. J., Barr, T., Baldini, F. and Helm, B. 2017. Elevated immune gene expression is associated with poor reproductive success of urban blue tits. - *Frontiers in Ecology and Evolution*. 5: 1–13.
Carroll, M. C. and Prodeus, A. P. 1998. Linkages of innate and adaptive immunity. - Current opinion in immunology. 10: 36–40.
Clutton-Brock, T. 2007. Sexual selection in males and females. - *Science* (80-.). 318: 1882–1885.
Cook, M. I., Beissinger, S. R., Toranzos, G. A. and Arendt, W. J. 2005a. Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection. - *Ecology Letters*. 8: 532–537.
Cook, M. I., Beissinger, S. R., Toranzos, G. A., Rodriguez, R. A. and Arendt, W. J. 2005b. Microbial infection affects egg viability and incubation

behavior in a tropical passerine. - *Behavioral Ecology*. 16: 30–36.
Cramp, S. 1998. The complete birds of the Western Paleartic. - Oxford University Press.
Dale, J., Dey, C. J., Delhey, K., Kempenaers, B. and Valcu, M. 2015. The effects of life history and sexual selection on male and female plumage colouration. - *Nature* 527: 367–370.
Douglas, A. E. 2018. Fundamentals of Microbiome Science: How Microbes Shape Animal Biology. - Princeton University Press.
Doutrelant, C., Grégoire, A., Grnac, N., Gomez, D., Lambrechts, M. M. and Perret, P. 2008. Female coloration indicates female reproductive capacity in blue tits. - *Journal of evolutionary biology*. 21: 226–233.
Gómez, D. 2006. AVICOL, a program to analyse spectrometric data, free program available from the author at dodogomez@yahoo.fr. in press.
Gyles, C. L., Prescott, J. F., Songer, . G. and Thoen, C. O. 2010. Pathogenesis of bacterial infections in animals (CL Gyles, JF Prescott, . G. Songer, and CO Thoen, Eds.). - Blackwell Publishing.
Habig, B. and Archie, E. A. 2015. Social status, immune response and parasitism in males: A meta-analysis. - *Philosophical Transactions of the Royal Society B: Biological Sciences*. in press.
Haribal, M., Dhondt, A. A., Rosane, D. and Rodriguez, E. 2005. Chemistry of preen gland secretions of passerines: Different pathways to same goal? why? - *Chemoecology* 15: 251–260.
Harshman, L. G. and Zera, A. J. 2007. The cost of reproduction: the devil in the details. - *Trends in Ecology & Evolution* 22: 80–86.
Horváthová, T., Nakagawa, S. and Uller, T. 2011. Strategic female reproductive investment in response to male attractiveness in birds. - *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 279: 163–170.
Ibáñez-Álamo, J. D., Ruiz-Rodríguez, M. and Soler, J. J. 2014. The mucous covering of fecal sacs prevents birds from infection with enteric bacteria. - *Journal of avian biology*. 45: 354–358.
Jacob, J. and Ziswiler, V. 1982. The uropygial gland. - In: Frander, D. S. et al. (eds), Avian Biology. Academic Press, pp. 199–324.
Keusch, G. T., Douglas, S. D. and Ugurbil, K. 1975. Intracellular bactericidal activity of leukocytes in whole blood for the diagnosis of chronic granulomatous disease of childhood. - *Journal of Infectious Diseases*. 131: 584–587.
Loyau, A., Saint Jalme, M., Cagniant, C. and Sorci, G. 2005. Multiple sexual advertisements honestly reflect health status in peacocks (*Pavo cristatus*). - *Behavioral ecology and Sociobiology*. 58: 552–557.
Martín-Platero, A. M., Valdivia, E., Ruíz-Rodríguez, M., Soler, J. J., Martín-Vivaldi, M., Maqueda, M. and Martínez-Bueno, M. 2006. Characterization of antimicrobial substances produced by Enterococcus faecalis MRR 10-3, isolated from the uropygial gland of the hoopoe

(*Upupa epops*). - *Applied and Environmental Microbiology* . 72: 4245–4249.

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

Martín-Vivaldi, M., Peña, A., Peralta-Sánchez, J. M., Sánchez, L., Ananou, S., Ruiz-Rodríguez, M. and Soler, J. J. 2010. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. - *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 277: 123–130.

Martin, L. B., Weil, Z. M. and Nelson, R. J. 2008. Seasonal changes in vertebrate immune activity: Mediation by physiological trade-offs. - *Philosophical Transactions of the Royal Society B: Biological Sciences*. 363: 321–339.

Matson, K. D., Ricklefs, R. E. and Klasing, K. C. 2005. A hemolysis-hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. - *Developmental & Comparative Immunology*. 29: 275–286.

Matson, K. D., Tieleman, B. I. and Klasing, K. C. 2006. Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds. - *Physiological and Biochemical Zoology*. 79: 556–564.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E., Rawls, J. F., Reid, A., Ruby, E. G., Rumpho, M., Sanders, J. G., Tautz, D. and Wernegreen, J. J. 2013. Animals in a bacterial world, a new imperative for the life sciences. - *Proceedings of the National Academy of Sciences U. S. A*. 110: 3229–3236.

McGarigal, K., Cushman, S. and Stafford, S. 2000. Multivariate statistics for wildlife and ecology research. - Springer Verlag Heideberg.

Merchant, M. E., Roche, C., Elsey, R. M. and Prudhomme, J. 2003. Antibacterial properties of serum from the American alligator (*Alligator mississippiensis*). - *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 136: 505–513.

Merino, S., Møller, A. P. and de Lope, F. 2000. Seasonal changes in cell-mediated immunocompetence and mass gain in nestling barn swallows: a parasite-mediated effect? - Oikos 90: 327-332file:///C:/Users/magda/Documents/ARTÍCULOS/.

Millet, S., Bennet, J., Lee, K. A., Hau, M. and Klasing, K. C. 2007. Quantifying and comparing constitutive immunity across avian species. - *Developmental & Comparative Immunology*. 31: 188–201.

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

Møller, A. P. and Pomiankowski, A. 1993. Why have birds got multiple sexual ornaments? - *Behavioral ecology and sociobiology*. 32: 167–176.

Møller, A. P., Czirjak, G. Á. and Heeb, P. 2009. Feather micro-organisms and uropygial antimicrobial defences in a colonial passerine bird. - *Functional Ecology*. 23: 1097–1102.

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

Moreno-Rueda, G. 2017. Preen oil and bird fitness: a critical review of the evidence. - *Biological Reviews*. 92: 2131–2143.

Murphy, T. G., Hernández-Muciño, D., Osorio-Beristain, M., Montgomerie, R. and Omland, K. E. 2009a. Carotenoid-based status signaling by females in the tropical streak-backed oriole. - *Behavioral Ecology*. 20: 1000–1006.

Murphy, T. G., Rosenthal, M. F., Montgomerie, R. and Tarvin, K. A. 2009b. Female American goldfinches use carotenoid-based bill coloration to signal status. - *Behavioral Ecology*. 20: 1348–1355.

Navarro, C., Pérez-Contreras, T., Avilés, J. M., Mcgraw, K. J. and Soler, J. J. 2010. Beak colour reflects circulating carotenoid and vitamin A levels in spotless starlings (*Sturnus unicolor*). - *Behavioral ecology and Sociobiology*. 64: 1057–1067.

Neve, L. De, Soler, J. J., Ruiz-Rodríguez, M., Martín-Gálvez, D., Pérez-Contreras, T. and Soler, M. 2007. Habitat-specific effects of a food supplementation experiment on immunocompetence in Eurasian Magpie *Pica pica* nestlings. - *Ibis* (Lond. 1859). 149: 763–773.

Ochsenbein, A. F. and Zinkernagel, R. M. 2000. Natural antibodies and complement link innate and acquired immunity. - *Immunology today*. 21: 624–630.

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

Pike, N. 2011. Using false discovery rates for multiple comparisons in ecology and evolution. - *Methods in Ecology and Evolution*. 2: 278–282.

Price, J. J. 2015. Rethinking our assumptions about the evolution of bird song and other sexually dimorphic signals. - *Frontiers in Ecology and Evolution*. 3: 1–6.

Reid, R. R., Prodeus, A. P., Khan, W., Hsu, T., Rosen, F. S. and Carroll, M. C. 1997. Endotoxin shock in antibody-deficient mice: unraveling the role of natural antibody and complement in the clearance of lipopolysaccharide.

-*The Journal of Immunology*. 159: 970–975.
Robson, T. E., Goldizen, A. W. and Green, D. J. 2005. The multiple signals assessed by female satin bowerbirds: Could they be used to narrow down females' choices of mates? - *Biology Letters*. 1: 264–267.
Roitt, I. 1997. Roitt's Essential Immunology. - Blackwell Science.
Romano, A., Costanzo, A., Rubolini, D., Saino, N. and Møller, A. P. 2017. Geographical and seasonal variation in the intensity of sexual selection in the barn swallow *Hirundo rustica*: a meta-analysis. - *Biological Reviews*. 92: 1582–1600.
Rosenberg, E. and Zilber-Rosenberg, I. 2016. Microbes drive evolution of animals and plants: The hologenome concept. - MBio 7: 1–8.
Roulin, A., Riols, C., Dijkstra, C. and Ducrest, A. L. 2001. Female plumage spottiness signals parasite resistance in the barn owl (*Tyto alba*). - *Behavioral Ecology*. 12: 103–110.
Ruiz-Rodriguez, M., Møller, A. P., Mousseau, T. A. and Soler, J. J. 2017. Capacity of blood plasma is higher in birds breeding in radioactively contaminated areas. - *PLoS One* 12: 1–12.
Ruiz-Rodríguez, M., Valdivia, E., Soler, J. J., Martin-Vivaldi, M., Martín-Platero, A. M. and Martínez-Bueno, M. 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. - *Journal of Experimental Biology*. 212: 3621–3626.
Ruiz-Rodríguez, M., Martínez-Bueno, M., Martín-Vivaldi, M., Valdivia, E. and Soler, J. J. 2013. Bacteriocins with a broader antimicrobial spectrum prevail in enterococcal symbionts isolated from the hoopoe's uropygial gland. - *FEMS microbiology ecology.* 85: 495–502.
Ruiz-Rodríguez, M., Tomás, G., Martín-Gálvez, D., Ruiz-Castellano, C. and Soler, J. J. 2015. Bacteria and the evolution of honest signals. The case of ornamental throat feathers in spotless starlings. - *Functional Ecology*. 29: 701–709.
Schmid-Hempel, P. 2003. Variation in immune defence as a question of evolutionary ecology. - *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 270: 357–366.
Shawkey, M. D., Pillai, S. R. and Hill, G. E. 2003. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. - *Journal of avian biology*. 34: 345–349.
Sheldon, B. C. 2000. Differential allocation: tests, mechanisms and implications. - Trends in Ecology & Evolution 15: 397–402.
Soler, J. J. and Moreno, J. 2012. Evolution of sexual dichromatism in relation to nesting habits in European passerines: A test of Wallace's hypothesis. - *Journal of evolutionary biology*. 25: 1614–1622.
Soler, J. J., De Neve, L., Pérez-Contreras, T., Soler, M. and Sorci, G. 2003. Trade-off between immunocompetence and growth in magpies: An experimental study. - *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 270: 241–248.

Soler, J. J., Navarro, C., Contreras, T. P., Avilés, J. M. and Cuervo, J. J. 2008. Sexually selected egg coloration in spotless starlings. - *The American Naturalist.* 171: 183–194.

Soler, J. J., Peralta-Sánchez, J. M., Martín-Platero, A. M., Martín-Vivaldi, M., Martínez-Bueno, M. and Møller, A. P. 2012. The evolution of size of the uropygial gland: Mutualistic feather mites and uropygial secretion reduce bacterial loads of eggshells and hatching failures of European birds. - *Journal of evolutionary biology*. 25: 1779–1791.

Soler, J. J., Ruiz-Rodríguez, M., Martín-Vivaldi, M., Peralta-Sánchez, J. M., Ruiz-Castellano, C. and Tomás, G. 2015. Laying date, incubation and egg breakage as determinants of bacterial load on bird eggshells: experimental evidence. - *Oecologia*. 179: 63–74.

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

Soler, J. J., Morales, J., Cuervo, J. J. and Moreno, J. 2019. Conspicuousness of passerine females is associated with the nest-building behaviour of males. - *Biological Journal of the Linnean Society*. 126: 824–835.

Sorci, G., Soler, J. J. and Moller, A. P. 1997. Reduced immunocompetence of nestlings in replacement clutches of the European magpie (*Pica pica*). - *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 264: 1593–1598.

Thornton, B. P., Vetvicka, V. and Ross, G. D. 1994. Natural antibody and complement-mediated antigen processing and presentation by B lymphocytes. -*The Journal of Immunology*. 152: 1727–1737.

Tobias, J. A., Montgomerie, R. and Lyon, B. E. 2012. The evolution of female ornaments and weaponry: Social selection, sexual selection and ecological competition. - *Philosophical Transactions of the Royal Society B: Biological Sciences*. 367: 2274–2293.

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

Torres, R. and Velando, A. 2005. Male preference for female foot colour in the socially monogamous blue-footed booby, *Sula nebouxii*. - *Animal Behaviour*. 69: 59–65.

Vincze, O., Vágási, C. I., Kovács, I., Galván, I. and Pap, P. L. 2013. Sources of variation in uropygial gland size in european birds. - *Biological Journal of the Linnean Society*. 110: 543–563.

Webb, W. H., Brunton, D. H., Aguirre, J. D., Thomas, D. B., Valcu, M. and Dale, J. 2016. Female song occurs in songbirds with more elaborate female coloration and reduced sexual dichromatism. - *Frontiers in Ecology and Evolution*. 4: 1–8.

Westneat, D. F. 2012. Evolution in response to social selection: The importance of interactive effects of traits on fitness. - *Evolution* (N. Y). 66: 890–895.

Annex

Table A1. GLM results of the association between the uropygial gland (amount of secretion and gland size) and the different PCAs representing the measured colour variables. Significant Q-values are in bold.

			F	df	p	beta	SE (beta)	Q
Back	PCA1	Secretion volume	11.23	1,20	<0.001	1.19	0.36	**0.04**
		Uropygial gland size	5.87	1,20	0.03	0.37	0.15	0.13
		Year	7.82	1,20	0.01	0.56	0.20	0.09
		Individual	1.59	74,20	0.12	-0.08	0.14	0.31
	PCA2	Secretion volume	0.02	1,20	0.89	0.06	0.42	0.93
		Uropygial gland size	0.48	1,20	0.49	0.13	0.18	0.73
		Year	0.0002	1,20	0.99	0.00	0.24	0.99
		Individual	1.4	74,20	0.20	-0.03	0.17	0.44
Leg	PCA1	Secretion volume	0.12	1,20	0.74	-0.16	0.46	0.86
		Uropygial gland size	1.68	1,20	0.21	0.26	0.20	0.44
		Year	4.94	1,20	0.04	-0.58	0.26	0.14
		Individual	1.01	74,20	0.52	0.12	0.18	0.73
Throat	PCA1	Secretion volume	2.77	1,20	0.11	-0.74	0.45	0.30
		Uropygial gland size	0.01	1,20	0.90	0.02	0.19	0.93
		Year	5.35	1,20	0.03	-0.58	0.25	0.14
		Individual	1.21	74,20	0.32	-0.29	0.18	0.61
	PCA2	Secretion volume	0.29	1,20	0.59	0.42	-0.64	0.76
		Uropygial gland size	0.63	1,20	0.44	0.18	-0.52	0.73
		Year	0.04	1,20	0.84	0.24	-0.44	0.93
		Individual	1.53	74,20	0.14	0.16	-0.36	0.34
Beak base	PCA1	Secretion volume	0.93	1,20	0.35	-0.25	0.26	0.62
		Uropygial gland size	30.83	1,20	<0.001	-0.63	0.11	**<0.001**
		Year	18.13	1,20	<0.001	-0.63	0.15	**<0.001**
		Individual	2.48	74,20	0.01	0.09	0.10	0.07
	PCA2	Secretion volume	1.11	1,20	0.30	-0.37	0.35	0.60
		Uropygial gland size	0.31	1,20	0.59	-0.09	0.15	0.76
		Year	0.42	1,20	0.53	-0.13	0.20	0.73
		Individual	2.18	74,20	0.03	-0.08	0.14	0.14
Beak tip	PCA1	Secretion volume	0.02	1,20	0.89	-0.07	0.50	0.93
		Uropygial gland size	0.45	1,20	0.51	0.15	0.22	0.73
		Year	0.48	1,20	0.49	-0.20	0.28	0.73
		Individual	0.85	74,20	0.71	0.08	0.20	0.86
	PCA2	Secretion volume	3.29	1,20	0.08	-0.55	0.30	0.24
		Uropygial gland size	0.12	1,20	0.74	-0.04	0.13	0.86
		Year	4.74	1,20	0.04	0.37	0.17	0.14
		Individual	1.85	74,20	0.06	-0.18	0.12	0.20

Table A2. GLM results of the association between the plasma immune response (lysis and agglutination) and the different PCAs representing the measured colour variables. Significant Q-values are in bold.

			F	df	p	beta	SE (beta)	Q
Back	PCA1	Lysis	1.06	1,15	0.32	0.23	0.22	0.52
		Agglutination	1.69	1,15	0.21	0.37	0.28	0.41
		Breeding status	2.29	2,15	0.14	-0.48	0.24	0.39
		Individual	0.92	50,15	0.61	-0.38	0.20	0.71
	PCA2	Lysis	0.22	1,15	0.65	-0.08	0.17	0.73
		Agglutination	0.00	1,15	0.98	0.01	0.22	0.98
		Breeding status	0.53	2,15	0.60	0.13	0.18	0.71
		Individual	1.64	50,15	0.15	0.02	0.16	0.39
Leg	PCA1	Lysis	3.25	1,15	0.09	0.22	0.12	0.29
		Agglutination	11.45	1,15	<0.001	0.52	0.15	**<0.001**
		Breeding status	19.40	2,15	<0.001	-0.78	0.13	**<0.001**
		Individual	3.08	50,15	0.01	-0.29	0.11	0.07
Throat	PCA1	Lysis	0.61	1,15	0.45	0.17	0.22	0.62
		Agglutination	1.77	1,15	0.20	-0.38	0.28	0.41
		Breeding status	1.10	2,15	0.36	0.20	0.24	0.56
		Individual	0.94	50,15	0.59	-0.13	0.20	0.71
	PCA2	Lysis	0.09	1,15	0.77	0.04	0.13	0.84
		Agglutination	1.35	1,15	0.26	0.20	0.17	0.45
		Breeding status	3.85	2,15	0.04	-0.39	0.14	0.16
		Individual	2.74	50,15	0.02	0.06	0.12	0.10
Beak base	PCA1	Lysis	0.35	1,15	0.56	-0.13	0.22	0.71
		Agglutination	0.70	1,15	0.41	0.23	0.28	0.59
		Breeding status	5.85	2,15	0.01	0.70	0.24	0.07
		Individual	0.66	50,15	0.86	-0.14	0.20	0.91
	PCA2	Lysis	1.54	1,15	0.23	0.18	0.14	0.41
		Agglutination	2.05	1,15	0.17	-0.26	0.18	0.41
		Breeding status	0.94	2,15	0.41	-0.16	0.15	0.59
		Individual	2.59	50,15	0.02	0.11	0.13	0.10
Beak tip	PCA1	Lysis	0.37	1,15	0.55	0.07	0.12	0.71
		Agglutination	0.02	1,15	0.88	0.02	0.16	0.91
		Breeding status	15.33	2,15	0.00	-0.62	0.13	**<0.001**
		Individual	2.38	50,15	0.03	-0.05	0.11	0.14
	PCA2	Lysis	2.42	1,15	0.14	0.26	0.17	0.39
		Agglutination	1.58	1,15	0.23	-0.27	0.21	0.41
		Breeding status	2.99	2,15	0.08	0.33	0.18	0.29
		Individual	1.51	50,15	0.19	-0.14	0.15	0.41

Table A3. GLM results of the association between the plasma antagonistic index and the different PCAs representing the measured colour variables. Significant Q-values are in bold.

			F	df	p	beta	SE (beta)	Q
Back	PCA1	Antagonistic index	0.15	1,35	0.71	-0.09	0.25	0.77
		Year	0.36	1,35	0.55	-0.14	0.24	0.68
		Clutch order	2.37	2,35	0.11	-0.32	0.20	0.43
		Individual	0.97	73,35	0.56	-0.20	0.16	0.68
	PCA2	Antagonistic index	0.43	1,35	0.52	-0.15	0.22	0.68
		Year	0.76	1,35	0.39	-0.19	0.22	0.66
		Clutch order	0.07	2,35	0.93	-0.05	0.18	0.96
		Individual	1.59	73,35	0.06	-0.07	0.15	0.33
Legs	PCA1	Antagonistic index	0.34	1,35	0.56	-0.13	0.23	0.68
		Year	8.84	1,35	0.01	-0.65	0.22	0.10
		Clutch order	5.27	2,35	0.01	-0.43	0.19	0.12
		Individual	1.24	73,35	0.24	0.20	0.15	0.58
Throat	PCA1	Antagonistic index	0.15	1,35	0.70	-0.10	0.27	0.77
		Year	0.97	1,35	0.33	-0.25	0.25	0.66
		Clutch order	0.20	2,35	0.82	0.12	0.21	0.87
		Individual	1.03	73,35	0.48	-0.23	0.17	0.68
	PCA2	Antagonistic index	0.92	1,35	0.34	0.21	0.22	0.66
		Year	0.48	1,35	0.49	0.14	0.21	0.68
		Clutch order	0.01	2,35	0.99	-0.02	0.18	0.99
		Individual	1.83	73,35	0.03	0.03	0.14	0.18
Beak base	PCA1	Antagonistic index	0.75	1,35	0.39	0.17	0.20	0.66
		Year	1.66	1,35	0.21	-0.25	0.19	0.53
		Clutch order	15.76	2,35	0.00	0.70	0.16	**<0.001**
		Individual	1.06	73,35	0.44	0.05	0.13	0.68
	PCA2	Antagonistic index	0.79	1,35	0.38	0.19	0.21	0.66
		Year	3.05	1,35	0.09	0.35	0.20	0.40
		Clutch order	2.17	2,35	0.13	-0.12	0.17	0.46
		Individual	1.83	73,35	0.03	-0.10	0.14	0.18
Beak tip	PCA1	Antagonistic index	0.71	1,35	0.40	0.20	0.24	0.66
		Year	0.18	1,35	0.68	-0.10	0.23	0.77
		Clutch order	2.07	2,35	0.14	-0.21	0.19	0.46
		Individual	1.02	73,35	0.48	0.20	0.16	0.68
	PCA2	Antagonistic index	1.94	1,35	0.17	-0.27	0.20	0.52
		Year	4.59	1,35	0.04	0.41	0.19	0.24
		Clutch order	1.04	2,35	0.36	0.14	0.16	0.66
		Individual	1.29	73,35	0.21	-0.21	0.13	0.53

CAPÍTULO 4

Interspecific variation in deterioration and degradability of avian feathers: The evolutionary role of microorganisms

Abstract

Feathers are essential for avian life, and factors affecting their integrity are important to understand their evolution. These factors should depend on, among other traits, species-specific bacterial environments and life-history characteristics. However, interspecific variation in feather deterioration, feather susceptibility to degradation by keratinolytic bacteria (degradability), and bacterial environment, have rarely been quantified. Here, we did so by measuring deterioration and degradability of wing feathers of fledglings in 16 bird species, and characterizing the bacterial environment where they developed. We found statistically significant interspecific variation for all considered variables. On average, non-melanised were more deteriorated than melanised feathers, but differences depended on the species. Moreover, nest bacterial loads were related to feathers wear, but the sign of the association depended on the bacterial group considered and on feather pigmentation. We also found a positive association of feather degradability with wear of non-melanised feathers, and with bacterial loads. These results suggest that bacterial environments determine the integrity of fledgling feathers as well as their resistance to bacterial degradation, which implies a preponderant role of bacteria in driving the evolution of avian feathers.

Keywords: Feather degrading bacteria, Feather wear, Interspecific comparisons, Keratinolytic bacteria, Nest bacterial environment.

Introduction

The presence of feathers is one of the defining characteristics of the Class Aves and has historically attracted the attention of evolutionary biologists trying to understand the extremely high variation in avian morphology, structure and coloration (Hanson 2011). Classic adaptive explanations for the existence of feathers include insulation (i.e., thermoregulation or waterproofing) and flight (Rayner 1988, Stettenheim 2000), but they also function in different scenarios of social communication and sexual selection (Andersson 1994, Møller et al. 1998, Senar 2006, Hanson 2011, Roulin 2016). Thus, feather characteristics providing the holder with advantages in the above mentioned scenarios have been studied as possible key adaptations driving the evolution of birds (Rayner 1988, Stettenheim 2000).

Factors affecting the integrity of feathers are therefore important to understand their evolution. Feather integrity might for instance depend on physiological conditions of individuals during feather growth, which could result in feather malformations (e.g. fault bars) that enhance feather deterioration and breakage (Jovani and Rohwer 2017). Among factors directly promoting feather damage, the physical abrasive wear due to friction with fluids while flying or diving, or deterioration due to contacts with distinct materials while walking or perching, are well known since many years (Francis and Wood 1989, Swaddle et al. 1996). Chemical abrasion of feathers has also received attention, especially during the last decade, focussing on that induced by keratinase-producing bacteria (Gunderson 2008). All these factors negatively affect feather integrity and, thus, their functioning in thermoregulation, communication and flight, resulting in severe fitness consequences. Risk of feather deterioration would in any case depend on life style or life history characteristics such as migratory or foraging behaviours (Møller et al. 2012). This scenario therefore raises the expectation that the

degree of feather deterioration should be a species-specific character, a prediction that to the best of our knowledge has never been explored.

Feather degradation by keratinolitic bacteria occurs in wild birds (Leclaire et al. 2014, Kent and Burtt 2016, but see Cristol et al. 2005), which for instance, might have consequences in scenarios of sexual selection (Shawkey et al. 2007, 2009, Ruiz-Rodríguez et al. 2015) and survival (Møller et al. 2012). Moreover, bacterial community of avian feathers vary interspecifically (Javůrková et al. 2019). Detecting evidence of interspecific variation in feather wear in standard conditions could suggest the existence of interspecific variation in environmental factors associated with the risk of feather deterioration, including bacterial environment (Kent and Burtt 2016). Moreover, since these environmental factors likely vary among species (see above), natural selection could have favoured the evolution of characteristics that counteract or reduce feather degradation by keratinolitic bacteria in environments that are distinctive of each species (Burtt Jr 2009, Burtt et al. 2011, Javůrková et al. 2019). If this was the case, we should find interspecific variation in traits functioning in preventing feathers' wear (Burtt Jr 2009) such as feather susceptibility to degradation by keratinolitic bacteria (hereafter, feather degradability) (Ruiz-de-Castaneda et al. 2015, Ruiz-de-Castañeda et al. 2012, Ruiz-Rodríguez et al. 2016).

The structure and chemical composition of feathers are known to affect feather wear of wild birds (Ruiz-de-Castañeda et al. 2012). Although the main chemical component of feathers is β-keratine, a slight variation in pigment composition may influence feather degradability. We know for instance that indentation hardness of melanic keratin is greater than that of non-melanic keratin (Bonser 1995), and that rates of wear (Burtt 1979) and of breakage (Kose and Møller 1999) are lower in melanic than in non-melanic feathers. Furthermore, melanised feathers resist bacterial degradation better than pale, unmelanised feathers (Goldstein et al. 2004, Gunderson et al. 2008, Ruiz-de-

Castañeda et al. 2012). Moreover, in comparisons with pale birds, dark birds often live in humid habitats (Delhey 2017), where more active feather degrading bacilli are abundant (Burtt and Ichida 2004), which suggests a link between habitat-related risk of feather degradation and feather characteristics that reduce such a risk. This scenario therefore suggests that feather degradability should vary between melanised and unmelanised feathers within the same species, and that a considerable interspecific variation should exist in such feather characteristics. Although variation in degradability has been studied intraspecifically in relation to feather pigmentation (Burtt et al. 2011, Ruiz-de-Castañeda et al. 2012) and habitat characteristics (Ruiz-Rodríguez et al. 2016), interspecific variation in feather degradability has only been explored for 13 parrot species (Burtt et al. 2011).

Here, we have quantified intra and interspecific variation in feather wear of nestlings' birds close to abandoning the nest (hereafter fledglings). We did so by microscopically quantifying deterioration of wing feathers, mostly secondary-covert-wing feathers, in 16 species of birds. We also estimated feather degradability by quantifying keratin degradation by the well-known feather degrading bacteria *Bacillus licheniformis* (Kent and Burtt 2016). We expected to find significant interspecific variation in feather wear and in feather degradability, and that these two traits would positively covary interspecifically. By using feathers of nestlings we restricted (i.e., standardized) factors that could have affected feather deterioration state to those occurring within the nest environment. Moreover, independently of the altricial bird species, feathers of nestlings close to abandon the nests are at a similar state of development and, thus, are interspecifically comparable. Nestling feathers are often of lower quality than adult feathers (Callan et al. 2019). Nestling feathers might play pivotal roles in social communication during nestlings (Morales et al. 2019) and post-fledging period (Moreno and Soler 2011), and even explain probability of predation (Callan et al. 2019) and

body condition (Minias et al. 2015) during the post-fledging and first winter periods. Thus, although post-juvenile moult is likely related to quality of fledgling feathers (Minias et al. 2015), exploring factors affecting integrity of nestling feathers is important to understand factors affecting their survival.

Within nests, the bacterial environment would largely determine feather bacterial loads (Jacob et al. 2018) and, thus, risk of bacterial degradation of feathers. Previous studies have detected interspecific variation in nest bacterial environments by sampling the microbiome of the eggshells of different species (Peralta-Sánchez et al. 2018). Moreover, interspecific variation in eggshell bacterial load is associated with life history characteristics (Peralta-Sánchez et al. 2012) and, thus, it is possible that nest bacterial environments determine wear and degradability of nestling feathers. Here, we characterised bacterial loads of the same nests where we collected nestling feathers to evaluate their deterioration state. However, the effects of nest bacterial environments on feather wear is not straightforward. It for instance depends on the presence of bacteria with keratinolytic activity (i.e. able to degrade feather tissue) that will positively affect feather deterioration. Antimicrobial activity (against feather-degrading bacteria) of bacteria within the nest community would also determine feather degradation; the higher the density of these bacteria the lower the effect on feather deterioration. Finally, it is also possible that the physical structure of feathers was adapted to species-specific nest bacterial communities, which might result in these two variables being not related to each other at the interspecific level. Thus, we explored the association of nest bacterial density with feather deterioration and degradability by distinguishing different groups of bacteria.

Material and Methods

Study areas and fieldwork

Fieldwork were carried out in the Hoya de Guadix (37º 18' N; 3º11' W), southern Spain, and in the forest of Valsaín (40°54'N, 04° 01'W), central Spain, during the 2015 and 2016 breeding seasons. For a detailed description of the southern and central Spain study areas see Martín-Vivaldi et al. (2006), Soler and Avilés (2010), and González-Braojos et al. (2017). We collected information for 16 species that included hole and non-hole nesting species (Table 1). The nests of hole-nesting species were mostly located within nest boxes installed in the study areas, while nests of the other species were found by systematic search of appropriate habitats within the study areas. Samples (wing feathers and nest bacterial samples) were collected at a standard relative age during their ontogeny when less than 10% of the primary feathers were covered by the cylindrical sheaths (i.e., when they were in the last fifth of its normal nestling development, close to abandoning the nest). Moreover, for a subsample of species, we captured adults during the breeding season and collect the same kind of feathers than in nestlings. We used these samples to validate the use of nestling feathers to explore interspecific differences in feather characteristics.

We collected a minimum of two wing feathers of at least two randomly selected nestlings in each sampled nest. For most sampled species, we collected the third secondary covert feathers. Whenever possible, we collected wing feathers that had both dark (i.e with relatively high concentration of eu- and/or pheo-melanin) and pale (i.e with relatively low if any concentration of eu- and/or pheo-melanin) areas, or different feathers that were either dark or pale. Feather darkness is usually related to presence of melanin (Goldstein et al. 2004, Gunderson et al. 2008, Ruiz-de-Castañeda et al. 2012) and, thus, we call them as melanised and non-melanised feathers, respectively. To cut the

feathers at the upper part of the quill (to avoid bled) we used scissors previously washed with 95% ethanol and disinfected with a Bunsen burner. Feathers of each nestling were kept in new zipper plastic bags and stored in the lab at dark and 4 ºC until the analyses within the following ten months. The same day of feather collection, during the last days of the nesting period, we sampled nest bacterial loads by gently rubbing the nest-cup material, or nest surface where nestlings were located, with a sterile swab (Sterile R, Nuova Aptaca S.R.L.) slightly wetted with sterile sodium phosphate buffer (0,2 M; pH = 7,2) during 10 seconds. Meanwhile, nestlings were kept in a cotton bag. Afterwards, we kept the swab in a microcentrifuge tube with 1.2 ml of phosphate buffer at 4-6 ºC, in a portable refrigerator until being processed in the lab within the next 4 days.

Table 1: Sample sizes to estimate degradation of pigmented and unpigmented feathers, feathers degradability and nest bacterial loads of different species.

	N = Nests (feathers)		N = Nests	N = Nests
	Feather wear		Feather Degradability	Bacterial loads
	Melanised	Non-melanised		
Athene noctua	13	13	9	13
Clamator glandarius	9	9	15	9
Coracias garrulus	6	6		6
Corvus monedula	6		6	6
Cyanistes caeruleus	5	5	10	5
Ficedula hypoleuca	23	25	10	24
Hirundo rustica	3	0		3
Oenanthe leucura	3	0		3
Otus scops	12	12	5	11
Parus major	19	18	18	17
Passer domesticus	8	8		8
Petronia petronia		3	4	3
Pica pica	17	17	15	17
Pyrrhocorax pyrrhocorax	5		11	5
Sturnus unicolor	5		9	5
Upupa epops	26	26	8	26

Feather wear

The level of feather wear was estimated following the protocol described in Ruiz-Rodríguez et al. (2015). Briefly, feathers were examined and photographed (2x) under a binocular lens (Nikon SMZ1500, Melville, NY, USA) connected to a camera (Nikon Digital Sight DS Fi1). Measurements from pictures were taken using the software NIS Elements F 3.1. Following Ruiz-Rodríguez et al. (2015), we considered: (i) whether or not the tip of the feather was incomplete; (ii) the length (mm) of the feather tip that showed clear signs of wear (apical part); and (iii) the number of barbules that were degraded in 20 randomly chosen barbs of the basal part (see Figure 3 in Ruiz-Rodríguez et al. (2015)). Wear levels were ranked from 0 (no degradation) to 3 (more than 2/3 of the feather damaged). Two lab technicians, who were unaware of the feather origin (i.e., species, body part, etc.) or hypotheses tested, evaluated all photographs. Whenever possible, we distinguished wear of melanised and of unmelanised feathers (or parts of the same feather). Repeatability of visual estimations was calculated by comparing both assessments in a one-way ANOVA. Repeatability was relatively high ($F = 7.32$, $df = 2599, 2600$, $P < 0.0001$, repeatability index = 0.76). Feather wear varied significantly among nests of the same species (GLM, effect of nest identity nested within species identity, $F = 1.88$, $df = 151,177.8$, $P < 0.001$), but not among nestlings within the same nest (GLM, effect of nestling identity nested within nest and species identities, $F = 0.99$, $df = 227,1648$, $P = 0.53$). Thus, we used average values of evaluated feathers from the same nest in subsequent analyses.

Feather degradability

Feather degradability was explored by using complete feathers and following the protocol described elsewhere (Ruiz-Rodríguez et al. 2015, Ruiz-Rodríguez et al. 2009, Gunderson et al. 2008, Ruiz-de-Castañeda et al. 2012). It is worth

mentioning here that because degradability was estimated for complete feathers, we were not able to distinguish between degradability of the pigmented and non-pigmented parts of feathers. Moreover, because pigmentation (melanised vs non-melanised) of the collected wing feathers does not vary within species, we did not contemplate pigmentation as a factor when analysing possible interspecific differences in feather degradation. Finally, a single feather per sampled nests were analysed.

A brief description of the protocol is as follows: Previously separated and weighed feathers were sterilized in the autoclave before the experiment. Afterward, each feather was included in a previously sterilized experimental glass tube containing 4 mL of a PBS buffer (9.34 mM NH4Cl, 8.55 mM NaCl, 1.72 mM K2HPO4, 2.92 mM KH2PO4, 0.49 mM MgCl2-6H2O and 0.01% yeast extract in 100 mL of distilled water). Moreover, a colony of *Bacillus licheniformis* D13 previously isolated from TSA plates was introduced in each of the experimental glasses with a sterile loop. After vortexing, we collected 1 mL from each tube as a basal measurement, and kept it at 4 ºC until measurement in the spectrophotometer. Experimental tubes were incubated at 37 ºC in constant agitation at 120 rpm in an orbital agitator (VWR, Spain). 1 mL was collected from each experimental tube after 21 days of incubation. Collected samples were centrifuged to remove bacterial cells, and absorbance of the supernatant was then estimated using a spectrophotometer (Helios Zeta UV-Vis, Thermo Scientific, United Kingdom) at 230 nm (Goldstein et al. 2004). The oligopeptide concentration in the supernatant mainly originates from keratin degradation and thus it is directly related to the amount of feather degraded. Calibration curves of absorbance and oligopeptide concentration (from 0 to 300 μg x mL1) were obtained by using bovine serum albumin (BSA) (R2 = 0.98 in both curves), which allowed us to extrapolate the absorbance to values of oligopeptide concentration.

To assure that oligopeptide measurements were due to the degradation of feathers by *Bacillus licheniformis* exclusively, we also prepared the following control samples: (i) tubes containing the buffer, (ii) tubes containing the buffer and the bacteria, but without the feathers, and (iii) tubes containing the feathers but without the bacteria. After 21 days of incubation, oligopeptide concentrations increased significantly (Repeated Measures ANOVA, effect of incubation, $F = 803.2$, $df = 1,360$, $P < 0.0001$), mainly in experimental tubes (Repeated Measures ANOVA, interaction between incubation and treatment effect, $F = 140.3$, $df = 1,1080$, $P < 0.0001$). Although intermediate level of oligopeptide concentrations were detected in tubes containing feathers without bacteria that were incubated during 21 days, control tubes had significantly less oligopeptide concentration than experimental tubes (Repeated Measures ANOVA, effect of experimental treatments, $F = 206.2$, $df = 1,1080$, $P < 0.0001$, ESM Fig 1). Thus, for subsequent analyses, as a measure of feather degradability, we conservatively used differences in oligopeptide concentration between experimental tubes containing feather and bacteria and control tubes containing feathers but not bacteria. These measures were standardized by feather weight (precision scale Mettler AB135-5/FACT, accuracy 000001g).

Nest bacterial loads

In the lab, tubes containing the bacterial samples were vigorously shaken in a vortex (Boeco V1 Plus) to remove bacteria from swabs. Afterward, we spread homogeneously 100 µl of serially diluted samples until 10−4 in Tryptic Soy Agar (TSA) and 10-2 in the others media (see below). Plates were incubated at 37 ºC during 72 hours, and then the number of colonies on each plate was counted. Following the method of Peralta-Sánchez et al. (2010), we used four different solid media (Scharlau Chemie S.A., Barcelona) to grow bacterial

samples. We used TSA, a broadly used general medium to grow mesophilic bacteria, and three specific media: Kenner Fecal Agar (KF) for growing bacteria belonging to the genus *Enterococcus*; Vogel-Johnsson Agar (VJ) for bacteria of the genus *Staphylococcus*; and Hecktoen Enteric Agar (HK) for Gram-negative bacteria of the family *Enterobacteriaceae*. Bacterial load of nests for each bacterial group was then expressed as the number of colony forming units (CFU) per ml (Nº colonies * 10dilution factor) / 100 µl spread).

Sample sizes and statistical analyses

We considered 16 species for which we collected information of feather wear for more than two nests (Table 1). For 11 of these species we successfully collected information of wear of pigmented (melanised) and unpigmented (parts of) feathers. However, because of malfunctioning of the spectrophotometer determining keratin degradation, we lost all feathers collected for this purpose during 2015. Thus, during 2016, we again collected feathers of most (11) species with information of feather wear. Nest bacterial loads were estimated for the 16 species with information of feather wear; most of the samples were in fact from the same nests where we collected the feathers to analyse their deterioration state.

Interspecific variation in feather wear of melanised and non-melanised feathers was explored in separate univariate ANOVAs with average values per nest as dependent variable and species identity as the unique independent factors. Possible differential wear between melanised and non-melanised feathers was explored with a subset of species with information for both kinds of feathers in a Repeated Measures ANOVA. Average values of feather wear per nest of melanised and non-melanised were used as repeat measures. Thus, feather pigmentation was the within independent factor and species identity as the between independent factor.

Interspecific variation in feather degradability was explored by using average nest values as the dependent variable and species identity as the only independent factor. Interspecific variation in nest bacterial loads (log(log(X)) transformed data) were explored in a MANOVA, with bacterial loads of mesophilic bacteria, enterobacteria, staphylococci and enterococci as multiple dependent variables and species identity as the independent factor.

The association between feather wear and nest bacterial loads was explored in General Linear Models (GLM) that did include species identity as additional independent factor to those reflecting nest bacterial load (i.e., the four bacterial groups). The expected associations were explored separately for melanised and non-melanised feathers, but also for the subset of species with information on degradation of melanised and non-melanised feathers in a repeated measures design. In this case, values for pigmented and unpigmented feathers were included as dependent variables in a Repeated Measures ANOVA (i.e. pigmentation as repeated measure), and bacterial loads of mesophilic bacteria, staphylococci, enterococci and enterobacteria as independent covariables. This model also included species identity as an additional independent factor. For all these models trying to explore the expected association between bacterial loads and feather wear, we adopted a backward stepwise procedure for model selection where factors with the largest p-values were removed one by one up to a p-value lower than 0.1. Final reduced models coincide with best models from AIC criteria (results not shown).

To explore the associations between feather degradability and feather wear, and between feather degradability and nest bacterial loads, we performed regression analyses. Since feather degradability and feather degradation were estimated from samples collected in different nests, but bacterial environment were estimated from the nests where feathers for estimating degradation were collected, we used average species values of feather degradability, but nest

values for bacterial environment and feather wear. The analyses including information of feather degradability did not include species identity as independent factor because no within species variance exists in feather degradability.

The effects of age (independent factor) on feather degradability (dependent variables) were explored in General Linear Models that also included species identity as additional independent factor. Feather wear was estimated for several nestling within the same nest and, thus, the effects of age on this variable were analysed in General Linear Mixed Models that included species identity as additional independent fixed factor and nest identity nested within the interaction between species identity and age as the random factor. We estimated the interaction between age and species identity in separate models that also included main affects, while main effects were estimated in models that did not include interactions.

Finally, expected association between feather deterioration or feather susceptibility to degradation and nest bacterial loads were also explored in weighted phylogenetic linear models (wPGLS) with average per species values. As far as we know, available methodologies to consider within species variance into the PGLS models is restricted to one of the predictor variables (Garamszegi and Møller 2010, Garamszegi 2014) and, thus, degrees of freedom were reduced to those reflecting number of species. Briefly, we used consensus phylogenetic trees from 1000 downloaded trees from birdtree.org (Jetz et al. 2012) and used wPGLS following recommendations by Garamszegi (2014) in R (version 3.6.1) with libraries ape (Paradis and Schliep 2018) and nlme (Pinheiro et al. 2019).

All statistical tests were performed in Statistica V13 (Statsoft-Inc. 2011).

Results

Feather wear; interspecific comparisons

We found interspecific differences in wear of secondary covert feathers of nestling birds, both for melanised ($F = 7.24$, $df = 14, 146$, $P < 0.0001$) and non-melanised feathers ($F = 11.50$, $df = 10, 132$, $P < 0.0001$). Blue tits (*Cyanistes caeruleus*) and black wheatears (*Oenanthe leucura*) were the species with most damaged melanised feathers, while those of pied flycatchers (*Ficedula hypoleuca*) and magpies (*Pica pica*) were the least damaged (Fig. 1A). When considering non-melanised feathers, the blue tit had again the highest feathers wear, while non-melanised feathers of hoopoes (*Upupa epops*) were by far those least damaged (Fig 1A). Finally, degradation of non-melanised feathers (mean (SE) = 1.57 (0.04)) did not differ significantly from that of melanised feathers (mean (SE) = 1.51 (0.04); Repeated measures, pigmentation effects: $F = 1.15$, $df = 1, 128$, $P = 0.28$). However, in some species the melanised feathers were more degraded that the unpigmented ones, while in some other species the tendency was the opposite resulting in a statistically significant interaction between feather pigmentation and species identity (Fig 1A; Repeated measures, $F = 7.15$, $df = 9,128$, $P < 0.0001$). These differences are mainly due to hoopoes and pied flycatchers (Fig 1A).

Feather degradability; interspecific comparisons

We found interspecific differences in feather degradability ($F = 5.26$, $df = 11, 107$, $P < 0.0001$), even after controlling for the positive influence of feather mass used (Beta (SE) = 0.83 (0.18), $F = 21.78$, $df = 1, 107$), $P < 0.0001$). Covert feathers of nestlings of little owls (*Athene noctua*), pied flycatchers, great tits (*Parus major*), hoopoes and blue tits, were the most easily degraded, while those of choughs (*Pyrrhocorax pyrrhocorax*), jackdaws (*Corvus monedula*)

and magpies (*Pica pica*) were the most resistant to bacterial degradation (Fig. 1B).

Nest bacterial loads; interspecific comparisons

Estimated nest bacterial loads of different groups of bacteria covaried with each other (R(Max-Min) = 0.65-0.46, N = 161, $P < 0.0001$). We detected a large interspecific variation (MANOVA, Wilks' $\lambda = 0.26$, $F = 3.80$, df = 60, 556.5, $P < 0.0001$; univariate results: $F > 3.018$, df = 15, 145, $P < 0.0003$) that depended on the considered bacterial group (Fig. 1C). However, independently of the bacterial group considered, nests of some species consistently harboured the lowest or the largest densities. For example, little owls, jackdaws and hoopoes were those with higher mesophilic bacterial loads, while nests of swallows (*Hirundo rustica*), choughs, and magpies harboured the lowest densities of this bacterial group. When considering *Enterobacteriaceae* and *Staphylococcu*s, nests of hoopoes were those with the highest density, while those of swallows harboured the lowest densities of these bacterial groups. Finally, when considering *Enterococcus*, nests of rollers (*Coracias garrulus*) and of hoopoes were those with the highest bacterial density, and nests of swallows again showed the lowest densities (Fig. 1C).

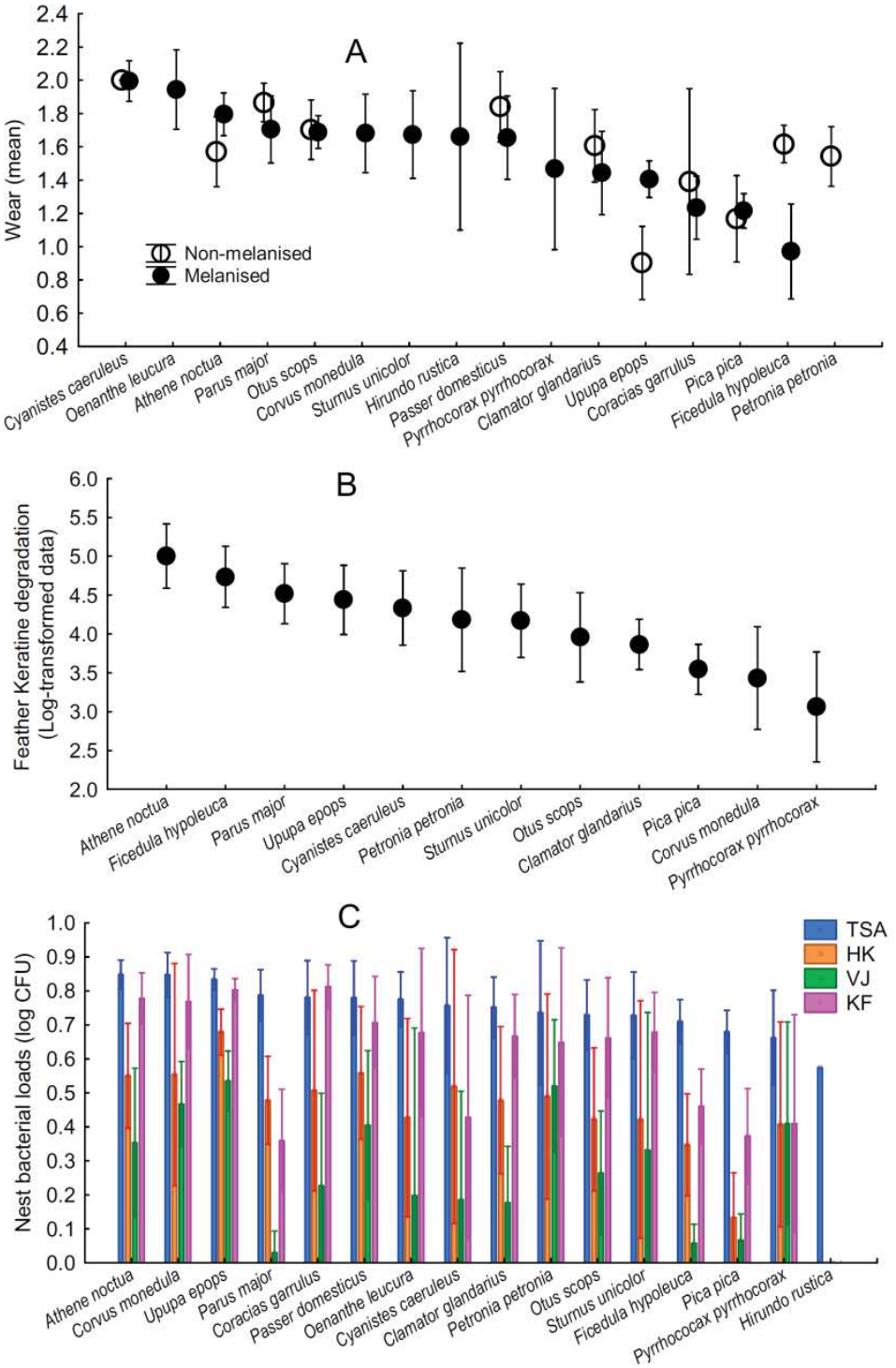


Fig. 1: Wear of melanised and non-melanised parts of secondary-covert wing feathers (A), feather degradability in terms of feather keratine degradability (B), and nest bacterial environment (C) estimated as density of mesophilic bacteria (TSA), enterobacteria (HK), staphylococci (VJ) and enterococci (KF). Values are averages ± 95% CI and species are ordered from the highest to the lowest value of each of the considered variables (density of mesophilic bacteria in the case of nest bacterial environment).

Feather wear and nest bacterial loads

Nest bacterial loads predicted wear of nestling feathers, (mainly those melanised). After controlling for the effect of species identity, the density of enterobacteria resulted positively associated with melanised feathers wear (Table 2, Fig. 2). Interestingly, in final-reduced models, density of mesophilic bacteria resulted negatively associated with melanised feather wear (Table 2, Fig. 2). When considering non-melanised feathers, no bacterial counts explained a significant proportion of variance of feather wear ($F < 2.16$, $df = 1,124$, $P > 0.14$), even after correcting for the effect of species identity ($F = 10.15$, $df = 10,124$, $P < 0.0001$).

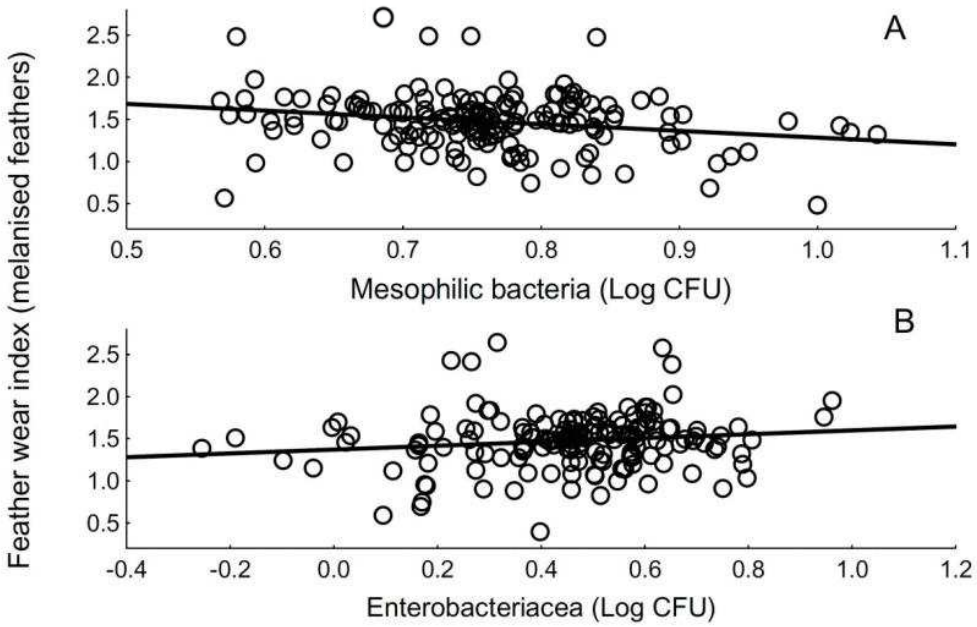


Fig 2: Association between feather wear of melanised feathers and nest bacterial environment estimated as densities of mesophilic bacteria and enterobacteria. Bacterial load and wear values are corrected for species identity. Lines are regression lines.

Differences in feather wear between non-melanised and melanised feathers (i.e. only species with these two kinds of feathers considered) depend on nest bacterial environment (enterobacteria (negatively, Fig. 2) and mesophilic bacterial loads (positively, Fig. 2)). Moreover, after controlling for

nest bacterial environment, within-nest differences in degradation of non-melanised minus melanised feathers resulted significantly different from zero; non-melanised were more degraded than melanised feathers (see within nest repeated measures in Table 2). That was the case after correcting for the effect of species identity (Table 2)

Feather degradability, feather wear and nest bacterial loads

Average degradability of feathers of different species tended to be positively related to wear of non-melanised feathers (Beta(SE) = 0.16(0.09), F = 3.27, df = 1,126, P = 0.073), but not to that of melanised (Beta(SE) = 0.01(0.08), F = 0.01, df =1,138, P = 0.958). Moreover, in the final model, feather degradability resulted negatively related to staphylococci bacterial loads and tended to be positively related to mesophilic and enterobacteria loads of sampled nests (see feather degradability in Table 2).

When considering both factors in the same models, feather degradability and nest bacterial environment explained feather wear. Feather degradability did not explain significant proportion of variance of wear index of melanised feathers (Beta(SE) = -0.01(0.08), F = 0.02, df = 130, P = 0.895), but bacterial environment did after controlling for species identity as showed in Table 2 (see wear of melanised feathers). On the other hand, wear of non-melanised feathers was positively associated with feather degradability and negatively, but not significantly, with staphylococci and enterococci bacterial loads of the nests (see wear of melanised feathers and feather degradability in Table 2).

All statistically significant associations disappeared when considering mean values per species of feather degradation, feather degradability and nest bacterial environment, and controlled for phylogenetic association among them (PGLS, P > 0.07,ESM, Table 1).

Wear and degradability of adults and nestling feathers

For the seven species with information on feather wear, interspecific variation of adults and nestlings followed a similar pattern (see the effect of age in Table 3). Only in hoopoes, feather wear of nestlings was higher than that detected in adult hoopoes (Fig 3A). Moreover, feathers of adult individuals were more resistant to bacterial degradation than those of nestlings. However, rank positions of feather degradability of adults and nestlings coincided except for hoopoes (Fig. 3B), which are the responsible of the significant interaction between species and age (Table 3).

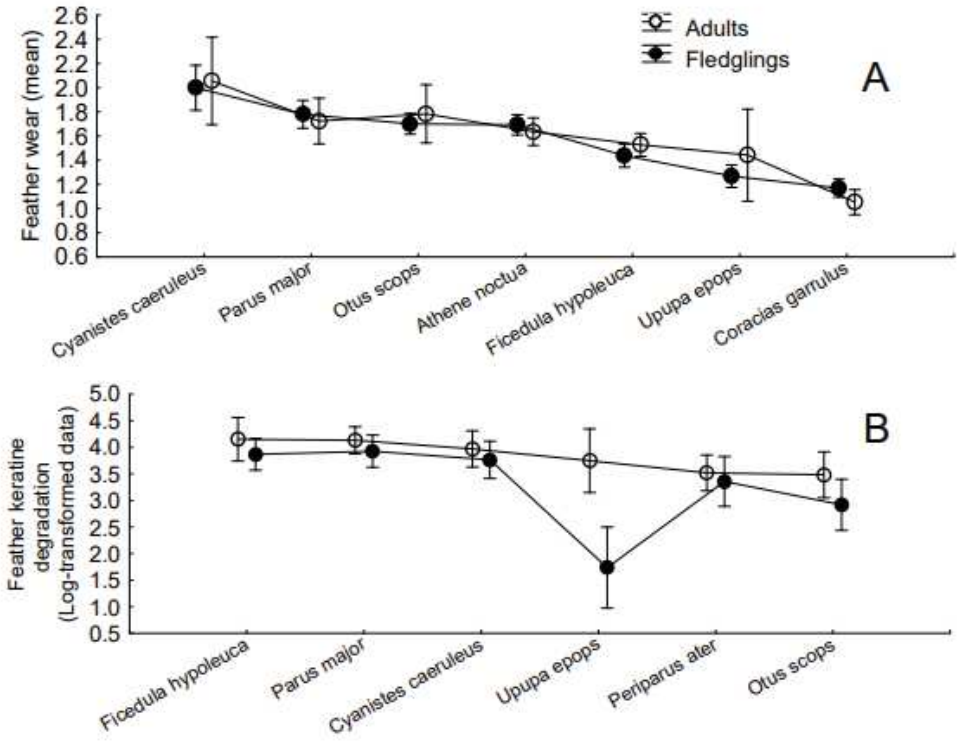


Fig 3: Wing feathers wear and degradability of 7 and 6 species, respectively, with information for fledging (filled dots) and adult (open circles) birds. Values are Least square means ± 95% CI. Species are ordered from the largest to lowest fledglings' values of the considered variable.

Table 2: Results from GLM models exploring the effects of nest bacterial environment (estimated as density of mesophilic bacteria, enterobacteria, staphylococci and enterococci) on (1) wear of melanised feathers, on (2) differences in wear of melanised and non-melanised of the same individual (repeated measures), after controlling for the effect of species identity. The effects of nest bacterial environment on feather degradability (average species values) were also explored in (3) GLM models. Finally, Results from GLM models exploring the effect of both bacterial environment and feather degradability (average species values) on wear of melanised feathers are also showed (4). We show statistics associated with full and final models (i.e., after a backward procedure, eliminating one by one the independent variable with the larger p-value up to 0.1. Variables associated with p-values smaller than 0.1 are showed in bold fonts to facilitated interpretation to readers.

	Full models				Final models			
	Beta(SE)	F	df	P	Beta(SE)	F	df	P
Wear of melanised feathers (1)								
Mesophilic bacteria	**-0.179(0.098)**	**3.31**	**1,137**	**0.07**	**-0.238(0.092)**	**6.75**	**1,139**	**0.010**
Enterobacteria	**0.206 (0.099)**	**4.30**	**1,137**	**0.04**	**0.157(0.095)**	**2.75**	**1,139**	**0.099**
Staphylococci	-0.094(0.095)	0.97	1,137	0.33				
Enterococci	-0.132(0.108)	1.49	1,137	0.22				
Species identity		**7.19**	**14,137**	**< 0.0001**		**7.00**	**14,139**	**< 0.0001**
Feather wear in relation to feather pigmentation (within-nests Repeated Measures) (2).								
Pigm		**3.32**	**1,120**	**0.071**		**4.34**	**1,122**	**0.039**
Pigm x Mesophilic bacteria		**4.27**	**1,120**	**0.041**		**6.99**	**1,122**	**0.009**
Pigm x Enterobacteriacea		**6.03**	**1,120**	**0.015**		**5.52**	**1,122**	**0.020**
Pigm x Staphylococci		1.78	1,120	0.184				
Pigm x Enterococci		0.05	1,120	0.823				
Pigm x Species identity		**6.22**	**9,120**	**< 0.0001**		**6.70**	**9,122**	**< 0.0001**

	Full models				Final models			
	Beta(SE)	F	df	P	Beta(SE)	F	df	P
Feathers degradability (3)								
Mesophilic bacteria	0.175(0.110)	2.53	1,136	0.114	**0.190(0.106)**	**3.17**	**1,137**	**0.077**
Enterobacteria	0.181 (0.112)	2.60	1,136	0.109	**0.196(0.109)**	**3.24**	**1,137**	**0.074**
Staphylococci	**-0.229(0.103)**	**4.94**	**1,136**	**0.028**	**-0.211(0.096)**	**4.79**	**1,137**	**0.030**
Enterococci	0.056(0.111)	0.26	1,136	0.614				
Wear of melanised feathers and feather degradability (4)								
Mesophilic bacteria	0.590(0.432)	1.86	1,119	0.175				
Enterobacteria	-0.191(0.193)	0.98	1,119	0.325				
Staphylococci	**-0.345(0.193)**	**3.18**	**1,119**	**0.077**	**-0.328(0.185)**	**3.15**	**1,121**	**0.078**
Enterococci	**-0.373(0.209)**	**3.20**	**1,119**	**0.076**	**-0.355(0.188)**	**3.54**	**1,121**	**0.062**
Feather degradability	**0.238(0.102)**	**5.47**	**1,119**	**0.021**	**0.241(0.098)**	**5.98**	**1,121**	**0.016**

Table 3: Results from General Linear Mixed Models exploring the effects of age (fledglings vs adults) and species identity explaining feather wear and degradability by keratinolytic bacteria. The main effects were estimated in models that did not include the interaction, and the interaction was estimated in models that also included main effects. Since feather wear was estimated from several nestlings of the same nest, we included nest identity nested within the interaction between species and age as a random factor. Moreover, since estimates of feather degradability depend of feather mass, we included such information as a covariable in the model.

Factors	Effect	Ms effect	MS error	df	F	P
Feather wear						
Species	F	15.96	0.62	6, 211.4	25.64	< 0.0001
Age	F	0.01	0.57	1, 280.9	0.01	0.923
Species * Age	F	0.81	0.56	6, 313.0	1.45	0.197
Nest id(Species*Age)	Rnd	0.66	0.37	168, 1706	1.99	< 0.0001
Feather degradability						
Feather mass	F	4.28	0.27	1, 101	15.89	0.0001
Species	F	2.12	0.27	5, 101	11.99	< 0.0001
Age	F	3.14	0.27	1, 101	30.85	0.0009
Species * Age	F	0.98	0.27	5, 96	4.23	0.0016

Discussion

We here estimated feather wear and degradability in fledglings and nest bacterial loads in nests of 16 species of Palaearctic birds, and detected statistically significant interspecific variation for all considered variables. On average, non-melanised feathers were more deteriorated than melanised feathers, but it was only detected when comparing feathers of nestlings within the same nests, and after controlling for bacterial environment. Moreover, our results suggest that the feather deterioration state was explained by feather

degradability (only for non-melanised feathers) and nest bacterial environment. Feather degradability was also explained by nest bacterial environment, suggesting that the nest microbiome determines the integrity of fledgling feathers as well as their strength to resist bacterial degradation. These last results were however not controlled for phylogenetic effects. Below we discuss and offer several possible explanations for the sign of the detected associations.

Consistent with previous papers claiming that non-melanised feathers are more easily degraded by bacterial activity than melanised feathers (Goldstein et al. 2004, Gunderson et al. 2008, Ruiz-de-Castañeda et al. 2012), we found that non-melanised feathers of fledglings were more deteriorated than melanised feathers of the same individual. Feather deterioration was estimated for wing cover feathers and, thus, we assumed that melanised and non-melanised parts of feathers should have experienced a similar risk of deterioration. Moreover, the pigmentation effect on feather deterioration was only detected when comparing melanised and non-melanised feathers within the same nest (i.e. repeated measures). These results suggest that the detected differences are due to differential effects of nest bacterial environment on melanised and non-melanised feathers, which might be explained by melanin conferring resistance (Bonser 1995) to feathers or/and different feather microstructure, which would confer different resistance to melanised and non-melanised part of feathers (Ruiz-de-Castañeda et al. 2012, Ruiz-Rodríguez et al. 2015). This general pattern of non-melanised feathers being more deteriorated does not occur in all studied species, with hoopoes showing the opposite patterns, and some other species showing no apparent differences. We did not consider the possibility that different species varied in the quality and quantity of pigments in analysed feathers, which might partially explain interspecific differences in the effect of feather pigmentation. Future work directed to explore interspecific differences in pigmentation and

microstructure of feathers would clarify the importance of such characters in explaining detected interspecific differences in feather wear.

The most robust result is the detected interspecific differences in all considered variables. Interspecific differences in nest bacterial environment estimated as eggshell bacterial loads were first detected by Peralta-Sánchez et al. (2012), whom also demonstrated associations with life history characteristics and hatching success (Peralta-Sánchez et al. 2018). Here, we directly sampled nest materials and for the first time confirm interspecific variation in nest bacterial environment that should affect probability of bacterial contamination of eggs, nestlings and adults. Moreover, we also found significant interspecific variation in feathers' susceptibility to bacterial degradation, which was first suggested by Burtt et al. (2011) in association with interspecific differences feathers' pigmentation of 13 parrot species. Interestingly these authors suggested that interspecific differences in feather degradability should be explained by risk of feather degradation typically experienced by each species. If this was the case, interspecific differences in degradation state of feathers collected from the same body parts (i.e. wing feathers) and at similar stage (similar nestling developmental stage) should be close to zero. However, our results did not fit this prediction, but pointed out interspecific differences in feather susceptibility to degradation. Our results, therefore, suggest that feather resistance do not completely adapt to risk of bacterial degradation, and that some other factors will play a role.

Interspecific differences in factors affecting feather wear could explain the detected differences in deterioration of fledgling feathers. We characterized interspecific differences in feather degradability and in nest bacterial environment to explore whether these characteristics were responsible of the wear variability of fledgling feathers, and it resulted associated with bacterial load of nests where sampled fledglings grew. However, the sign of the association depended of feather pigmentation and of the bacterial group

considered. Negative associations between bacterial loads of nests and feather deterioration can be explained in several ways. First, feathers of species developing in nests with higher bacterial densities could be more resistant to bacterial degradation. Partly in accordance with this *a posteriori* explanation, we found that feather degradability was lower in species with a high density of staphylococci in their nests. However, the association turned to be positive when considering mesophilic bacteria or enterobacteria, which suggest that the possible influences of nest bacterial environment on the evolution of feather resistance to bacterial deterioration would depend on the bacterial group considered and of characteristics of the bacterial community in relation to the risk of feather deterioration by microorganisms. This speculative inference links the first to the second possibility explaining the detected negative association between bacterial loads of nests and feather wear index. This second explanation posits that characteristics of the bacterial community, or that some bacteria in avian nests, could protect nestling feathers from feather-degrading microorganisms. If that was the case, feathers of species with protecting bacteria in their nests should be more susceptible to bacterial degradation. On the one hand, nest environments with higher bacterial densities might harbour keratinolytic bacteria in lower proportion than those with lower bacterial density. This possibility is however unlikely since mesophilic and keratinolytic bacterial densities are positively correlated, at least in starling feathers (Ruiz-Rodríguez et al. 2015). It might also be possible that bacteria producing antibiotics against keratinolitic ones occur at higher relative density in nests with higher bacterial densities. Bacterial antimicrobials function by reducing competition for space and resources against other bacterial strains (Ji et al. 1997, Riley and Wertz 2002), and bacterial density is one of the factors known to enhance antimicrobial production (Riley and Wertz 2002). Thus, it is possible that high-density bacterial communities also produce high level of antimicrobials against keratinolytic bacteria able to colonize feather tissues, resulting in lower levels

of feather degradation. However, we did not quantify the density of keratinolytic bacteria in nest environments nor in nestling feathers, which is necessary to test and discuss this possibility.

Another possibility that could explain the negative association between nest-bacterial density and wear of unpigmented feathers is that this part of nestling feathers function honestly reflecting (i.e., signalling) individual abilities of nestlings to maintain plumage integrity during the last days of the nestling stage (Morales et al. 2019). Reliability of signals partly depends on costs associated with their production, showiness and/or maintenance (Maynard-Smith and Harper 2003), and fledglings showing non-degraded plumage, or plumage of a particular coloration, might signal its phenotypic quality to conspecifics (López-Idiáquez et al. 2018). The role of bacteria in the evolution of feather characteristics that honestly signal phenotypic quality of holders has been previously demonstrated in adult males of the spotless starling (*Sturnus unicolor*), which wear delicate, long sexual feathers that are easily degraded by bacteria and that harbour bacteria at a higher density than non-sexual feathers (Ruiz-Rodríguez et al. 2015). Only males with a higher capacity to prevent feather wear by bacteria will show non-deteriorated feathers. Similarly, only good quality fledglings would show non-degraded white patches on their feathers. Signalling roles of fledgling plumage in different scenarios of social interaction have been recently proposed to explain juvenile plumage patterns (Moreno and Soler 2011, Ligon and Hill 2013, Fargallo et al. 2014, Romano et al. 2016, Galván 2017, Morales et al. 2019). Our results show that feather degradability explains feather wear, but mainly that of non-melanised feathers, which is in accordance with the proposed scenario. However, melanised feathers were less deteriorated than non-melanised feathers in some, but not all species, which is contrary to the proposed scenario. Future work should concentrate on exploring this hypothesis intra- and interspecifically.

Detected associations that included feather degradability should be considered cautiously since they are not controlled by species identity, and the effects disappeared when using mean values per species in phylogenetically controlled analyses. These analyses however included only 11 species, which is a small sample size to reach strong conclusions.

Summarising, we found clear interspecific differences in the deterioration state of fledgling feathers that tended to parallel interspecific differences in feather resistance to bacterial degradation, and in nest bacterial environment, which suggest a role of bacteria in determining such interspecific variation. The sings of the association between nest bacterial loads and feather deterioration depend on the bacterial group considered so the different possible explanations offered should be considered as preliminary and further tested both at the intra- and interspecific levels.

Acknowledgments

We thank Estefanía López Hernández and Pilar Fuentetaja Casado for assistance in laboratory work and Natalia Juárez García-Pelayo for assistance in fieldwork. We benefited from comments by two anonymous referees that improved the quality of the manuscritp. This work was supported by the currently named Ministerio de Ciencia, Innovación y Universidades and European (FEDER) funds (CGL2013-48193-C3-1-P, CGL2013-48193-C3-2-P, CGL2013-48193-C3-3-P, CGL2017-83103-P). MAG and SDL were financed by predoctoral contracts (BES-2014-068661, BES-2014-069116) from the Spanish Ministerio de Economía y Competitividad. The authorization for carrying out the experimental work on animals in the field was granted by the Dirección General de Gestión del Medio Natural of the Consejería de Medio Ambiente of Junta de Andalucía (Guadix) and by the Dirección General del Medio Natural of Junta de Castilla y León (Valsaín).

References

Andersson, M. 1994. *Sexual selection,* Princeton, Princeton University Press.

Bonser, R. H. C. 1995. Melanin and the abrasion resistance of feathers. -*Condor,* 97: 590-591.

Burtt, E. H. 1979. Tips on wings and other things. In: BURTT, E. H. (ed.) *The Behavioural Significance of Color.* Garland Publishing Inc., New York, pp. 75-100.

Burtt, E. H. and Ichida, J. M. 2004. Gloger's rule, feather-degradlng bacteria, and color variation among song sparrows. -*Condor,* 106: 681-686.

Burtt, E. H., Schroeder, M. R., Smith, L. A., Sroka, J. E. and Mcgraw, K. J. 2011. Colourful parrot feathers resist bacterial degradation. -*Biology Letters,* 7: 214-216.

Burtt Jr, E. 2009. A future with feather-degrading bacteria. -*Journal of Avian Biology,* 40: 349-351.

Callan, L. M., La Sorte, F. A., Martin, T. E. and Rohwer, V. G. 2019. Higher Nest Predation Favors Rapid Fledging at the Cost of Plumage Quality in Nestling Birds. -*American Naturalist,* 193: 717-724.

Cristol, D. A., Armstrong, J. L., Whitaker, J. M. and Forsyth, M. H. 2005. Feather-degrading bacteria do not affect feathers on captive birds. -*Auk,* 122: 222-230.

Delhey, K. 2017. Gloger's rule. -*Current Biology,* 27: R689-R691.

Fargallo, J. A., Velando, A., Lopez-Rull, I., Ganan, N., Lifshitz, N., Wakamatsu, K. and Torres, R. 2014. Sex-specific phenotypic integration: endocrine profiles, coloration, and behavior in fledgling boobies. -*Behavioral Ecology,* 25: 76-87.

Francis, C. M. and Wood, D. S. 1989. Effects of age and wear on wing length of wood warblers. -*Journal of Field Ornithology,* 60: 495-503.

Galván, I. 2017. Condition-dependence of pheomelanin-based coloration in nuthatches Sitta europaea suggests a detoxifying function: implications for the evolution of juvenile plumage patterns. -*Scientific Reports,* 7: 9138.

Garamszegi, L. 2014. Uncertainties Due to Within-Species Variation in Comparative Studies: Measurement Errors and Statistical Weights. pp. 157-199.

Garamszegi, L. Z. and Møller, A. P. 2010. Effects of sample size and intraspecific variation in phylogenetic comparative studies: a meta-analytic review. -*Biological Reviews,* 4: 797-805.

Goldstein, G., Flory, K. R., Browne, B. A., Majid, S., Ichida, J. M. and Burtt, E. H. 2004. Bacterial degradation of black and white feathers. -*Auk,* 121: 656-659.

González-Braojos, S., Sanz, J. J. and Moreno, J. 2017. Decline of a montane Mediterranean pied flycatcher Ficedula hypoleuca population in relation to climate. -*Journal of Avian Biology,* 48: 1383-1393.

Gunderson, A. R. 2008. Feather degrading bacteria: a new frontier in avian and host-parasite research? -*Auk,* 125: 972-979.

Gunderson, A. R., Frame, A. M., Swaddle, J. P. and Forsyth, M. H. 2008. Resistance of melanized feathers to bacterial degradation: is it really so black and white? -*Journal of Avian Biology,* 39: 539-545.

Hanson, T. 2011. *Feathers: the evolution of a natural miracle,* New York, Basic Books.

Jacob, S., Salle, L., Zinger, L., Chaine, A. S., Ducamp, C., Boutault, L., Russell, A. F. and Heeb, P. 2018. Chemical regulation of body feather microbiota in a wild bird. -*Molecular Ecology,* 27: 1727-1738.

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

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

Ji, G., Beavis, R. and Novick, R. P. 1997. Bacterial interference caused by autoinducing peptide variants. -*Science,* 276: 2027-2030.

Jovani, R. and Rohwer, S. 2017. Fault bars in bird feathers: mechanisms, and ecological and evolutionary causes and consequences. -*Biological Reviews,* 92: 1113-1127.

Kent, C. M. and Burtt, E. H. 2016. Feather-degrading bacilli in the plumage of wild birds: Prevalence and relation to feather wear. -*Auk,* 133: 583-592.

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

Leclaire, S., Pierret, P., Chatelain, M. and Gasparini, J. 2014. Feather bacterial load affects plumage condition, iridescent color, and investment in preening in pigeons. -*Behavioral Ecology,* 25: 1192-1198.

Ligon, R. A. and Hill, G. E. 2013. Is the juvenal plumage of altricial songbirds an honest signal of age? Evidence from a comparative study of thrushes (Passeriformes: Turdidae). -*Journal of Zoological Systematics and Evolutionary Research,* 51: 64-71.

López-Idiáquez, D., Fargallo, J. A., López-Rull, I. and Martínez-Padilla, J. 2018. Plumage coloration and personality in early life: sexual differences in signalling. -*Ibis,* 0.

Martín-Vivaldi, M., Ruiz-Rodríguez, M., Mendez, M. and Soler, J. J. 2006. Relative importance of factors affecting nestling immune response differs between junior and senior nestlings within broods of hoopoes *Upupa epops*. -*Journal of Avian Biology,* 37: 467-476.

Maynard-Smith, J. and Harper, D. 2003. *Animal Signals,* Oxford, Oxford University Press.

Minias, P., Wlodarczyk, R., Surmacki, A. and Iciek, T. 2015. Silver spoon effects on plumage quality in a passerine bird. -*R Soc Open Sci,* 2: 140459.

Møller, A. P., Barbosa, A., Cuervo, J. J., De Lope, F., Merino, S. and Saino, N. 1998. Sexual selection and tail streamers in the barn swallow. -*Proceedings of the Royal Society of London B, Biological Sciences,* 265: 409-414.

Møller, A. P., Peralta-Sánchez, J. M., Nielsen, J. T., López-Hernández, E. and Soler, J. J. 2012. Goshawk prey have more bacteria than non-prey. -*Journal of Animal Ecology,* 81: 403-410.

Morales, J., Cuervo, J. J., Moreno, J. and Soler, J. J. 2019. Juvenile plumage whiteness is associated with the evolution of clutch size in passerines. -*Behavioral Ecology*.

Moreno, J. and Soler, J. J. 2011. Sources of distinctness of juvenile plumage in Western Palearctic passerines. -*Biological Journal of the Linnean Society,* 102: 440-454.

Paradis, E. and Schliep, K. 2018. ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. -*Bioinformatics (Oxford, England),* 35.

Peralta-Sánchez, J. M., Martín-Platero, A. M., Wegener-Parfrey, L., Martínez-Bueno, M., Rodríguez-Ruano, S., Navas-Molina, J. A., Vázquez-Baeza, Y., Martín-Gálvez, D., Martín-Vivaldi, M., Ibáñez-Álamo, J. D., Knight, R. and Soler, J. J. 2018. Bacterial density rather than diversity correlates with hatching success across different avian species. -*FEMS Microbiology Ecology,* 94: fiy022-fiy022.

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

Peralta-Sánchez, J. M., Møller, A. P., Martín-Platero, A. M. and Soler, J. J. 2010. Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: an experimental study. -*Functional Ecology,* 24: 426-433.

Pinheiro, J., Bates, D., Debroy, S., Sarkar, D. and Team, R. C. 2019. {nlme}: Linear and Nonlinear Mixed Effects Models. R package version 3.1-141, https://CRAN.R-project.org/package=nlme.

Rayner, J. M. V. 1988. Form and function in avian flight. In: JOHNSTON, R. F. (ed.) *Current Ornithology.* Springer US, Boston, MA, pp. 1-66.

Riley, M. A. and Wertz, J. E. 2002. Bacteriocines: evolution, ecology, and application. -*Annual Review of Microbiology,* 56: 117-137.

Romano, A., Bazzi, G., Caprioli, M., Corti, M., Costanzo, A., Rubolini, D. and Saino, N. 2016. Nestling sex and plumage color predict food allocation by barn swallow parents. -*Behavioral Ecology,* 27: 1198-1205.

Roulin, A. 2016. Condition-dependence, pleiotropy and the handicap principle of sexual selection in melanin-based colouration. -*Biological Reviews,* 91: 328-348.

Ruiz-De-Castaneda, R., Burtt, E. H., Gonzalez-Braojos, S. and Moreno, J. 2015. Bacterial degradability of white patches on primary feathers is associated with breeding date and parental effort in a migratory bird. -*Ibis,* 157: 871-876.

Ruiz-De-Castañeda, R., Burtt Jr, E., González--Braojos, S. and Moreno, J. 2012. Bacterial degradability of an intrafeather unmelanized ornament: a role for feather-degrading bacteria in sexual selection? -*Biological Journal of the Linnean Society,* 105: 409-419.

Ruiz-Rodríguez, M., Møller, A. P., Mousseau, T. A. and Soler, J. J. 2016. Defenses against keratinolytic bacteria in birds living in radioactively contaminated areas. -*The Science of Nature,* 103: 1-8.

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

Ruiz-Rodríguez, M., Valdivia, E., Soler, J. J., Martin-Vivaldi, M., Martin-Platero, A. M. and Martinez-Bueno, M. 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. -*Journal of Experimental Biology,* 212: 3621-3626.

Senar, J. 2006. Color displays as intrasexual signal of agression and dominance In: HILL, G. E. and MCGRAW, K. J. (eds.) *Bird coloration: Function and evolution.* Harvard University Press, Cambridge, Massachusetts, pp. 87-136.

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

Shawkey, M. D., Pillai, S. R., Hill, G. E., Siefferman, L. M. and Roberts, S. R. 2007. Bacteria as an agent for change in structural plumage color: Correlational and experimental evidence. -*American Naturalist,* 169: S112-S121.

Soler, J. J. and Avilés, J. M. 2010. Sibling competition and conspicuousness of nestling gapes in altricial birds: a comparative study. -*PLoS ONE,* 5: e10509.

Statsoft-Inc. 2011. *STATISTICA (data analysis software system), version 10*, www.statsoft.com.

Stettenheim, P. R. 2000. The integumentary morphology of modern birds — An Overview. -*American Zoologist,* 40: 461-477.

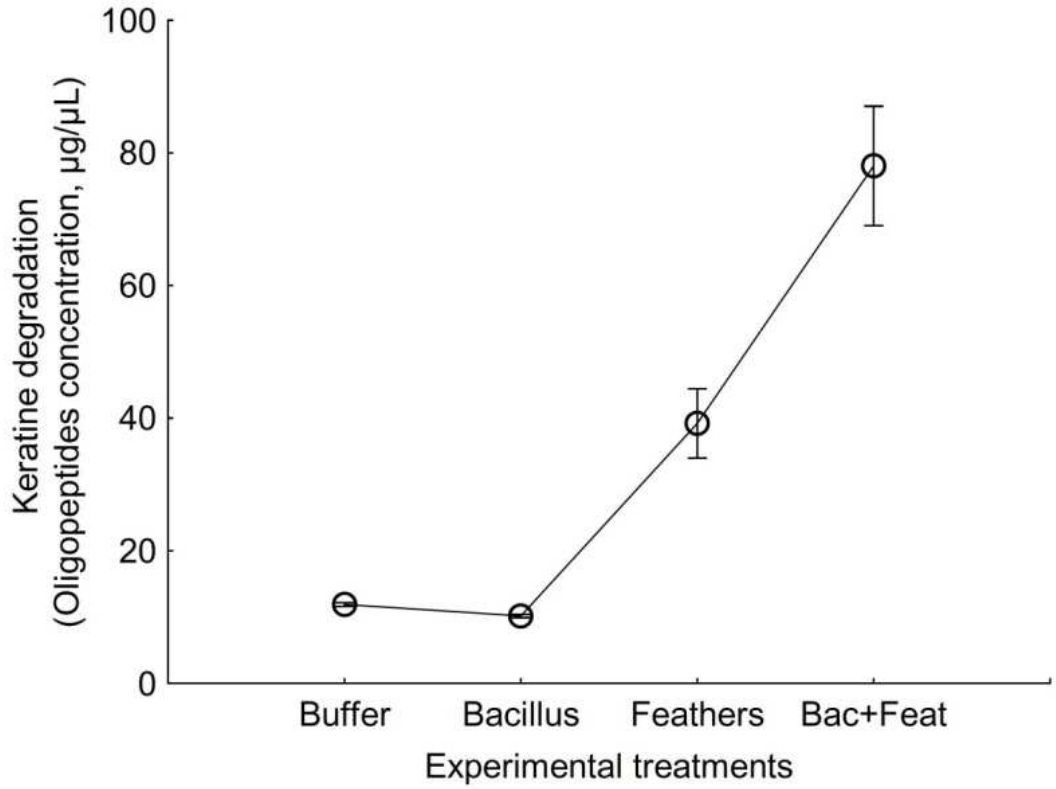
Swaddle, J. P., Witter, M. S., Cuthill, I. C., Budden, A. and Mccowen, P. 1996. Plumage condition affects flight performance in common starlings: Implications for developmental homeostasis, abrasion and moult. -*Journal of Avian Biology,* 27: 103-111

Annex

Table A1: Results from PGLM models exploring the effects of nest bacterial environment (estimated as density of mesophilic bacteria, enterobacteria, staphylococci and enterococci) on (1) wear of melanised feathers (2), and feather degradability (3) after correcting for phylogeny. We also explored the effect of both bacterial environment and feather degradability on wear of melanised (4) and non-melanised (5) feathers. For all the analyses we used average species values, which were by sample size (1/N).

	Full models			
	B (SE)	t	df	P
Wear of melanised feathers (1)				
Mesophilic bacteria	3.887(2.612)	1.49	10	0.17
Enterobacteria	-0.617(1.166)	0.53	10	0.61
Staphylococci	1.001(0.960)	1.05	10	0.32
Enterococci	-0.656(1.000)	0.66	10	0.53
Wear of non-melanised feathers (2)				
Mesophilic bacteria	-1.975(3.268)	0.60	6	0.57
Enterobacteria	1.926(1.435)	1.34	6	0.23
Staphylococci	-2.298(1.046)	2.20	6	0.071
Enterococci	0.814(1.235)	0.66	6	0.53
Feather degradability (3)				
Mesophilic bacteria	1.527(4.270)	0.358	7	0.73
Enterobacteria	1.594(1.925)	0.828	7	0.44
Staphylococci	2.330(1.726)	1.35	7	0.22
Enterococci	0.959(1.909)	0.50	7	0.63
Wear of melanised feathers and feather degradability (4)				
Mesophilic bacteria	3.226(3.101)	1.04	5	0.346
Enterobacteria	0.569(1.456)	0.39	5	0.71
Staphylococci	-0.096(1.492)	0.06	5	0.95
Enterococci	-0.361(1.429)	0.25	5	0.81
Feather degradability	-0.324(0.278)	1.16	5	0.30
Wear of non-melanised feathers and feather degradability (5)				
Mesophilic bacteria	2.100(4.159)	0.50	3	0.65
Enterobacteria	1.765(1.801)	0.98	3	0.40
Staphylococci	2.372(1.809)	1.31	3	0.28
Enterococci	0.881(2.033)	0.43	3	0.69
Feather degradability	0.036(0.342)	0.10	3	0.92

Fig. A1. Means ± CI 95% of oligopeptide concentration after 21 days of incubation of control tubes containing only buffer, the buffer plus the degrading bacteria *Bacillus licheniformis*, the buffer plus a piece (1 cm^2) of feather, or the buffer plus the bacteria plus the piece of feather.



CAPÍTULO 5

Length of ornamental throat feathers predicts telomere dynamic and hatching success in spotless starling (*Sturnus unicolor*) males

Abstract

Sexually selected signals are associated with variables that reliably reflect individual phenotypic or genetic quality, and, thus, survival prospects of holders. Telomere length is considered a good predictor of life expectancy. Consequently, exploring the links between telomere length and sexually selected traits is much needed to understand the underlying mechanisms explaining functional significance of secondary sexual traits. We manipulated the length of throat feathers in spotless starling (Sturnus unicolor) males (a sexually selected signal) before reproduction and explored its effects on telomere shortening and breeding performance in subsequent reproductive events. We did not detect any significant experimental effect, but males with longer throat feathers before the experiment had shorter telomeres that also shortened more slowly than those of males with shorter throat feathers. Moreover, length of throat feathers of males before manipulation was positively related to hatching success of second clutches. Thus, correlative but not experimental results support the expected associations. We discuss such results in scenarios of sexual selection where feather length reflects, but does not directly cause, telomere attrition and enhanced reproductive success. Males with longer throat feathers might be older, more experienced males (i.e., with shorter telomeres), able to buffer telomere shortening between reproductive events. Because of the absence of experimental effects, differential incubation effort of females cannot explain the detected association with hatching success, but other sexually selected traits that covary with throat-feather length could be responsible. Exploring those physiological and/or morphological

characteristics related to throat-feather length should therefore be the matter of future research.

Keywords: Hatching success, Sexual selection, Sexual signalling, Sturnus unicolor, Telomere dynamics, Throat-feathers length.

Introduction

The evolution of signals, defined as behavioural, morphological or physiological characteristics that function transferring information among organisms (Otte 1974), have attracted the attention of evolutionary biologists for decades (Maynard-Smith and Harper 2003). By definition, the use of signals must result in benefits for both the emitter and the receiver(s), which implies that the transmitted information should be reliable (Bradbury and Vehrencamp 1998; Searcy and Nowicki 2005). Extravagant secondary sexual characters of males are usually considered as examples of signals reflecting different components of their genetic and phenotypic quality (Andersson 1994). Females, by preferring males with traits that correlate with their genetic and /or phenotypic quality, will gain direct (good parents) or indirect (good genes) beneficial fitness effects (Hamilton 1990; Andersson 1994; Møller 1994; Møller and Jennions 2001; Jones and Ratterman 2009). On the other hand, by exaggerating attractive characters for females, males might gain breeding opportunities (Darwin 1871), and increased investment in reproduction by females (Burley 1988) that should result in higher reproductive success per reproductive event (Møller 1994; Sheldon 2000).

The above-described scenario for explaining the evolution of sexually selected traits in males predicts a positive association between the character and the reproductive success of both males and females. Although it was suggested that male traits that are selected by females do not need to be related to phenotypic or genetic quality of holders (e.g., runaway sexual selection process, Fisher 1930), it is now accepted that, mainly because of costs associated to intra-sexual competition, these traits (i.e. sensory traps) would rapidly evolve reflecting the quality of males (Macias García and Ramirez 2005). Thus, sexually selected traits should reliably reflect phenotypic or genetic quality of the holder. Reliability of the signals is mainly warranted by

associated costs that cheaters would not be able to afford (Zahavi 1975, 1977; Maynard-Smith and Harper 2003). These costs might refer to those of production (Hill 2000; Pardal et al. 2018), maintenance (e.g., in time and energy (Blount et al. 2003; Ruiz-Rodríguez et al. 2015), and showiness (social control (Tibbetts and Dale 2004; Webster et al. 2018) and detectability by parasites or predators (Stuart-Fox et al. 2003; Johnson and Candolin 2017; but see Webster et al. 2018)). Detecting such costs, and associations between the expression of the target trait and variables reflecting phenotypic or genetic quality of the holder, is essential to conclude in favour of the existence of sexually selected reliable signals.

Telomeres are likely candidates for maintaining reliability in sexually selected signals because their length is nowadays considered a good indicator of phenotypic quality (Bauch et al. 2013). Telomeres are regions of repeated noncoding DNA sequence (TTAGGG) that cap the end of eukaryotic chromosomes. Among other functions, telomeres protect wholeness of genetic information during cell division (Blackburn 1991). Because replication failures frequently occur at the end of the chromosomes, telomeres continuously shorten as a consequence of cell division (Boonekamp et al. 2017). Thus, telomere length is a good predictor of senescence, both at cellular and organism levels (Suram et al. 2012; Campisi 2013) and survival prospects (Monaghan and Haussmann 2006; Kotrschal et al. 2007; Soler et al. 2015). Moreover, telomere length and attrition apparently have significant genetic components (Dugdale and Richardson 2018) and are related to antioxidant capabilities (Beaulieu et al. 2011; Badás et al. 2015; Kim and Velando 2015) and level of stress suffered during development (Boonekamp et al. 2014; Herborn et al. 2014), including infection (Asghar et al. 2015; Eisenberg et al. 2017; 2018). Telomere length even predicted reproductive success in wild birds (Boonekamp et al. 2014; Parolini et al. 2017; Bauer et al. 2018) and, thus, detecting relationships between a sexually selected character and telomere

length and/or attrition would suggest that the trait reflects (i.e. signals) phenotypic and/or genetic quality of the holder.

As far as we know, the expected association between a sexually selected trait and telomere length and/or dynamic, has only been explored and detected in two bird species, the barn swallow (*Hirundo rustica*) (Parolini et al. 2017) and the common yellowthroat (*Geothlypis trichas*) (Taff and Freeman-Gallant 2017). In both studies, the focal sexually selected trait was plumage colouration. Telomere length of males and females correlated with sexually dimorphic colouration of swallows (Parolini et al. 2017). In the case of yellowthroats, plumage colouration was related to telomere length and rate of telomere loss between years, but only in males (Taff and Freeman-Gallant 2017). More recently, it has been detected that colouration of spotless starlings (*Sturnus unicolor*, hereafter starlings) eggshells predicts telomere length of fledglings (Soler et al. 2018). Since eggshell colouration is a secondary sexual trait of females in this species (Soler et al. 2008), these results also suggest a link between telomeres and sexually selected traits. These examples are correlative results dealing with sexually selected characters mediated by pigments. We here go a step forward and test the expected association between telomere length and sexually selected traits in an experimental framework, focusing on a sexual trait whose expression is not related to pigments.

We manipulated the length of the sexually selected throat feathers of starling males (Hiraldo and Herrera 1974; Ruiz-Rodríguez et al. 2015) and explored the effects on telomere dynamics, egg-laying dates and reproductive success. The apical part of these feathers is quite flexible and males exhibit them very conspicuously during courtship (Ruiz-Rodríguez et al. 2015). In males, the apical part of throat feathers harboured more feather degrading keratinolytic bacteria and degraded more quickly than its basal part, suggesting that males with less degraded feathers are better able to control bacterial infections (Ruiz-Rodríguez et al. 2015). Moreover, length of the throat feathers

is positively related with degree of individual homozygosity (Aparicio et al. 2001), and with the ability of counteracting the immunosuppressive effects of testosterone (Gil and Culver 2011). Thus, there is reasonable evidence supporting the signalling nature of throat feathers of starling males that females use in mate choice (Aparicio et al. 2001).

We manipulated the length of the throat feathers of males just before reproduction and explored its effects on laying date and success of subsequent reproductive events. Laying date has been traditionally used as a proxy for mating success and thus for sexual selection component of fitness (Møller et al. 2006; Romano et al. 2017). We also explored the effects of experimental manipulation on telomere shortening by analysing telomere length of males during subsequent reproductive seasons. Finally, we tested the relationship between length of throat feathers and telomere length and dynamics (as proxies of phenotypic and/or genetic quality) in males and females. Our expectation is that throat-feather length of starling males, but not that of females, should positively affect reproductive success, early pairing, and telomere length, reducing rate of telomere deterioration. Thus, we should find such associations in correlative frameworks, while the experimental reduction of throat feather length should result in lower reproductive success, later breeding dates and increased rates of telomere deterioration. If reduction of throat-feather length increases stress levels due to social interactions, its effect on telomere deterioration should be detected. By contrast, if it negatively affects mating success and thus reduces reproductive effort, telomere deterioration would be lower in males with experimentally shortened throat feathers.

Material and Methods

Study area and study species

The study was conducted during the years 2013, 2015, 2016 and 2017 in a south-eastern region of Spain (Hoya de Guadix, 37°15'N, 3°01'W) where nest-boxes attached to tree trunks or walls at 3–4 m above-ground are available for starlings to breed on (for further information on the study area see Soler et al. (2017)).

The spotless starling is a medium-sized, hole-nesting and polygynous passerine (Cramp 1998; Veiga and Polo 2016). Starlings are sexually dimorphic. Apart from slight differences in beak and plumage coloration between males and females (Navarro et al. 2010; Veiga and Polo 2016), the most apparent sexually dimorphic character are the throat feathers. Post-breeding moult in this species starts at the end of June, thus it may overlap with the nestling period, and it is completed between mid-September and mid-October (Veiga and Polo 2016).

In the studied population, the reproductive season starts in early April and most individuals lay a second clutch during May-June. The most common clutch size is 4-5 eggs. The incubation phase usually begins with the penultimate egg, which promotes that the last nestling hatches later than the others (Veiga and Polo 2016). Incubation is mostly carried out by females with sporadic help from males, and extends for around 14 days, while the nesting period lasts 21-22 days (Cramp 1998). Both male and female parents contribute to feeding the offspring and remove nestling faecal sacs (Cramp 1998). During the whole nesting process, adults bring feathers and aromatic plants to the nest, which have been shown to have antimicrobial-beneficial functions (Ruiz-Castellano et al. 2016; Soler et al. 2017). Reproductive success in the study area varies among years and breeding attempts, with second

clutches usually showing lower reproductive success than first clutches (pers. obs.).

Fieldwork and experimental procedure

In this population, courtship activity (e.g. singing, introducing fresh green plants and feathers in nest boxes) starts in February, more than one month before egg laying (pers. obs.). During this period, some birds roost in nest-boxes and we take advantage of this fact for conducting yearly bird trapping sessions in the study area (twice a year between February and mid-March). One hour before dawn, we closed the entrance of all nest boxes in the study area, and immediately after dawn, we captured by hand all individuals found roosting inside. Captured birds were kept individually in clean cotton bags hanging from a stick to keep birds quiet, and were released immediately after sampling. The maximum time that a captured starling was in the bag did never exceed 3 h. For additional details, see Ruiz-Rodriguez et al. (2015). This procedure does not imply apparent negative effects on breeding performance of captured birds (Soler et al. 2008).

Every year, we also captured birds during breeding, 4-5 days after the hatching date, by using nest-box traps for a maximum time of one hour. All captured adults were ringed with a numbered metal ring (if not already ringed) and with a unique combination of three colour rings. We also measured length of throat feathers of males and females with a ruler as shown in Fig. 1a. Three measurements were taken to estimate its repeatability. In addition, we collected 0.5-0.6 ml of blood by puncturing the brachial vein and approximately 0.1 ml of this blood was conserved in ethanol for further molecular analyses. Finally, captured males were alternately assigned to control or experimental treatment. With the aid of scissors, we cut the distal half portion of throat feathers of experimental individuals, while we handled control males in the same way but without cutting throat feathers. After manipulation, we again measured feather

length of experimental males, which on average were shortened by 1.5 cm (GLM of throat-feather lengths by treatment in his first capture; Least squares means ± standard error: experimental males: 1.8 ± 0.1 cm; control males: 3.2 ± 0.1; $F = 140.16$, $p < 0.0001$) (Fig. 1). Males that were recaptured during different study years were assigned each time to the same experimental treatment, repeating the same procedure on them, with the exception of three males. Treatment of these three males changed from control in the first to experimental in the second capture, one year later. The effects of treatment in these males were considered as independent information in the analyses.

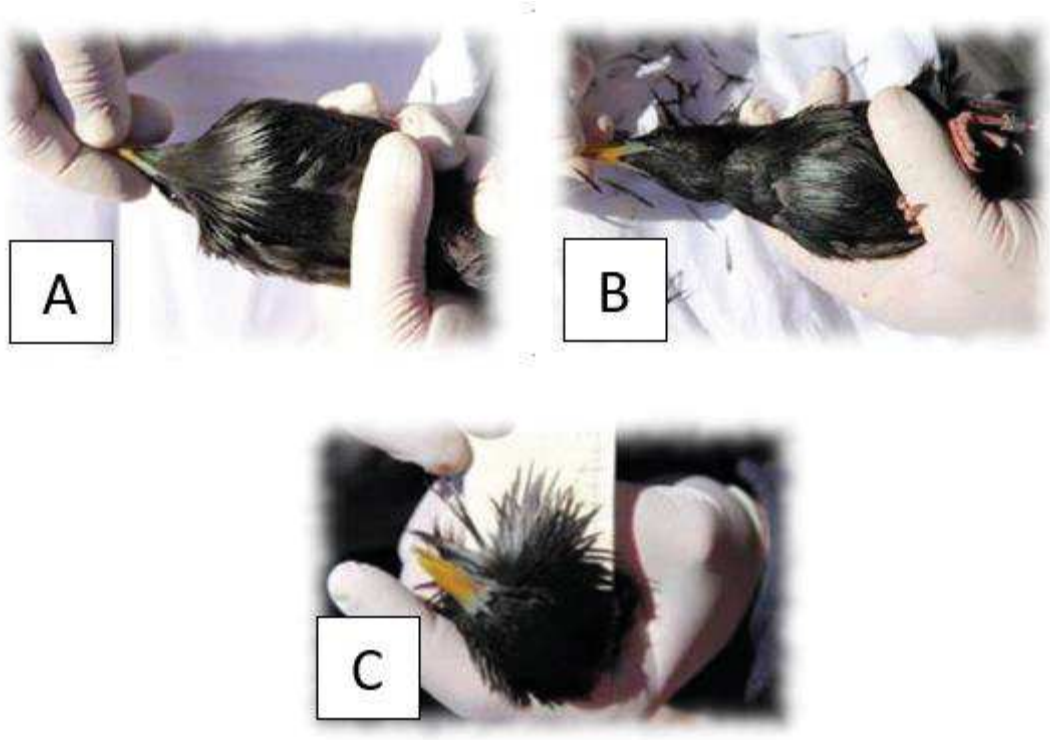


Fig. 1: Aspect of a control (A) and an experimental (B) individual, and the estimation of ornamental throat feathers length of a male spotless starling (C).

Starling reproduction was monitored by checking nest-boxes every three days since the beginning of April, which allowed us to accurately detect the date of first-egg laying and clutch size (i.e. maximum number of eggs detected). Afterwards, nest boxes with known laying dates were visited weakly until two days before the expected hatching date (14 days after the first egg

was laid), when we visited the nest every two days to estimate hatching success (percentage of laid eggs that hatched successfully). During the nestling phase, we visited the nest-boxes at an intermediate nestling age (approximately 8 days after hatching), and few days before fledging (14 days after hatching). During this last visit, the number of nestlings was recorded, and fledging success was estimated as the percentage of hatchlings that survive until this day.

Experimental males were identified during breeding by capture at the nest boxes during the nesting phase (see above), or by visualizing the unique combinations of colour rings in 1h video recordings of nest-box entrances during the second-third day after hatching. Males usually attend the same nest-box during the entire breeding season in our study area (e.g. in 63 of 65 nest boxes the male attending the first and the second clutch was the same, MAG, personal observation). Thus, for those males that were only recorded in one of the breeding attempts (either first or second), we reasonably assumed that they attended the same nest-box during the other. The two cases were we detected different males attending first and second breeding attempts in the same nest-box were not considered in the analyses.

Telomere length estimation

DNA was extracted from blood samples using a standard chloroform–isoamyl alcohol-based protocol (see Ferraguti et al. (2013)). DNA concentration was adjusted to 20 ng µL-1 using distilled water and conserved frozen until further analyses. Relative telomere length (hereafter telomere length) was estimated by q-PCR following the protocol and primers of Criscuolo et al. (2009). As control single-copy gene, we used the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to normalize the quantity of telomere sequence to the amount of DNA in the q-PCR. The final PCR volume was 20 µL containing 10 µL of LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH,

Mannheim, Germany) and 1 μL of DNA at 20 ng μL^{-1} of DNA. The reactions for telomeres or GAPDH were done in different plates due to the differential PCR conditions. Telomere PCR conditions were 10 min at 95 °C followed by 30 cycles of 1 min at 56 °C and 1 min at 95 °C. GAPDH PCR started with 10 min at 95 °C followed by 40 cycles of 1 min at 60 °C and 1 min at 95 °C, both performed in a LightCycler 480 RT-PCR System (Roche). Each sample was run in duplicate, and samples with a coefficient of variation higher to 5 % were removed from the analyses. Each 96-well plate included serial dilutions of DNA (40 ng, 10 ng, 2.5 ng, 0.66 ng of DNA per well) from a reference pool (the internal control) run in triplicate, which were used to generate the standard curves, and a blank control with no DNA. Quantification cycle values (Ct) were transformed into normalized relative quantities (NRQs) following Hellemans et al. (2007) procedure, which controls for the amplifying efficiency of each qPCR. Amplification efficiency for telomere products ranged between 1.92 and 2.07 and for the GAPDH product between 1.94 and 2.02. The melting curves of the control gene cycles confirmed no evidence of primer dimer or nonspecific amplification.

Telomere shortening was calculated by subtracting the NRQ value of samples from the last capture to those of the first capture. These differences were standardized by dividing telomere shortening for the length of telomere of first captures. Elapsed time between captures varied between 82 days (that included most of the breeding season) and two years. Unfortunately, due to blood conservation problems, samples from the 2013 breeding season could not be analysed.

Statistical analyses

The three measurements of throat feathers in males and females were highly repeatable ($r = 0.96$, $F = 51.0$, $df = 145,292$, $p < 0.0001$) and, consequently, we

used the mean value for further analyses. As approximations to the mating success of the males, we used date of laying of the first egg in the season. Hatching and fledging success were used as proxies of reproductive success in the analyses. Independently of the study year, the zero value of laying date corresponded to the 1st of April.

Telomere length was estimated only for individuals (males and females) that were caught at least twice, whose time between captures lasted more than 82 days and included at least one reproductive event. To explore the associations between length of throat feather (dependent variable) and telomere length (independent variable) we used General Linear Models (GLMs) that also included study year as discrete fixed independent factor. In this analysis we only considered information on first captures (i.e., before experimental manipulation). The association between throat-feather length and telomere shortening was also explored in GLMs that included telomere attrition as the dependent variable, throat-feather length at first capture and time elapsed between captures as continuous independent factors, and experimental treatment as fixed effect (in males). These associations were separately explored for males and females.

The associations between experimental manipulation of throat-feather length (independent factor) and variables reflecting mating (laying date) and breeding success (hatching success and fledgling success) (dependent variable) were also explored in GLMs. Capture event (first or second capture) and study year were included in the model as additional independent factors. Individual identity did not explain a significant proportion of variance in reproductive variables (within and between individuals variance was similar) and, thus, was not included in the models. Hatching and fledging success were analysed separately for first and second clutches. We did so because environmental conditions usually deteriorate during second clutches and, thus, the expected association with the sexually selected trait may be more easily detected in

second breeding attempts. In separate models we also explored the possibility that the expected experimental effects varied between captures by including the interaction between capture event and experimental treatment in the above described models. Moreover, we also tested the possibility that the relationship between throat-feather length and variables reflecting mating and breeding success differ for experimental and control males by exploring the interaction between throat-feather length and treatment in the previously described models. In no case, these interactions reached statistical significance ($p > 0.145$) and, thus, were not considered further. In addition, we explored whether the experimental shortening of throat feathers had any effect on feather size in subsequent moults. We conducted a GLM where the dependent variable was the difference in feather length between the second and first capture, with time elapsed in days between captures as a continuous variable and treatment as a discrete factor.

Sample sizes differ between analyses for several reasons. First, not all captured individuals were recaptured or identified during breeding. Second, a few genetic analyses failed or samples deteriorated before the analyses. Finally, information from control and experimental individuals was useful for testing some but not other predictions.

Residuals of all statistical models were plotted and visually checked for normality. All analyses were performed with Statistica V13 (Dell-Inc. 2015).

Results

Feather length and telomere length

Telomere length and throat-feather length were negatively related in males (Beta (SE) = -0.54(0.21), $F = 6.77$, $df = 1,14$, $p = 0.021$) and positively related

in females (Beta (SE) = 0.36(0.17), F = 4.76, df = 1,31, p = 0.037) (Fig. 2A), after controlling for the non-significant effect of study year (Males: F = 0.82, df = 2,14, p = 0.459; Females: F = 0.87, df = 2,31, p = 0.429). Telomere shortening was also negatively related with throat-feather length before the experiment (in his first capture), but only for males (Males: Beta (SE) = -0.54(0.18), F = 8.67, df = 1,17, p = 0.009; Females: Beta (SE) = 0.20(-0.17), F = 1.45, df = 1,34, p = 0.236) (Fig. 2B). Neither time elapsed between telomere length measurements (F = 2.75, df = 1,17, p= 0.11), nor experimental treatment (control (N=10): mean(SE) = 0.00(0.09), experimental (N=11): mean(SE) = -0.13(0.08), F = 1.19, df = 1,17, p = 0.291) explained additional proportion of variance in telomere shortening in males. Thus, males with longer throat feathers tended to have shorter telomeres but experienced lower rates of telomere shortening, while the manipulation of throat feathers did not affect rate of telomere shortening.

Feather length and reproductive performance

Length of throat feathers of males before manipulation was positively associated with hatching success of second breeding attempts after controlling for the non-significant effect of study year (Table 1, Fig. 3). However, no experimental effect was detected in any of the considered reproductive variables (Fig. 3).

There was no difference between treatments in the difference in feather length between first capture and subsequent moults (control (N=10): mean(SE) = 0.08(0.13), experimental (N = 9): mean(SE) = 0.34(0.14), df = 1,16, F = 1.66, p = 0.215).

Table 1: Effects of the experimental manipulation of throat-feather length in spotless starling males on laying date, hatching success and fledging success. Hatching and fledging success of first (1st) and second (2nd) breeding attempts are separately analysed. Significant results ($p < 0.05$) are in bold.

	Throat-feather length				Treatment						Year		First year/ Following year	
Dependent variables	Beta (SE)	DF	F	P	Mean $(SE)_{Ctrl}$	N	Mean $(SE)_{Exp}$	N	F	P	F	P	F	P
Laying date	0.10 (0.13)	1,63	0.65	0.423	0.09 (0.18)	33	-0.06 (0.18)	34	0.37	0.543	-	-	0.001	0.971
Hatching success (1st)	-0.00 (0.14)	1,54	0.00	0.971	0.82 (0.04)	32	0.86 (0.04)	29	0.80	0.374	2.01	0.124	0.29	0.591
Hatching success (2nd)	**0.39 (0.16)**	**1,36**	**6.31**	**0.017**	0.76 (0.07)	21	0.73 (0.06)	22	0.19	0.668	0.89	0.453	1.58	0.217
Fledging success (1st)	-0.10 (0.16)	1,46	0.45	0.504	0.78 (0.05)	28	0.89 (0.05)	25	2.60	0.114	0.81	0.495	1.19	0.282
Fledging success (2nd)	-0.30 (0.25)	1,16	1.46	0.245	0.75 (0.11)	10	0.76 (0.09)	13	0.01	0.940	1.23	0.330	0.26	0.613

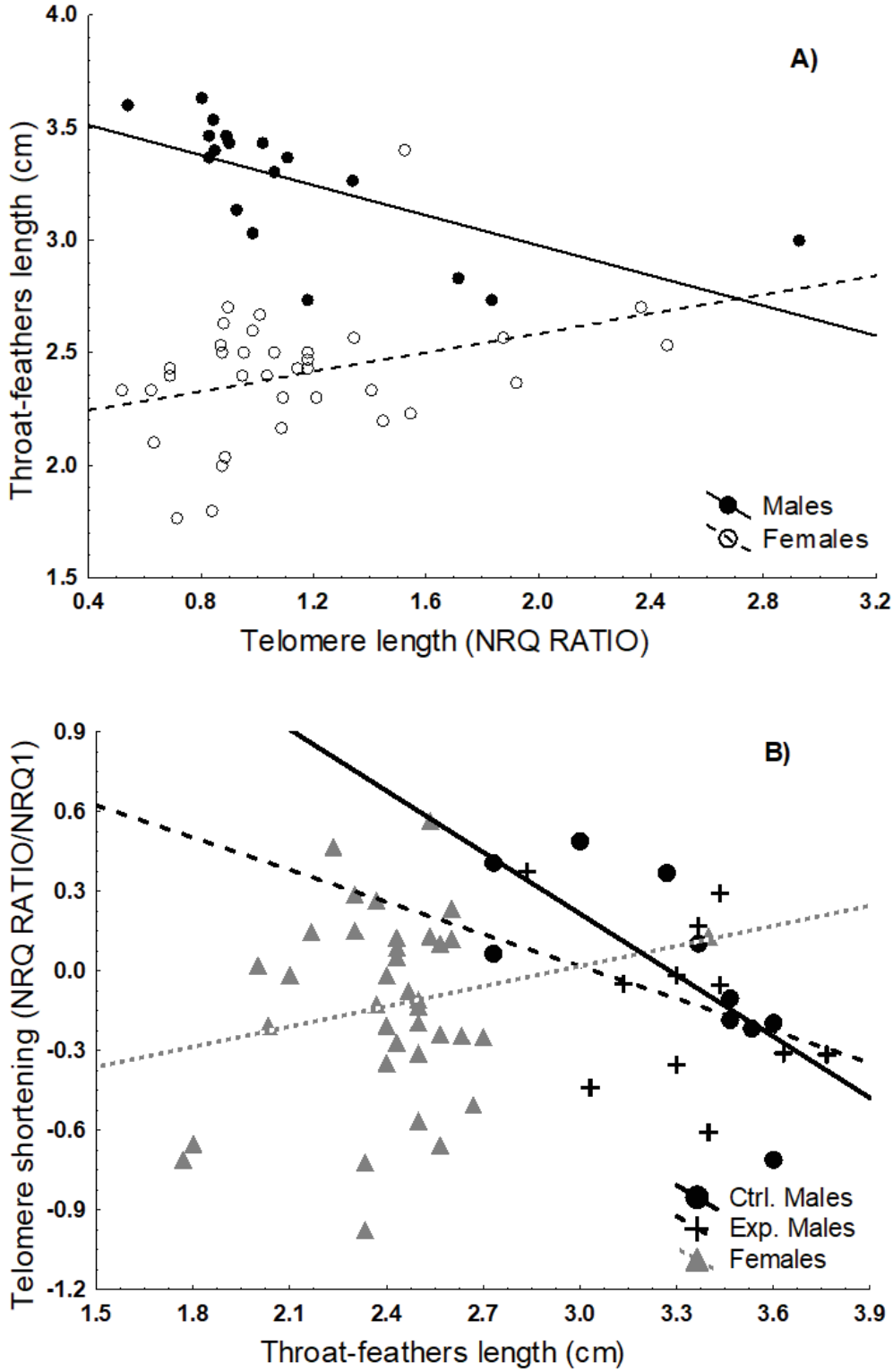


Fig.2: Relationship between telomere length and the average length of the throat feathers in males and females (A), and between telomere shortening and the average length of the throat feathers in control (Ctrl. N=10) and experimental (Exp. N= 11) males, and in female spotless starlings (B). Lines are regression lines.

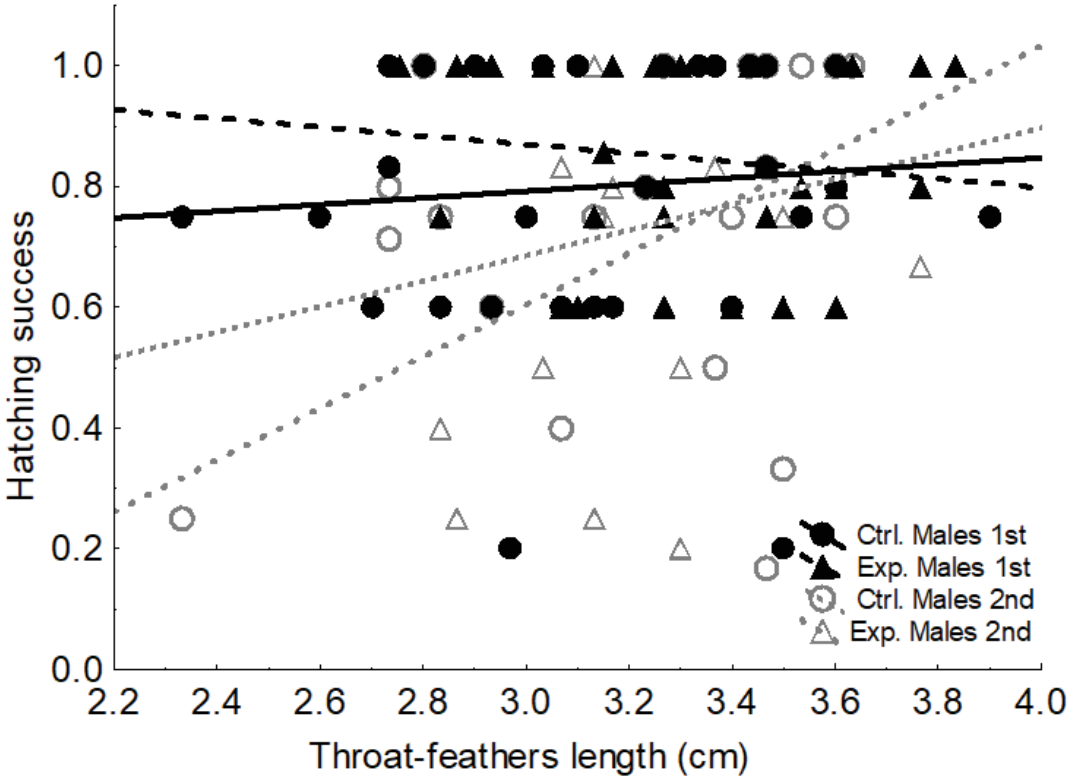


Fig.3: Relationship between average length of the throat feathers of males and hatching success for experimental and control spotless starling males in first (N_{Ctrl}= 32; N_{Exp} = 29) and second (N_{Ctrl}= 21; N_{Exp} = 22) breeding attempts.

Discussion

Our main results are that spotless starling males with longer throat- feathers had shorter telomeres that deteriorate more slowly than those of males with shorter throat feathers. Experimental shortening of throat feathers of males did not affect telomere deterioration rate, suggesting that the correlative association was not due to feather length but to correlated variables, or that time elapsed between captures was not enough for feather length to exert an effect on telomere deterioration. The association between length of throat feathers and telomere length also appeared in females, but in the opposite

direction than in males. These results therefore suggest a link between throat-feathers and telomere length and dynamics that differ between sexes. Moreover, we found a positive association between length of throat feathers of males and hatching success of second clutches. Again, experimental manipulation of throat-feather length did not influence hatching success suggesting that the detected association were not directly mediated by feather length. Below we discuss such results in scenarios of sexual selection where feather length reflects phenotypic quality of males in terms of telomere length and/or attrition rates.

Feather length and telomere length

Older spotless starling males usually possess longer throat feathers (Hiraldo and Herrera 1974; Aparicio et al. 2001; Veiga and Polo 2016). Moreover, telomeres deteriorate with age (Haussmann and Vleck 2002; Haussmann et al. 2003; Pauliny et al. 2006). Thus, the detected negative association between feather length and telomere length might be simply due to length of throat feathers reflecting male age-related telomere shortening. Another non-exclusive possibility explaining this negative association is related to physiological costs derived from developing exaggerated ornaments that would imply elevated telomere deterioration. Extensive evidence suggests that high testosterone levels are required to develop exaggerated ornaments (Gonzalez et al. 2001; Fusani 2008). Elevated testosterone levels imply considerable costs in terms of, for instance, immunosuppression (Folstad and Karter 1992; Gil and Culver 2011) and increased oxidative stress (Blas et al. 2006; Alonso-Alvarez et al. 2007). Oxidative stress is one of the better-known causes of telomere deterioration (von Zglinicki 2002; Richter and von Zglinicki 2007) and thus, sexual ornamentation and telomere length might be negatively associated (Monaghan 2014), a prediction that our results fulfilled.

A positive association between sexually selected characters and telomere length can also be predicted because males of higher phenotypic and/or genetic quality are better able to cope with costs associated with the expression of these traits (Andersson 1994; Møller 1994). In accordance with this last scenario, previous works exploring associations between sexually selected traits and telomere length in birds found positive rather than negative relationships (Parolini et al. 2017, Taff and Freeman-Gallant 2017). In these cases, however, the considered secondary sexual trait did not change with age (Parolini et al. 2017, Taff and Freeman-Gallant 2017), in contrast to length of throat feathers in starlings (Veiga and Polo 2016). In addition, in accordance with sexually selected characteristics related to telomere length, Parolini et al. (2017) and Taff and Freeman-Gallant (2017) found that telomeres of males with more exaggerated sexual traits deteriorated at lower rates, a pattern that we also detected here. Telomere attrition conveys information on the capacity of individuals to maintain chromosome wholeness and, thus, it is a good predictor of survival and of maximum lifespan in birds (Tricola et al. 2018). Thus, since telomeres of starling males with longer throat feathers deteriorated at relatively lower rate, it is likely that the negative association between telomere and feather lengths was due to age being positively and negatively correlated with feather length and telomere length, respectively.

We experimentally reduced throat-feather length of male starling, but found no effect in telomere deterioration. Throat feathers are not involved in flight activity but in social communication (Ruiz-Rodríguez et al. 2015). Thus, this result suggests that the association with telomere length and shortening should be mainly related to physiological pleiotropic effects rather than to social environment. Further studies should explore the role of physiological characteristics related to throat-feather length (e.g. immunocompetence, (Gil and Culver 2011)) which may predict telomere length and shortening. Different associations between sexually dimorphic characteristics and

telomere dynamics of males and females have been reported (Parolini et al. 2017; Taff and Freeman-Gallant 2017). Thus, it is likely that sexual hormones mediate the association between sexually selected traits and telomeres. Here, we found that length of throat feathers and telomere length of females were significantly related, but in the opposite direction that in males. Moreover, in females, telomere shortening was not predicted by throat-feather length. Throat feathers of females are 1 cm shorter than those of males, and they are not considered a sexually selected trait, at least to the same extent as in males (Ruiz-Rodríguez et al. 2015). It is therefore likely that development of these feathers in females does not depend on age or sexual hormones and, thus, its length would not be associated with testosterone provoking oxidative stress and being mirrored in their telomeres. In this case, throat-feather length might reflect phenotypic conditions of females at the moulting time. Therefore, a positive rather than a negative association with telomere length could be expected as shown here.

Feather length and reproductive performance

Throat feathers of starling males are involved in sexual signalling (Aparicio et al. 2001; Gil and Culver 2011; Ruiz-Rodríguez et al. 2015). Here, we found a positive association between throat-feather length and hatching success of second reproductive attempts, which support the expected reproductive values of the secondary sexual traits. This association was only detected for second clutches, which usually take place under poorer environmental conditions (as for example, an increase in temperature and a decrease in food availability) that might increase the probability of hatching failures (Salaberria et al. 2014). Two different non-exclusive scenarios might explain the detected association between ornamentation of males and hatching success. First, females might invest more in incubation duties when mated to more attractive males with

longer throat feathers (differential allocation in relation to male ornamentation, Møller and Thornhill 1998; Harris and Uller 2009). However, the experimental reduction of throat-feather length did not cause a reduction of hatching success and thus feather length of males during the incubation period does not explain the detected association. The second possibility is that males with longer ornaments mated with females of superior genetic or phenotypic quality that improve hatching success. Previous studies in spotless starlings detected a positive association between hatching success and level of heterozygosity of females, but not of males (Cordero et al. 2004). Moreover, it is also known that males with longer throat feathers mate with highly heterozygous females (Aparicio et al. 2001). In our study, most experimental males were manipulated after mating but before reproduction started and, thus, it is possible that heterozygosity of females already mated with experimental males explained the detected association between feather length and hatching success.

Summarizing, we found that the length of throat feathers, a sexually selected trait in male spotless starlings, reflects telomere length and possibly the age of individuals as well as their phenotypic quality in terms of preventing telomere deterioration. We also found a positive association between throat-feather length and hatching success, which might be related to particularities of females mated to males with longer feathers. Interestingly, the experimental reduction of throat-feather length did not produce a more pronounced telomere deterioration, nor a reduction in the fitness related variables considered. Therefore, the interesting detected associations might not be due to the physical appearance of throat feathers but, perhaps, to other physiological and/or non-manipulated morphological characteristics that covaried with them. Future studies should explore possible candidates that included antioxidant capability or related morphological signals.

Acknowledgments

We thank Olga Corona Forero, Francisco Miranda and Olaya García for technical assistance with DNA extraction from blood samples and amplification for telomere length estimation. We also thank Angela Martinez-Garcia, Natalia Juárez García-Pelayo and Carmen Soler Zamora for the help in capturing birds and collecting data from the field.

References

Alonso-Alvarez C, Bertrand S, Faivre B, Chastel O, Sorci G, 2007. Testosterone and oxidative stress: the oxidation handicap hypothesis. -*Proceedings of the Royal Society B: Biological Sciences,* 274: 819-825.

Andersson M, 1994. Sexual selection. Princeton: Princeton University Press.

Aparicio JM, Cordero PJ, Veiga JP, 2001. A test of the hypothesis of mate choice based on heterozygosity in the spotless starling. -*Animal Behaviour*, 62: 1001-1006.

Asghar M, Hasselquist D, Hansson B, Zehtindjiev P, Westerdahl H, Bensch S, 2015. Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. -*Science,* 347: 436-438.

Asghar M, Yman V, Homann MV, Sonden K, Hammar U, Hasselquist D, Farnert A, 2018. Cellular aging dynamics after acute malaria infection: A 12-month longitudinal study. -*Aging Cell,* 17: 8.

Badás EP, Martínez J, Rivero de Aguilar Cachafeiro J, Miranda F, Figuerola J, Merino S, 2015. Ageing and reproduction: antioxidant supplementation alleviates telomere loss in wild birds. -*Journal of evolutionary biology,* 28: 896-905.

Bauch C, Becker PH, Verhulst S, 2013. Telomere length reflects phenotypic quality and costs of reproduction in a long-lived seabird. -*Proceedings of the Royal Society B: Biological Sciences,* 280.

Bauer CM, Graham JL, Abolins-Abols M, Heidinger BJ, Ketterson ED, Greives TJ, 2018. Chronological and Biological Age Predict Seasonal Reproductive Timing: An Investigation of Clutch Initiation and Telomeres in Birds of Known Age. -*The American Naturalist,* 191: 777-782.

Beaulieu M, Reichert S, Le Maho Y, Ancel A, Criscuolo F, 2011. Oxidative status and telomere length in a long-lived bird facing a costly reproductive event. -*Functional Ecology,* 25: 577-585.

Blackburn EH, 1991. Structure and Function of Telomeres. -*Nature,* 350: 569-573.

Blas J, Perez-Rodriguez L, Bortolotti GR, Vinuela J, Marchant TA, 2006. Testosterone increases bioavailability of carotenoids: Insights into the honesty of sexual signaling. -*Proceedings of the National Academy of Sciences of the United States of America,* 103: 18633-18637.

Blount JD, Metcalfe NB, Birkhead TR, Surai PF, 2003. Carotenoid modulation of immune function and sexual attractiveness in zebra finches. -*Science,* 300: 125-127.

Boonekamp JJ, Bauch C, Mulder E, Verhulst S, 2017. Does oxidative stress shorten telomeres? -*Biology letters,* 13: 20170164.

Boonekamp JJ, Mulder GA, Salomons HM, Dijkstra C, Verhulst S, 2014. Nestling telomere shortening, but not telomere length, reflects developmental stress and predicts survival in wild birds. -*Proceedings of the Royal Society B: Biological Sciences,* 281: 20133287.

Bradbury JW, Vehrencamp SL, 1998. Principles of animal communication: Sinauer Associates, Inc.

Burley N, 1988. The differential-allocation hypothesis: An experimental test. -*The American Naturalist,* 132: 611-628.

Campisi J, 2013. Aging, Cellular Senescence, and Cancer. In: Julius D, editor. Annual Review of Physiology, Vol 75 Palo Alto: *Annual Reviews*. p. 685-705.

Cordero PJ, Aparicio JM, Veiga JP, 2004. Parental genetic characteristics and hatching success in the spotless starling, Sturnus unicolor. -*Animal Behaviour,* 67: 637-642.

Cramp S, 1998. The complete birds of the Western Palearctic. Optimedia: Oxford University Press.

Criscuolo F, Bize P, Nasir L, Metcalfe NB, Foote CG, Griffiths K, Gault EA, Monaghan P, 2009. Real-time quantitative PCR assay for measurement of avian telomeres. -*Journal of Avian Biology,* 40: 342-347.

Darwin CR, 1871. The descent of man and selection in relation to sex. London: John Murray.

Dugdale HL, Richardson DS, 2018. Heritability of telomere variation: it is all about the environment! -*Philosophical Transactions of the Royal Society B: Biological Sciences,* 373: 14.

Eisenberg DTA, Borja JB, Hayes MG, Kuzawa CW, 2017. Early life infection, but not breastfeeding, predicts adult blood telomere lengths in the Philippines. -*American Journal of Human Biology,* 29: 11.

Ferraguti M, Martinez-de la Puente J, Ruiz S, Soriguer R, Figuerola J, 2013. On the study of the transmission networks of blood parasites from SW Spain: diversity of avian haemosporidians in the biting midge Culicoides circumscriptus and wild birds. -*Parasites & Vectors,* 6: 208.

Fisher RA, 1930. The genetical theory of natural selection. Oxford: Oxford University Press.

Folstad I, Karter AJ, 1992. Parasites, bright males, and the immunocompetence handicap. -*The American Naturalist,* 139: 603-622.

Fusani L, 2008. Testosterone control of male courtship in birds. -*Hormones and Behavior,* 54: 227-233.

Gil D, Culver R, 2011. Male ornament size in a passerine predicts the inhibitory effect of testosterone on macrophage phagocytosis. -*Functional Ecology,* 25: 1278-1283.

Gonzalez G, Sorci G, Smith LC, de Lope F, 2001. Testosterone and sexual signalling in male house sparrows (Passer domesticus). -*Behavioral Ecology and Sociobiology,* 50: 557-562.

Hamilton WD, 1990. Mate choice near or far. -*American Zoologist*, 30: 341-352.

Harris WE, Uller T, 2009. Reproductive investment when mate quality varies: differential allocation versus reproductive compensation. -*Philosophical Transactions of the Royal Society B: Biological Sciences,* 364: 1039-1048.

Haussmann MF, Vleck CM, 2002. Telomere length provides a new technique for aging animals. -*Oecologia,* 130: 325-328.

Haussmann MF, Winkler DW, O'Reilly KM, Huntington CE, Nisbet ICT, Vleck CM, 2003. Telomeres shorten more slowly in long-lived birds and mammals than in short-lived ones. -*Proceedings of the Royal Society B: Biological Sciences,* 270: 1387-1392.

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J, 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. -*Genome Biol,* 8: 1-14.

Herborn KA, Heidinger BJ, Boner W, Noguera JC, Adam A, Daunt F, Monaghan P, 2014. Stress exposure in early post-natal life reduces telomere length: an experimental demonstration in a long-lived seabird. -*Proceedings of the Royal Society B: Biological Sciences,* 281: 10.1098/rspb.2013.3151.

Hill GE, 2000. Energetic constraints on expression of carotenoid-based plumage coloration. -*Journal of Avian Biology,* 31: 559-566.

Hiraldo F, Herrera CM, 1974. Dimorfismo sexual y diferenciación de edades en Sturnus unicolor Temm. Doñana, Acta Vert 1: 149-170.

Johnson S, Candolin U, 2017. Predation cost of a sexual signal in the threespine stickleback. -*Behavioral ecology,* 28: 1160-1165.

Jones AG, Ratterman NL, 2009. Mate choice and sexual selection: What have we learned since Darwin? -*Proceedings of the National Academy of Sciences of the United States of America,* 106: 10001-10008.

Kim SY, Velando A, 2015. Antioxidants safeguard telomeres in bold chicks. -*Biology letters,* 11.

Kotrschal A, Ilmonen P, Penn DJ, 2007. Stress impacts telomere dynamics. -*Biology letters,* 3: 128-130.

Macias García C, Ramirez E, 2005. Evidence that sensory traps can evolve into honest signals. -*Nature,* 434: 501-505.

Maynard-Smith J, Harper D, 2003. Animal Signals. Oxford: Oxford University Press.

Møller AP, 1994. Sexual selection and the barn swallow. Oxford, UK: Oxford University Press.

Møller AP, Chabi Y, Cuervo JJ, Lope F, Kilpimaa J, Kose M, Matyjasiak P, Pap PL, Saino N, Sakraoui R, Schifferli L, Hirschheydt J, 2006. An analysis of continent-wide patterns of sexual selection in a Passerine bird. -*Evolution,* 60: 856-868.

Møller AP, Jennions MD, 2001. How important are direct fitness benefits of sexual selection? -*Naturwissenschaften,* 88: 401-415.

Møller AP, Thornhill R, 1998. Male parental care, differential parental investment by females and sexual selection. -*Animal Behaviour,* 55: 1507-1515.

Monaghan P, 2014. Organismal stress, telomeres and life histories. -*Journal of Experimental Biology,* 217: 57-66.

Monaghan P, Haussmann MF, 2006. Do telomere dynamics link lifestyle and lifespan? -*Trends in Ecology & Evolution,* 21: 47-53.

Navarro C, Pérez-Contreras T, Avilés JM, McGraw KJ, Soler JJ, 2010. Beak colour reflects circulating carotenoid and vitamin A levels in spotless starlings (*Sturnus unicolor*). -*Behavioral Ecology and Sociobiology,* 64: 1057-1067.

Otte D, 1974. Effects and functions in the evolution of signaling systems. -*Annual Review of Ecology and Systematics,* 5: 385-417.

Pardal S, Alves JA, Mota PG, Ramos JA, 2018. Dressed to impress: breeding plumage as a reliable signal of innate immunity. -*Journal of Avian Biology*, 49: 13.

Parolini M, Romano A, Costanzo A, Khoriauli L, Santagostino M, Nergadze SG, Canova L, Rubolini D, Giulotto E, Saino N, 2017. Telomere length is reflected by plumage coloration and predicts seasonal reproductive success in the barn swallow. -*Molecular Ecology,* 26: 6100-6109.

Pauliny A, Wagner RH, Augustin J, Szép T, Blomqvist D, 2006. Age-independent telomere length predicts fitness in two bird Species. -*Molecular Ecology,* 15: 1681-1687.

Richter T, von Zglinicki T, 2007. A continuous correlation between oxidative stress and telomere shortening in fibroblasts. -*Experimental gerontology,* 42: 1039- 1042.

Romano A, Costanzo A, Rubolini D, Saino N, Moller AP, 2017. Geographical and seasonal variation in the intensity of sexual selection in the barn swallow Hirundo rustica: a meta-analysis. -*Biological Reviews* 92: 1582-1600.

Ruiz-Castellano C, Tomás G, Ruiz-Rodríguez M, Martín-Galvez D, Soler JJ, 2016. Nest Material Shapes Eggs Bacterial Environment. -*PLoS One,* 11: e0148894.

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

Salaberria C, Celis P, Lopez-Rull I, Gil D, 2014. Effects of temperature and nest heat exposure on nestling growth, dehydration and survival in a Mediterranean hole-nesting passerine. -*Ibis,* 156: 265-275.

Searcy WA, Nowicki S, 2005. The evolution of animal communication. Reliability and deception in signaling systems. Princeton (NJ): Princeton UNiversity Press.

Sheldon BC, 2000. Differential allocation: tests, mechanisms and implications. -*Trends in Ecology & Evolution,* 15: 397-402.

Soler JJ, Navarro C, Pérez-Contreras T, Avilés JM, Cuervo JJ, 2008. Sexually selected egg coloration in spotless starlings. -*The American Naturalist,* 171: 183-194.

Soler JJ, Ruiz-Castellano C, Figuerola J, Martin-Vivaldi M, Martinez-de la Puente J, Ruiz-Rodriguez M, Tomas G, 2017. Telomere length and dynamics of spotless starling nestlings depend on nest-building materials used by parents. -*Animal Behaviour,* 126: 89-100.

Soler JJ, Ruiz-Castellano C, Figuerola J, Martinez-de la Puente J, Ruiz-Rodriguez M, Tomas G, 2018. Egg colouration predicts brood size, telomere length and body condition of spotless starling fledglings. -*Journal of Avian Biology*, 49: 12.

Soler JJ, Ruiz Castellano C, Martínez-de la Puente J, Tomás G, Ruiz-Rodríguez M, Figuerola J, 2015. Telomere dynamics in parasitic great spotted cuckoos and their magpie hosts. -*Journal of evolutionary biology,* 28: 1610-1617.

Stuart-Fox DM, Moussalli A, Marshall NJ, Owens IPF, 2003. Conspicuous males suffer higher predation risk: visual modelling and experimental evidence from lizards. -*Animal Behaviour,* 66: 541-550.

Suram A, Kaplunov J, Patel PL, Ruan HH, Cerutti A, Boccardi V, Fumagalli M, Di Micco R, Mirani N, Gurung RL, Hande MP, di Fagagna FD, Herbig U, 2012. Oncogene-induced telomere dysfunction enforces cellular senescence in human cancer precursor lesions. -*Embo Journal,* 31: 2839-2851.

Taff CC, Freeman-Gallant CR, 2017. Sexual signals reflect telomere dynamics in a wild bird. -*Ecology and Evolution,* 7: 3436-3442.

Tibbetts EA, Dale J, 2004. A socially enforced signal of quality in a paper wasp. -*Nature,* 432: 218-222.

Tricola GM, Simons MJP, Atema E, Boughton RK, Brown JL, Dearborn DC, Divoky G, Eimes JA, Huntington CE, Kitaysky AS, Juola FA, Lank DB, Litwa HP, Mulder EGA, Nisbet ICT, Okanoya K, Safran RJ, Schoech SJ, Schreiber EA, Thompson PM, Verhulst S, Wheelwright NT, Winkler DW, Young R, Vleck CM, Haussmann MF, 2018. The rate of telomere loss is related to maximum lifespan in birds. -*Philosophical Transactions of the Royal Society B: Biological Sciences,* 373: 11.

Veiga J, Polo P, 2016. Estornino Negro–Sturnus unicolor. En: Enciclopedia Virtual de los Vertebrados Españoles. Salvador, A., Morales, M. B. (Eds.): Museo Nacional de Ciencias Naturales, Madrid.

Von Zglinicki T, 2002. Oxidative stress shortens telomeres. -*Trends in biochemical sciences,* 27: 339-344.

Webster MS, Ligon RA, Leighton GM, 2018. Social costs are an underappreciated force for honest signalling in animal aggregations. -*Animal Behaviour,* 143: 167-176.

Zahavi A, 1975. Mate selection--A selection for a handicap. -*Journal of theoretical Biology,* 53: 205-214.

Zahavi A, 1977. Cost of honesty - (further remarks on handicap principle*).* -*Journal of theoretical Biology,* 67: 603-605.

CAPÍTULO 6

Beak colouration of starling (*Sturnus unicolor*) males depends on the length of their throat feathers

Abstract

Within the context of complex sexual signalling, most research has focussed on exploring the association between several signals and/or their relationship with different proxies of individual quality. However, few studies have focused on checking whether the expression of one signal is conditioned by the expression of the others. Here, by experimentally shortening the throat feathers in males, we evaluated the influence of this trait on the colour expression of the beak base in spotless starlings (*Sturnus unicolor*). In addition, we tested the relationship between these two secondary sexual characters with traits indicating individual quality such as body condition and colour reflectance at the wavelength related to carotenes in the tip of the beak. Our results show that the colouration of the beak base in males, but not in females, is positively related to body condition and to the length of ornamental throat feathers. Moreover, the experimental shortening of throat feathers in males had a negative effect on the blue chroma intensity of their beak base one year after manipulation. . These results support for the first time a causal link between the expressions of two sexually dimorphic characters, which is essential to understand their functionality in a multiple signalling framework.

Keywords: Beak colour, Body condition, Interacting signals, Multiple signals, Ornamental feathers length, Sexual selection.

Introduction

Animals use a wide array of signals to inform about their phenotypic or genetic quality to conspecifics in social interactions, in contexts such as mate choice or competition for resources (Lyon and Montgomerie 2012, Kokko 2003, Edward 2015, Andersson and Simmons 2006, Kraaijeveld et al. 2007). In contexts of sexual selection, males typically possess multiple traits that may convey independent information to receivers (Møller and Pomiankowski 1993). Although most research on the evolution and function of signals has focused on single traits, the importance of studying these characters within the theoretical and more realistic framework of multiple signals has been highlighted (Hebets and Papaj 2005, Candolin 2003). Different characters might, for instance, imply multiple or redundant messages (i.e. information), or might be more efficient in particular environments or in stimulating particular sensory senses (Hebets and Papaj 2005, Candolin 2003, Møller and Pomiankowski 1993). Hebets and Papaj (2005) developed a framework of testable hypotheses for explaining the evolution and functioning of multiple signals. They highlighted (i) the importance of considering complex signals and the unit of character selection; (ii) that complex signals include several characters that function together, either facilitating the transmission (e.g., using different sensory channels) or reinforcing transmitted information to receivers (i.e, redundant information); and (iii) that individual signals or components of complex signals do not necessarily function independently, but may interact in a functional way.

Most research on complex signalling has focussed on exploring the association between several signals (Bro-Jorgensen and Dabelsteen 2008, Chaine and Lyon 2015, Mason et al. 2014, Perrier et al. 2002, Girard et al. 2015), or between signals and different proxies of fitness including phenotypic quality (Martin and Lopez 2009, Balmford et al. 1992), mating success (Møller

and Pomiankowski 1993) and efficacy of signal transmission in different environments (Endler and Houde 1995). Even though the study of the interactions (i.e., associations) between different signalling characters is essential to know individual or complex signals functioning, it is one of the least explored areas within the field of signal evolution. The study of signal interactions shed some light on signal functioning because, for instance, detecting a positive association would suggest that transmitted information is redundant or complementary. Moreover, a negative association would indicate that a trade-off between signalling characters exists, while the absence of association between different signals would suggest that they convey different information to receivers (Candolin 2003, Hebets and Papaj 2005). In most instances, inter-signal interaction occurs when the presence of one signal or a signal component alters the response of the receiver to a second signal or component by amplifying or conditioning the information provided by each other.

Interactions between signals may also occur when the production of one signal influences the cost of production of another signal (Johnstone 1996, Candolin 2003). In this case, independently of the transmitted information, the phenotypic expression of one signal impinges on the resulting phenotype of the other signal. Signals are typically costly to produce (Hasson 1994, Salvador et al. 1996), to maintain (Ruiz-Rodríguez et al. 2015), or to show (i.e. social cost; Tibbetts and Dale 2004), and the expression of signals or signal components may be traded-off against each other. On the one hand, there could be a trade-off between two signals (e.g. by using the same resources as carotenes), so that a lesser expression of one increases the expression of the other (Andersson et al. 2002). On the other hand, it is also possible that the expression of one signal reduces the average costs due to social interactions (Morales and Velando 2018) and, thus, facilitates or enhances the expression of other signals. For instance, ornaments that develop before reproduction and

function in social contexts others than sexual (e.g. intra-sexual), and could serve to stablish social hierarchy, may reduce agonistic social interactions and mitigate subsequent energetic costs. Saved energy could thus enhance the production of some other sexual ornaments during courtship or reproduction and, therefore, the expression of ornaments developed before and during reproduction could be positively related. This might be the case of certain plumage characteristics of birds that reduce social costs before reproduction (Senar et al. 2000), and thus, could boost the expression of other sexually selected traits, such as song or other similar flexible dynamics traits, that are exclusively expressed during reproduction (Mason et al. 2014, Badyaev et al. 2002).

Detecting evidences supporting the hypothesis that the expression of one signal is conditioned by the expression of other signals can be challenging. A main reason is that sexual signals are typically condition-dependent (e.g. Saino et al. 1997, Soler et al. 2008, Velando et al. 2006). Thus, detecting positive or negative associations between the expression of different signals is not enough to infer causation. Rather, this hypothesis should be tested in experimental frameworks where the modification of one signal causes or explains the phenotypic expression of other signals. As far as we know, this hypothesis has been tested experimentally only once by Henderson et al. (2018), who manipulated plumage colouration of house finch (*Haemorhous mexicanus*) males before reproduction and detected an effect on male investment in song under captivity conditions. However, the effect was dependent on experimentally modified social context (feather colouration of neighbours) and, thus, it is not completely clear that the detected effects were exclusively caused by costs associated to plumage colouration. Here, we go a step further and look for experimental evidence supporting the hypothesis in the wild in spotless starlings (*Sturnus unicolor*, hereafter starlings).

Starlings are semi colonial and sexually dimorphic birds, with males showing elongated throat feathers (Hiraldo and Herrera 1974, Lezana et al. 2000) and conspicuous yellow beak with blue coloured basal part (Navarro et al. 2010). These two secondary sexual traits could harbour different kinds of information or, at least, information at different time scales. The apical part of these feathers is quite flexible, and males exhibit them very conspicuously during the entire year in social interactions, including courtship (Aparicio et al. 2001, Ruiz-Rodríguez et al. 2015). In addition, these feathers honestly reflect the phenotypic quality of individuals (Lezana et al. 2000, Lopez-Rull et al. 2007, Gil and Culver 2011, Ruiz-Rodríguez et al. 2015). On the other hand, during reproduction, the otherwise black coloured beak of starlings turn to yellow colouration in both sexes, while its basal part turn to blue in males and to pink in females (Cramp 1998) (Fig.1). Beak colour in starlings is a sexually dimorphic and dynamic trait that likely reflects antioxidant capacity (Navarro et al. 2010) and, accordingly, previous studies found that the yellow colour of the beak is related to the level of carotenoids and vitamin A in the plasma in both sexes (Navarro et al. 2010). The moult of throat feathers occurs in September-October (Veiga and Polo 2016), thus far before the reproductive period. Therefore, it is likely that these feathers serve to stablish social hierarchies within the population during the whole year, allowing to reduce agonistic interactions and to mitigate its associated costs (Andersson 1994). If that was the case, the length of the throat feathers could play an important role during the non-breeding season by affecting the acquisition and allocation of resources, which could be reflected in the intensity of beak colouration in starling males. Length of throat feathers can be easily manipulated (see Material and Methods), so the hypothesis that the expression of one signal (length of throat feathers) determines the expression of the other (beak colouration) can be experimentally tested.

We manipulated the length of the throat feathers of males by cutting-off approximately the half-distal portion of the feathers before reproduction, and explored its effects on the colour (chroma and brightness) of the base of the beak during reproduction. If this manipulation increases social agonistic interactions before reproduction, energy and resources available for developing sexual colouration during reproduction will be lower for experimental than for control males. This scenario therefore predicts a negative effect of experimentally shortened throat feathers on beak colour. An alternative scenario is that the experiment did not result in differential social costs for experimental and control males before reproduction. In this case, it is possible that experimental males compensate the loss of one sexual signal by increasing the expression of the other. This alternative scenario therefore predicts a positive effect of the manipulation on the expression of beak colouration.

We also tested correlative predictions of the hypothesis that considered these traits as sexual signals of males but not of females. Particularly, we expected that length of throat feathers and colour (chroma and/or brightness) of the base of the beak were positively related in starling males, but not in females. Moreover, we also expected that both traits are correlated with body condition, an indicator of the phenotypic quality of individuals.

Material and Methods

Study area and study species

The study was conducted during the years 2015, 2016 and 2017 in a south-eastern region of Spain (Hoya de Guadix, 37°15'N, 3°01'W), where nest-boxes attached to tree trunks or walls at 3–4 m above-ground are available for starlings to breed in (for further information on the study area see Soler et al. (2017)).

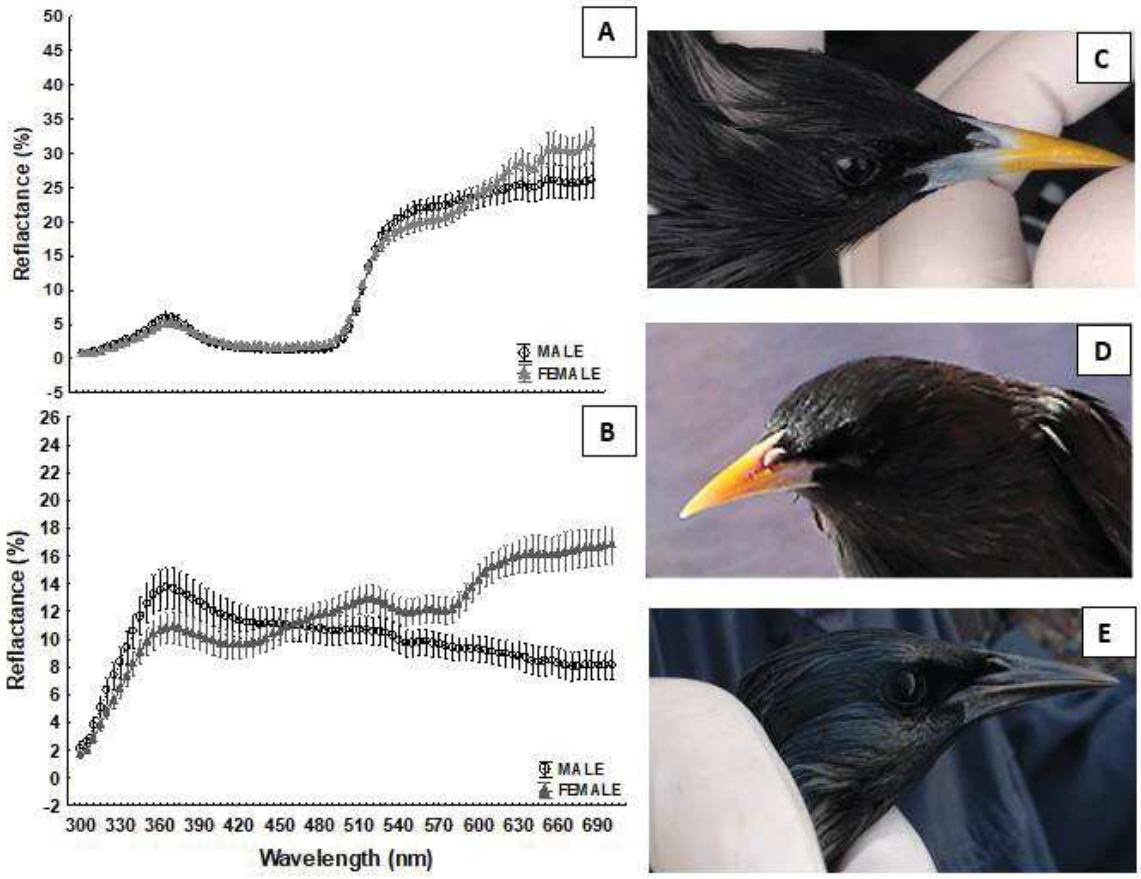


Fig. 1: Mean spectral reflectance of the tip (A) and base (B) of the beak in male (empty black circles) and female (filled grey triangles) spotless starlings during the breeding season. Vertical bars denote 95 % CI. Photographs show the typical colour of the beak of spotless starling males (C) and females (D) during the breeding season, as well as the black beak of starlings out of the breeding season (E).

In the studied population, the reproductive season starts in early April and most individuals lay a second clutch during May-June. The most common clutch size is 4-5 eggs. Incubation is mostly carried out by females with sporadic help from males, and extends for around 14 days, while the nesting period lasts 18-25 days (Cramp 1998). Both male and female parents contribute to feeding the offspring and remove nestling faecal sacs (Cramp 1998). During the whole nesting process, adults bring feathers and aromatic plants to the nest, which have been shown to have antimicrobial-beneficial functions (Ruiz-Castellano et al. 2016, Soler et al. 2017, Ruiz-Castellano et al. 2019). Reproductive success in the study area varies among years and breeding

attempts, with second clutches usually showing lower reproductive success than first clutches (unpublished data). Here, we will focus on the colouration of the base of the beak, a trait with a more marked sexual differentiation as we can see in its reflectance at different wavelengths (Fig. 1).

Fieldwork and experimental procedure

In this population, courtship activity (e.g. singing, introducing fresh green plants and feathers in nest boxes) starts in February, more than one month before egg laying (pers. obs.). During this period, some birds roost in nest-boxes and we take advantage of this fact for conducting yearly bird trapping sessions in the study area (twice a year between February and mid-March). One hour before dawn, we closed the entrance of all nest boxes in the study area, and immediately after dawn, we captured by hand all individuals found roosting inside. Captured birds were kept individually in clean cotton bags hanging from a stick to keep birds quiet, and were released immediately after sampling. The maximum time that a captured starling was in the bag did never exceed three hours. For additional details, see Ruiz-Rodríguez et al. (2015). This procedure does not imply apparent negative effects on breeding performance of captured birds (Soler et al. 2008).

Every year, we also captured birds during breeding, 4-5 days after the hatching date, by using nest-box traps operated for a maximum time of one hour. All captured adults (males and females) were ringed with a numbered metal ring (if not already ringed) and with a unique combination of three colour rings. They were weighed with a hanging scale (Pesola 0–300 g, accuracy 2 g), and their tarsus, wing, and beak length measured with a digital calliper (accuracy 0.01 mm) and a ruler (accuracy 1 mm), respectively. We also measured three times the length of throat feathers of males and females with a ruler. In addition, the colour of the base of the beak was measured with a

spectrometer (see below). Finally, captured males were alternately assigned to control or experimental treatment. With the aid of scissors, we cut the distal half portion of throat feathers of experimental individuals, while we handled control males in the same way but without cutting throat feathers (Fig. 2). After manipulation, we again measured feather length of experimental males, which on average were shortened by 1.5 cm (GLM of throat-feather lengths by treatment in his first capture; Least squares means ± SE: experimental males: 1.8 ± 0.1 cm; control males: 3.2 ± 0.1; $F_{1,52} = 140.16$, $p < 0.0001$). Males that were recaptured during different study years were assigned each time to the same experimental treatment, repeating the same procedure on them, with the exception of three males. Treatment of these three males changed from control in the first to experimental in the second capture, one year later. The effects of treatment in these males were considered as independent information in the analyses.

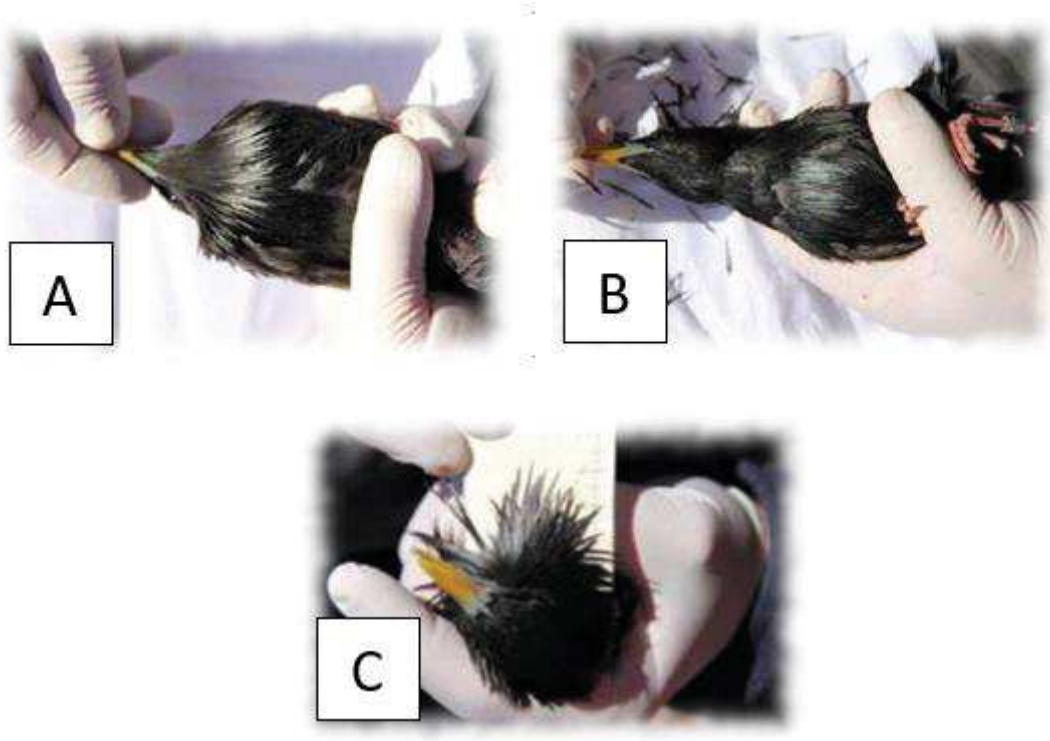


Fig. 2: Aspect of a control (A) and an experimental (B) individual, and the estimation of ornamental throat feathers length of a male spotless starling (C).

Colour measurements

The colour of the base of the beak was measured in males and females with an Ocean Optics S2000 spectrometer in the field. It was connected to a deuterium-halogen light (D2-W, Mini). To standardize ambient light conditions, we used a black bag that wrapped the tip of the optical fibre and the beak and made the measurements inside a dim area (nearby building or in a tent). Before the measurement of each individual, we calibrated the spectrometer using a standard white and black reference. We obtained reflectance spectra at 10 nm intervals from 300-700 nm for all individuals. The colour was measured three times on the base of the beak, and average values were obtained.

We estimated the proportion of total reflectance within the blue range (colour to which the base of the males beak tends) of chroma of the spectrum (λ = 400-475 nm, hereafter blue colour intensity). We did the same with the yellow-red range of chroma of the spectrum (λ = 570-700nm, hereafter yellow-red colour intensity). This range coincides with the typical gradual increasing colour spectral shape of phaeomelanins (Navarro et al. 2010) and in which the reflectance values of males and females are more differentiated at the base of the beak (Fig. 1). In addition, it covers the range of red to which the base of the beak usually tends in females. We also calculated the average brightness over the entire range of the spectrum (300-700 nm). Finally, we also calculated the chroma reflectance values at the carotenoids' wavelengths (450-570 nm). We obtained this variable using the shape model in the Avicol V.6 software (Gomez 2006). Prior to all analyses, negative values were set to zero and reflectance curves were corrected for noise using triangular smoothing (Gomez 2006).

Statistical analyses

The three measurements of the length of throat feathers in males and females were highly repeatable ($R = 0.96$, $F_{145,292} = 51.0$, $p < 0.0001$) and, consequently, we used the mean value for further analyses. Body condition was estimated as residuals of body mass after correcting for tarsus length ($R = 0.151$, $F_{1,241} = 5.66$, $p = 0.018$). However, residuals from this regression were positively correlated with other body size indicator, the wing length ($R = 0.289$, $F_{1,241} = 21.91$, $p < 0.00001$) and, therefore, we included this variable in the model (multiple $R = 0.323$, $F_{2,240} = 13.95$, $p < 0.0001$; partial regression coefficients: tarsus length = 0.127, $t_{240} = 2.08$, $p = 0.039$; wing length = 0.286, $t_{240} = 4.66$, $p < 0.0001$) to estimate body condition (Green 2001). Residuals of this model no longer correlated with other body size indicator variables such as beak length ($R = 0.059$, $F_{1,241} = 0.84$, $p = 0.359$).

The effect of the experimental treatment on males was estimated only for individuals that were caught at least twice. The time between captures lasted more than 315 days (N = 22), with the exception of 5 individuals, whose second captures were performed 14 and 97 days after the first ones. We repeated the analyses by removing these 5 individuals and the results were qualitatively the same, so we show here the results with the larger sample sizes to increase statistical power since beak colour changes may occur in days or hours (Iverson and Karubian 2017). In all the analyses with the colour variables, we took into account only the captures made at the breeding season. The date of capture (day in which the measure of colour was taken) was included in the models, since beak colours may vary throughout the reproductive season (Navarro et al. 2010). However, the date of capture was not included in the analyses related to the length of the throat feathers because no moulting or any major changes in feather length are expected to occur along the breeding season. Independently of the study year, the zero value of capture date corresponds to the 1st of April.

To explore the associations between length of throat feathers (dependent variable) and body condition (independent variable) we used General Linear Models (GLMs) that also included study year as discrete fixed independent factor. We performed this analysis for both sexes, only considering information on first captures (i.e., before manipulation). We used similar models to calculate the relationship between different beak colour variables (blue and yellow-red colour intensity and brightness; dependent variables) and body condition (independent variable) where, in addition to the year (discrete fixed independent factor), we added the date of capture within the season as an independent co-variable.

On the other hand, we explored the association between different beak colour variables (blue and yellow-red colour intensity and brightness; dependent variables) and length of throat feathers (independent variable). We added the study year as fixed factor. Because of the bimodal distribution for males and females of length of throat feathers and variables describing beak colouration, these models were ran separately for each sex. In addition, we performed GLMs to explore the relationships among 1) blue and yellow-red chroma at the base of the beak (controlled by year and date of capture), and 2) base and tip beak colours.

Finally, the effects of the experimental manipulation (i.e., shortening the length of throat feathers) on the beak base colour and on body condition in males were tested by mean of repeated-measures ANOVAs separately, with first and last capture as the within factor (i.e. dependent variable), and experimental treatment as the categorical predictor. The date of first and last captures, as well as number of days between captures were included as continuous independent variables in the statistical models. In addition, we checked whether the experiment did affect length of throat feathers after moult, by carrying out repeated-measures ANOVAs. In these models, the feather length at first and last captures were the dependent variables (repeated

measures), the experimental treatment was the categorical predictor, and the number of days between captures was the continuous independent variable. Residuals of all statistical models were plotted and visually checked for normality. All analyses were performed with Statistica V13 (Dell-Inc. 2015).

Results

The blue chroma and the yellow-red chroma of the starlings' beak-base are negatively related in both males (Beta(SE) = -0.68(0.08), $F_{1,54} = 74.64$, $p < 0.001$) and females (Beta(SE) = -0.92(0.03), $F_{1,100} = 933.58$, $p < 0.001$). Moreover, the base (400-475 nm and 570-700 nm) and tip (450-570 nm) beak colours were not significantly associated in males ($blue_{400-475}$: Beta(SE) = 0.02(0.13), $F_{1,54} = 0.03$, $p = 0.867$; yellow-red $_{570-700}$: Beta(SE) = -0.08(0.11), $F_{1,54} = 0.49$, $p = 0.487$), but a tendency (positive for blue and negative for yellow-red chroma) was detected in females ($blue_{400-475}$: Beta(SE) = 0.14(0.07), $F_{1,100} = 3.92$, $p = 0.050$; $yellow\text{-}red_{570-700}$: Beta(SE) = -0.14(0.07), $F_{1,100} = 3.77$, $p = 0.055$).

Body condition was positively and negatively related to intensity of blue and yellow-red colouration of males' beak, respectively (Table 1, Fig. 3). Neither, the brightness of males' beak nor the length of their throat feathers were related to body condition (Table 1). In females, none of these variables predicted body condition (Table 1). Similarly, length of throat feathers of males, but not that of females, was positively and negatively related to blue and yellow-red colour intensity of males' beak, respectively (Table 1, Fig. 3). Beak brightness did not predict length of throat feathers of males or females (Table 1). Thus, the blue colour intensity of males' beak co-varied with the length of throat feathers, which might inform females on the phenotypic quality (body condition) of males.

Importantly, the experimental shortening of throat feathers in males provoked a reduction in the intensity of the blue, but no other, colouration of their beaks (measured one year after manipulation of throat feathers) (Table 2, Fig. 4). Moreover, the experimental manipulation did not affect body condition or the length of throat feathers in subsequent captures (Table 2). These results suggest a direct link between length of throat feathers and beak colouration of males, which is independent of the association of both characters with phenotypic condition of males.

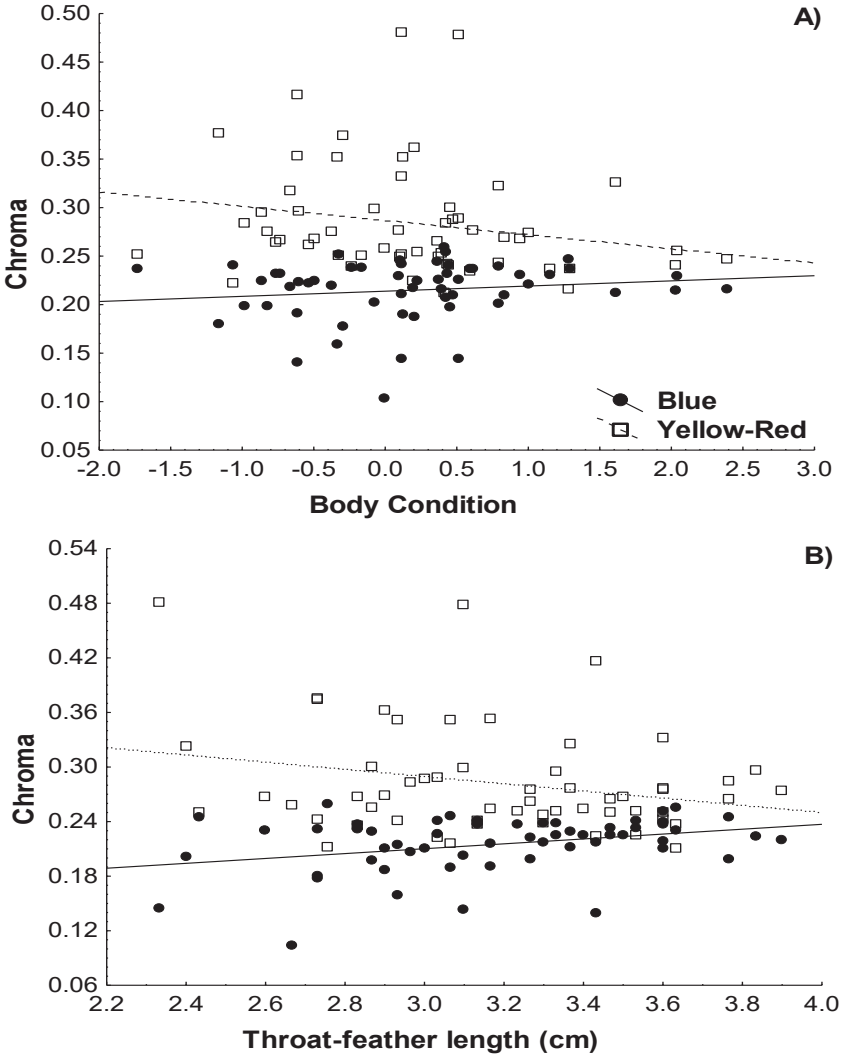


Fig. 3: Relationships between (A) body condition (residuals of body mass after controlling for tarsus and wing length) and (B) length of throat feathers with blue and yellow-red colour intensity of the base of the beak of starling males. Lines are regression lines.

Table 1: Results from GLMs exploring the associations between body condition (dependent variable, top) and length of the throat feathers (dependent variable, down) with the different colour variables (blue (400-475 nm) and yellow-red (570-700 nm) chroma and brightness (300-700 nm)) and the length of throat feathers in the case of body condition (feathers) (independent variables) of males and females. P-values lower than 0.05 are in bold.

	Males				Females			
Variables	Beta(SE)	F	df	p	Beta(SE)	F	df	p
Body condition								
Blue	0.37(0.12)	9.72	1,52	**0.003**	0.01(0.09)	0.01	1,96	0.917
Yellow-red	-0.30(0.14)	4.72	1,52	**0.034**	-0.04(0.10)	0.16	1,96	0.686
Brightness	0.10(0.14)	0.56	1,52	0.456	-0.03(0.09)	0.14	1,96	0.71
Feathers	-0.08(0.14)	0.28	1,52	0.596	0.10(0.10)	0.98	1,96	0.326
Length of throat feathers								
Blue	0.32(0.13)	5.89	1,53	**0.019**	0.15(0.10)	2.29	1,98	0.134
Yellow-red	-0.29(0.14)	4.39	1,53	**0.041**	-0.14(0.10)	2.09	1,98	0.151
Brightness	0.16(0.13)	1.34	1,53	0.251	0.09 (0.10)	0.76	1,98	0.385

Table 2: Results from Repeated Measures ANOVAs exploring the effects of experimental shortening of throat feathers (control (Ctrl.) vs experimental (Exp.)) on the body condition, feather growth (feathers) and beak colouration (blue (400-475 nm) and yellow-red (570-700 nm) chroma and brightness (300-700 nm)) among captures (repeated measures). P-values lower than 0.05 are in bold.

	First Capture		Second Capture				
	Mean $_{Ctrl.}$ (SE)	Mean $_{Exp.}$ (SE)	Mean $_{Ctrl.}$ (SE)	Mean $_{Exp.}$ (SE)	df	F	p
Blue	0.23 (0.004)	0.23 (0.004)	0.23 (0.008)	0.20 (0.008)	1,17	7.13	**0.016**
Yellow-Red	0.26 (0.008)	0.25 (0.008)	0.26 (0.016)	0.29 (0.016)	1,17	3.09	0.097
Brightness	11.80 (0.990)	11.40 (0.990)	11.42 (0.877)	10.31 (0.877)	1,17	0.10	0.753
Body condition	0.30 (0.235)	0.28 (0.221)	0.48 (0.189)	0.43 (0.177)	1,12	0.01	0.934
Feather	3.24 (0.123)	3.38 (0.140)	3.20 (0.132)	3.09 (0.150)	1,13	1.00	0.336

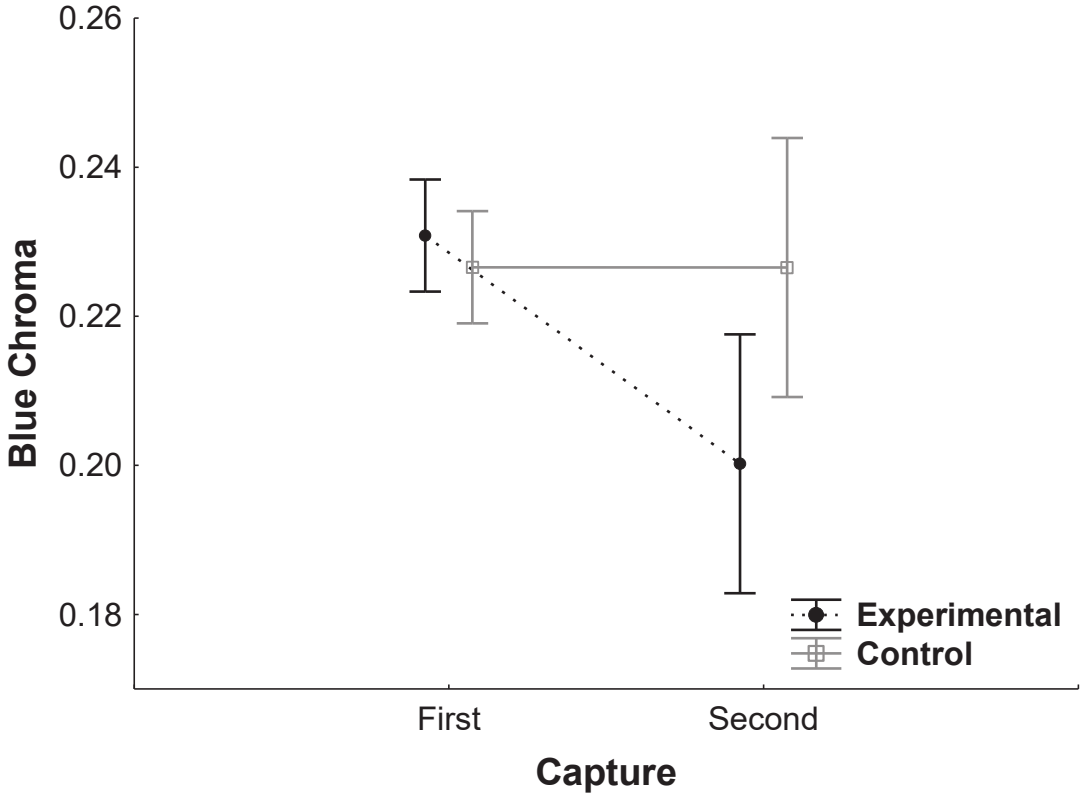


Fig. 4: Blue colour intensity of the base of the beak of males that were (experimental, N = 11) or were not (control, N = 11) subjected to the experimental shortening of throat feathers. Beak colouration was measured during the first capture, before the experimental manipulation, and in second captures, after the manipulation. Vertical lines denote ± 95 % CI

Discussion

Our main results are that (i) intensity of colouration of the beak base of spotless starling males, but not that of females, was positively related to body condition; (ii) beak colouration of males was positively related to the length of their ornamental throat feathers, and (iii) the experimental shortening of throat feathers in males had a negative effect on the blue chroma intensity of males one year after manipulation. Length of throat feathers and beak colouration are two sexually dimorphic traits that reflect phenotypic quality of males (Aparicio et al. 2001, Navarro et al. 2010) and, thus, our results demonstrate a direct

connection between these two traits suggesting that they may function as a whole in a multiple signalling framework.

Starlings have several known sexually dimorphic traits and are an appropriate model system to explore functional interactions between sexual signals. Most studies on sexual signals in this species are focussed on the length of throat feathers of males, which predicts mating success (Aparicio et al. 2001), genetic heterozigosity (Aparicio et al. 2001), immune response (Gil and Culver 2011) and telomere length (Azcárate-García et al. submitted). Bill colouration on the distal yellow part has also been studied as a sexually selected trait of the species because it is related to carotenoid and vitamin A concentration in the blood of males and females, but only during the mating period (Navarro et al. 2010). Sexual differences are however more apparent at the basal part of the beak (Fig 1), and we concentrated on this trait to experimentally explore the possible association with the length of throat feathers. In agreement with the assumption that the blue colouration of the basal part of the beak has a sexual-signalling function, we found that its blue-colour intensity was positively related with both body condition and the length of the throat feathers. Thus, exploring the interaction between these two traits is justified.

Length of throat feathers and beak coloration of starling males provide information at different time scales. Black feathers are relatively static and would provide information of the phenotypic condition and quality of males at the time of moulting (Hebets and Papaj 2005, Badyaev and Hill 2000). Moreover, feather deterioration would also provide information on feather quality and on ability of males reducing feather degradation (Shawkey et al. 2007, Shawkey et al. 2009, Ruiz-de-Castañeda et al. 2012, Ruiz-Rodríguez et al. 2015). Thus, length of throat feathers might even include different kinds of information at a long-term scale. The beak colouration, however, should function at a short time scale. Like for the colouration of other bare parts of

birds, beak colouration has the potential to change within weeks, days, hours, or even seconds (Iverson and Karubian 2017). Thus, this kind of dynamic characters should be continuously evaluated by receivers (Velando et al. 2006, Simons and Verhulst 2011, Dey et al. 2015). As far as we know, associations between these two types of sexual traits have never been assessed.

Our results showed that male body condition at the time of mating was related to blue colouration of the beak, but not to the length of throat feathers, suggesting that both signals do not provide identical but, perhaps, complementary information. However, length of throat feathers was positively related to the intensity of blue colouration of the beak and, thus, it is possible that both traits convey redundant information to females. In agreement with the possibility that these two traits transfer complementary information to females, we experimentally showed a negative effect of length of throat feathers on the intensity of the blue chroma of the beak base of males several months after the manipulation. We know that colouration of the tip of the beak reflects the antioxidant capacity of starlings (Navarro et al. 2010). The association between beak coloration and carotenoids' concentration in the blood has also been detected in other species (Faivre et al. 2003). We did not measure concentration of carotenoids in the blood in this study and, thus, cannot explore whether this association exists for the blue coloration of the beak base of males. Moreover, colour reflectance of the beak tip at the carotenoid wavelength, which resulted positively related to carotenoid level in starlings (Navarro et al. 2010), was not related to colouration of the base of the beak of males. Consequently, the colouration of the beak base is unlikely conveying information on antioxidant capacity to females. Thus, our experimental results should be interpreted as length of throat feathers functioning during the non-reproductive period and determining phenotypic conditions of males during mating.

As some other signals operating in non-sexual scenarios such as parent-offspring communication (Morales and Velando 2018), or sibling negotiation (Soler and Avilés 2010, Johnstone and Roulin 2003), including those mediated by feather colourations (Senar 2006), the length of throat feathers of males might serve to stablish some kind of social hierarchy between males that reduce the probability of agonistic interactions among individuals of different status (Rohwer 1975, Senar 1999, McGraw and Hill 2000). Starlings moult throat feathers several months before reproduction, and males frequently display these feathers while singing in high visible places during non-reproductive periods (pers. obs.), which might have a functional significance in a context of social interactions. In some bird species, probability of social aggression by conspecifics is related to feather characteristics signalling bird status (Senar 1990, Chaine and Lyon 2008, McGraw et al. 2007)). Moreover, aggressions are more common among individuals showing similar status (Midamegbe et al. 2011), with individuals harbouring signals of higher quality eliciting lower level of aggressiveness (Lopez-Idiaquez et al. 2016). Thus, it is possible that starling males with longer throat feathers experienced lower rates of social aggressions during the non-reproductive period. In this scenario, experimental starling males should have experienced higher costs than control males. These costs can affect the expression of some other traits related to phenotypic condition, including immune responses (Hawley et al. 2006), oxidative status (Galván and Alonso-Alvarez 2009) or the expression of sexual signals (Møller et al. 2000). Although we have no data on probability of aggression or social interactions in general in relation to length of throat feathers in starlings, we think that social costs associated to the experimental reduction of length of throat feathers during the non-breeding period is the most likely explanation for the detected experimental effects on beak colouration during reproduction. However, this mechanistic explanation deserves further research exploring for instance the

expected association between feather length and aggression during the non-reproductive period.

Whatever the mechanistic explanations, our experimental results strongly suggest a causal link between expression of two sexually dimorphic traits in spotless starlings. As far as we know, causal links between two sexually selected traits have only been detected in another bird species, the house finch, a highly social species in which head and breast feathers of males show great variability from red to yellow colouration (Henderson et al. 2018). Henderson et al. (2018) found that red-feathered males are more attractive and sing more than yellow-feathered males but, when yellow males were housed with red males, they sang more than when housed with equally unattractive yellow males. Thus, males adapted their singing effort to the social environment (attractiveness) determined by the plumage coloration of the social groups. Therefore, the detected link was explained, not as a direct consequence of one of the traits, but indirectly by the social environment in terms of level of attractiveness of neighbours, which was also manipulated. Our experimental results therefore contrast with those of Henderson et al. (2018) by showing a direct causal effect of length of throat feathers on the expression of the colouration of the base of the beak of spotless starling males, a trait that is only expressed during the reproductive period.

To conclude, we demonstrate for the first time a causal link between the expression of two sexually dimorphic characters, which is essential to understand their functionality in a multiple signalling framework. This type of interactions between sexually selected signals might be widespread in nature and could be more easily detected when considering signals that, like feather coloration or morphological traits, have signalling functions in non-reproductive contexts.

Acknowledgments

We thank Natalia Juárez García-Pelayo and Carmen Soler Zamora for the help in capturing birds and collecting data from the field. The research group benefits from facilities, including and apartment, provided by the city hall of Guadix where a small lab to quickly proceed the samples was installed.

References

Andersson, M. 1994. Sexual selection, Princeton, Princeton University Press.

Andersson, M. and Simmons, L. W. 2006. Sexual selection and mate choice. -*Trends in ecology & evolution*, 21: 296-302.

Andersson, S., Pryke, S. R., Ornborg, J., Lawes, M. J. and Andersson, M. 2002. Multiple receivers, multiple ornaments, and a trade-off between agonistic and epigamic signaling in a widowbird. -*American Naturalist*, 160: 683-691.

Aparicio, J. M., Cordero, P. J. and Veiga, J. P. 2001. A test of the hypothesis of mate choice based on heterozygosity in the spotless starling. -*Animal Behaviour*, 62: 1001-1006.

Badyaev, A. V. and Hill, G. E. 2000. Evolution of sexual dichromatism: contribution of carotenoid versus melanin-based coloration. -*Biological Journal of the Linnean Society*, 69: 153-172.

Badyaev, A. V., Hill, G. E. and Weckworth, B. V. 2002. Species divergence in sexually selected traits: Increase in song elaboration is related to decrease in plumage ornamentation in finches. -*Evolution*, 56: 412-419.

Balmford, A., Rosser, A. M. and Albon, S. D. 1992. Correlates of female choice in resource-defending antelope. -*Behavioral Ecology and Sociobiology*, 31: 107-114.

Bro-Jorgensen, J. and Dabelsteen, T. 2008. Knee-clicks and visual traits indicate fighting ability in eland antelopes: multiple messages and back-up signals. -*Bmc Biology*, 6: 9.

Candolin, U. 2003. The use of multiple cues in mate choice. -*Biological Reviews*, 78: 575-595.

Chaine, A. S. and Lyon, B. E. 2008. Intrasexual selection on multiple plumage ornaments in the lark bunting. -*Animal Behaviour*, 76: 657-667.

Chaine, A. S. and Lyon, B. E. 2015. Signal architecture: temporal variability and individual consistency of multiple sexually selected signals. -*Functional Ecology*, 29: 1178-1188

Cramp, S. 1998. Cramp's the complete birds of the Western Palearctic, Oxford, Optimedia, Oxford University Press.

Dey, C. J., Valcu, M., Kempenaers, B. and Dale, J. 2015. Carotenoid-based bill coloration functions as a social, not sexual, signal in songbirds (Aves: Passeriformes). -*Journal of Evolutionary Biology*, 28: 250-258.

Edward, D. A. 2015. The description of mate choice. -*Behavioral Ecology*, 26: 301-310.

Endler, J. A. and Houde, A. E. 1995. Geographic-variation in female preferences for male traits in *poecilia-reticulata*. -*Evolution*, 49: 456-468.

Faivre, B., Gregoire, A., Preault, M., Cezilly, F. and Sorci, G. 2003. Immune activation rapidly mirrored in a secondary sexual trait. -*Science*, 300: 103-103.

Galván, I. and Alonso-Alvarez, C. 2009. The expression of melanin-based plumage is separately modulated by exogenous oxidative stress and a melanocortin. -*Proceedings of the Royal Society B: Biological Sciences,* 276: 3089-3097.

Gil, D. and Culver, R. 2011. Male ornament size in a passerine predicts the inhibitory effect of testosterone on macrophage phagocytosis. -*Functional Ecology*, 25: 1278-1283.

Girard, M. B., Elias, D. O. and Kasumovic, M. M. 2015. Female preference for multi-modal courtship: multiple signals are important for male mating success in peacock spiders. -*Proceedings of the Royal Society B-Biological Sciences*, 282: 10.

Gomez, D. 2006. AVICOL, a program to analyse spectrometric data, free program available from the author at dodogomez@yahoo.fr.

Green, A. J. 2001. Mass/length residuals: measures of body condition or generators of spurious results? -*Ecology*, 82: 1473-1483.

Hasson, O. 1994. Cheating signals. -*Journal of Theoretical Biology*, 167: 223-238.

Hawley, D. M., Lindstrom, K. and Wikelski, M. 2006. Experimentally increased social competition compromises humoral immune responses in house finches. -*Hormones and Behavior*, 49: 417-424.

Hebets, E. A. and Papaj, D. R. 2005. Complex signal function: developing a framework of testable hypotheses. -*Behavioral Ecology and Sociobiology*, 57: 197-214.

Henderson, L. J., Brazeal, K. R. and Hahn, T. P. 2018. Plumage coloration and social context influence male investment in song. -*Biology Letters*, 14: 4.

Hiraldo, F. and Herrera, C. M. 1974. Dimorfismo sexual y diferenciación de edades en Sturnus unicolor Temm. -Doñana, Acta Vert, 1: 149-170.

Iverson, E. N. K. and Karubian, J. 2017. The role of bare parts in avian signaling. -*Auk*, 134: 587-611.

Johnstone, R. A. 1996. Multiple displays in animal communication: 'Backup signals' and 'multiple messages'. -*Philosophical Transactions of the Royal Society B-Biological Sciences*, 351: 329-338.

Johnstone, R. A. and Roulin, A. 2003. Sibling negotiation. -*Behavioral Ecology*, 14: 780-786.

Kokko, H. 2003. The evolution of mate choice and mating biases. -*Proceedings of the Royal Society of London B, Biological Sciences*, 270: 653-664.

Kraaijeveld, K., Kraaijeveld-Smit, F. J. L. and Komdeur, J. 2007. The evolution of mutual ornamentation. -*Animal Behaviour*, 74: 657-677.

Lezana, L., Miranda, R., Campos, F. and Peris, S. J. 2000. Sex differentiation in the spotless starling (*Sturnus unicolor*, Temminck 1820). -*Belgian Journal of Zoology,* 130: 139-142.

Lopez-Idiaquez, D., Vergara, P., Fargallo, J. A. and Martinez-Padilla, J. 2016. Female plumage coloration signals status to conspecifics. -*Animal Behaviour*, 121: 101-106.

Lopez-Rull, I., Celis, P. and Gil, D. 2007. Egg colour covaries with female expression of a male ornament in the spotless starling (*Sturnus unicolor*). -*Ethology*, 113: 926-933.

Lyon, B. E. and Montgomerie, R. 2012. Sexual selection is a form of social selection. -*Philosophical Transactions of the Royal Society B-Biological Science*s, 367: 2266-2273.

Martin, J. and Lopez, P. 2009. Multiple color signals may reveal multiple messages in male Schreiber's green lizards, Lacerta schreiberi. -*Behavioral Ecology and Sociobiology*, 63: 1743-1755.

Mason, N. A., Shultz, A. J. and Burns, K. J. 2014. Elaborate visual and acoustic signals evolve independently in a large, phenotypically diverse radiation of songbirds. -*Proceedings of the Royal Society B-Biological Sciences*, 281: 9.

Mcgraw, K. J. and Hill, G. E. 2000. Carotenoid-based ornamentation and status signaling in the house finch. -*Behavioral Ecology*, 11: 520-527.

Mcgraw, K. J., Medina-Jerez, W. and Adams, H. 2007. Carotenoid-based plumage coloration and aggression during molt in male house finches. -*Behaviour*, 144: 165-178.

Midamegbe, A., Gregoire, A., Perret, P. and Doutrelant, C. 2011. Female-female aggressiveness is influenced by female coloration in blue tits. -*Animal Behaviour*, 82: 245-253.

Møller, A. P., Biard, C., Blount, J. D., Houston, D. C., Ninni, P., Saino, N. and Surai, P. F. 2000. Carotenoid-dependent signals: Indicators of foraging

efficiency, immunocompetence or detoxification ability? -*Avian and Poultry Biology Reviews*, 11: 137-159.

Møller, A. P. and Pomiankowski, A. 1993. Why have birds got multiple sexual ornaments. -*Behavioral Ecology and Sociobiology*, 32: 167-176.

Morales, J. and Velando, A. 2018. Coloration of chicks modulates costly interactions among family members. -*Behavioral Ecology*, 29: 894-903.

Navarro, C., Pérez-Contreras, T., Avilés, J. M., Mcgraw, K. J. and Soler, J. J. 2010. Beak colour reflects circulating carotenoid and vitamin A levels in spotless starlings (Sturnus unicolor). -*Behavioral Ecology and Sociobiology*, 64: 1057-1067.

Perrier, C., De Lope, F., Møller, A. P. and Ninni, P. 2002. Structural coloration and sexual selection in the barn swallow *Hirundo rustica*. -*Behavioral Ecology*, 13: 728-736.

Rohwer, S. 1975. Social significance of avian winter plumage variability. -*Evolution*, 29: 593-610.

Ruiz-Castellano, C., Ruiz-Rodriguez, M., Tomas, G. and Soler, J. J. 2019. Antimicrobial activity of nest-lining feathers is enhanced by breeding activity in avian nests. -*FEMS microbiology ecology*, 95.

Ruiz-Castellano, C., Tomás, G., Ruiz-Rodríguez, M., Martín-Galvez, D. and Soler, J. J. 2016. Nest Material Shapes Eggs Bacterial Environment. -*PLoS One*, 11: e0148894.

Ruiz-De-Castañeda, R., Burtt Jr, E., González--Braojos, S. and Moreno, J. 2012. Bacterial degradability of an intrafeather unmelanized ornament: a role for feather-degrading bacteria in sexual selection? -*Biological Journal of the Linnean Society*, 105: 409-419.

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

Saino, N., Bolzern, A. M. and Møller, A. P. 1997. Immunocompetence, ornamentation, and viability of male barn swallows (Hirundo rustica). -*Proceedings of the National Academy of Sciences of the USA*, 94: 549-552.

Salvador, A., Veiga, P., Martín, J., López, P., Abelenda, M. and Puerta, M. 1996. The cost of producing a sexual signal: testoterone increases tha susceptibility of male lizards to ectoparasitic infestation. -*Behavioral Ecology*, 7: 145-150.

Senar, J. 1990. Agonistic communication in social species: what is communicated? -*Behaviour*, 112: 270-283.

Senar, J. 1999. Plumage coloration as a signal of social status. Proceedings of the International Ornithological Congress, 1999. BirdLife South Africa, pp. 1669-1686.

Senar, J. 2006. Color displays as intrasexual signal of agression and dominance In: HILL, G. E. and MCGRAW, K. J. (eds.) Bird coloration: Function and evolution. Harvard University Press, Cambridge, Massachusetts, pp. 87-136.

Senar, J. C., Polo, V., Uribe, F. and Camerino, M. 2000. Status signalling, metabolic rate and body mass in the siskin: the cost of being a subordinate. -*Animal Behaviour*, 59: 103-110.

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

Shawkey, M. D., Pillai, S. R., Hill, G. E., Siefferman, L. M. and Roberts, S. R. 2007. Bacteria as an agent for change in structural plumage color: Correlational and experimental evidence. -*American Naturalist*, 169: S112-S121.

Simons, M. J. P. and Verhulst, S. 2011. Zebra finch females prefer males with redder bills independent of song rate-a meta-analysis. -*Behavioral Ecology*, 22: 755-762.

Soler, J. J. and Avilés, J. M. 2010. Sibling competition and conspicuousness of nestling gapes in altricial birds: a comparative study. -*PLoS ONE*, 5: e10509.

Soler, J. J., Navarro, C., Pérez-Contreras, T., Avilés, J. M. and Cuervo, J. J. 2008. Sexually selected egg coloration in spotless starlings. -*American Naturalist*, 171: 183-194.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martin-Vivaldi, M., Martinez-De La Puente, J., Ruiz-Rodriguez, M. and Tomas, G. 2017. Telomere length and dynamics of spotless starling nestlings depend on nest-building materials used by parents. -*Animal Behaviour*, 126: 89-100.

Tibbetts, E. A. and Dale, J. 2004. A socially enforced signal of quality in a paper wasp. -*Nature*, 432: 218-222.

Veiga, J. and Polo, P. 2016. Estornino Negro–Sturnus unicolor. En: Enciclopedia Virtual de los Vertebrados Españoles. Museo Nacional de Ciencias Naturales, Madrid, Salvador, A., Morales, M. B. (Eds.).

Velando, A., Beamonte-Barrientos, R. and Torres, R. 2006. Pigment-based skin colour in the blue-footed booby: an honest signal of current condition used by females to adjust reproductive investment. -*Oecologia*, 149: 535-542.

PRINCIPALES RESULTADOS Y DISCUSIÓN INTEGRADORA

En la naturaleza, los animales utilizan una compleja red de comunicación por la que son capaces de obtener información social en distintos contextos, tales como depredación (Templeton and Greene 2007, Parejo et al. 2012), infestación por ectoparásitos (Tomás and Soler 2016), o parasitismo de cría (Tomás et al. 2017). Detectar esta información tiene un gran valor adaptativo para los receptores, ya que les permitirá optimizar su toma de decisiones. En esta tesis, presentamos novedosos resultados que contribuyen a entender la comunicación animal. Desde una aproximación experimental (capítulos 1, 2, 5 y 6) y correlativa (capítulos 3 y 4), analizamos la información emitida por distintas pistas y señales en el estornino negro. Por un lado, ponemos de manifiesto el uso de las motas de los huevos por parte de las hembras parásitas de cría para evaluar el riesgo de ectoparasitismo de los nidos hospedadores (capítulo 1), y por otro, que el comportamiento de los adultos de retirar los sacos fecales de los pollos, ha podido evolucionar como un mecanismo para evitar dejar pistas que faciliten la detección del nido por depredadores y ectoparásitos (capítulo 2).

Además, hemos estudiado la posible emisión de información a través de señales visuales como son la coloración de distintas partes del cuerpo y la longitud de los ornamentos, relacionándolos con la capacidad de los individuos de defenderse frente a las bacterias (capítulo 3) y con su calidad genética (capítulo 5). También, estudiamos cómo estos dos tipos de señales se relacionan entre sí, y la influencia de la longitud del ornamento en la expresión del color del pico (capítulo 6). Por último, en una aproximación comparativa, hemos estudiado la posible influencia de la presión ejercida por el ambiente bacteriano de los nidos en el desarrollo de las plumas en los pollos (capítulo

4). A continuación, discutiremos los principales resultados en distintos escenarios de comunicación y de uso de información.

1. El uso de pistas como fuente de información

La manipulación experimental de las motas que hay sobre la cáscara de los huevos de estornino como consecuencia de la actividad parasitaria de las moscas *Carnus* (capítulo 1), reveló que estas motas constituyen unas pistas que pueden ser utilizadas por otras hembras parásitas de cría para seleccionar el nido hospedador, prefiriendo aquellos con una menor cantidad de motas (es decir, los que tendrían menos ectoparasitismo). Curiosamente, no hubo un efecto del color de las motas experimentales en la elección de los nidos, por lo que concluimos que las hembras parásitas utilizan el componente acromático de las motas. El uso del moteado de los huevos como pista para elegir el nido a parasitar resulta especialmente útil a las hembras parásitas, ya que con su decisión disminuyen el riesgo de sufrir los efectos negativos causados por los ectoparásitos (por ejemplo, Soler et al. 1999, Martín-Vivaldi et al. 2006, Avilés et al. 2009, Hoi et al. 2010, Hoi et al. 2018) en sus pollos. Además, los nidos con huevos moteados pueden indicar también que ya ha comenzado la incubación (López-Rull et al. 2007b, Tomás et al. 2017), lo que supondría una disminución del éxito de eclosión y de cría de los huevos y pollos parásitos (Wiley 1988, Kattan 1997, Soler et al. 2015b). Por lo tanto, el uso del moteado de los huevos como pista que informa sobre la intensidad del parasitismo y el inicio de la incubación sería una ventaja selectiva para las hembras parásitas. Esta es la primera demostración experimental de que las hembras parásitas de cría utilizan manchas y/o patrones de color de la cáscara de los huevos para seleccionar los potenciales nidos hospedadores. Pero además, estas pistas podrían ser también usadas por los adultos de cada nido, que tienen la

oportunidad de ajustar el esfuerzo de alimentación de los pollos a la densidad de las manchas de la cáscara de los huevos (Avilés et al. 2009).

Por otro lado, la información aportada por las pistas (ya sean visuales, sonoras u olfativas) puede ser también recibida por heterospecíficos, como depredadores o ectoparásitos (Petit et al. 1989, Hart 1997, Lang et al. 2002, Quan et al. 2015, Ibáñez-Álamo et al. 2016). En el capítulo 2 simulamos los costos que tendría el hecho de no retirar los sacos fecales en un grupo de nidos rompiéndolos para evitar su retirada, y comprobamos cómo aumentó la depredación y, en menor medida, el ectoparasitismo por *Carnus*. Por lo tanto, el comportamiento de los adultos de retirar los sacos ha evolucionado probablemente por la presión que supone para el nido dejar las pistas que acarrean los excrementos. Además, comprobamos que en los nidos con excrementos aumentó la carga bacteriana. Estas bacterias podrían ser responsables, al menos en parte, de la producción de volátiles (Lemfack et al. 2014) atrayentes para depredadores y ectoparásitos. Trabajos previos han puesto de manifiesto la importancia de los volátiles producidos por las bacterias en diferentes escenarios ecológicos, incluyendo la comunicación social (por ejemplo, Theis et al. 2013, Ezenwa and Williams 2014, Leclaire et al. 2017), e interacciones depredador-presa (Fogarty et al. 2017, Mihailova et al. 2018), o parásito-huésped (Bucher 1988, Gow et al. 2015, Schulte et al. 2016).

Al aumentar la carga bacteriana de los nidos impidiendo la retirada de los excrementos, los pollos tendrían que enfrentarse a una mayor densidad de bacterias potencialmente patógenas (Devriese et al. 1991, Bruce y Drysdale 1994, Benskin et al. 2009). Esto, junto con la mayor tasa de ectoparásitos, puede explicar que los pollos en los nidos experimentales tuvieran tarsos más cortos que los de nidos controles. Este resultado apoya la importancia de la presión selectiva ejercida por el ambiente bacteriano del nido en el crecimiento de los pollos, anteriormente demostrado con la selección del material

antimicrobiano (Mennerat et al. 2009b, Soler et al. 2017) y la densidad de bacterias (Jacob et al. 2015) en los nidos. De este modo, la presión ejercida por las bacterias podría también haber contribuido a la evolución del comportamiento de higiene de los nidos para reducir los efectos negativos de los microorganismos.

Los métodos utilizados para caracterizar las comunidades bacterianas han sido los clásicos de cultivo. Aunque estas técnicas resultan fiables para este tipo de estudios (González-Braojos et al. 2012), el uso de técnicas moleculares permitirán en un futuro determinar los grupos bacterianos responsables de las relaciones encontradas en este estudio, y es sin duda el siguiente paso en esta línea de investigación.

2. Información transmitida a través de señales

2.1 Relación de las bacterias con las señales visuales

Debido a la importante presión selectiva que ejercen las bacterias sobre las aves, estas han desarrollado numerosas estrategias para hacerles frente (Ruiz-Rodríguez et al. 2009, Martín-Vivaldi et al. 2010, Brandl et al. 2014, Ibáñez-Álamo et al. 2014). Así, la capacidad individual para defenderse de las infecciones bacterianas puede ser una característica importante en el emparejamiento. Por ejemplo, los machos de estornino muestran su habilidad para regular la carga bacteriana soportada a través del estado de las plumas ornamentales de la garganta (Ruiz-Rodríguez et al. 2015). En la presente tesis, hemos estudiado la posible variación estacional de algunos mecanismos de defensa en hembras de estornino frente a infecciones bacterianas. Además, estudiamos la relación de dichas variables con la coloración de distintas partes del cuerpo, y con su éxito reproductor (Capítulo 3). Encontramos que las hembras de estornino (i) varían su inversión en defensas antimicrobianas entre

la época de emparejamiento y la de pollos, (ii) que su capacidad de defensa está relacionada con su éxito reproductor, y (iii) que su capacidad antimicrobiana se relaciona con diferentes coloraciones. Probablemente, la señalización de sus capacidades antimicrobianas funciona en contextos de selección sexual, ya que las hembras pueden indicar a través de sus coloraciones su calidad y potencial reproductor. El estudio de la relación de caracteres indicativos de calidad con la coloración de las hembras en la época de emparejamiento, resulta novedoso ya que la mayoría de estudios de este tipo se han realizado en machos (Webb et al. 2016).

Sabemos que, debido a los costos de la reproducción, el nivel de inmunocompetencia de las hembras tiende a disminuir a lo largo de la temporada de cría (Ardia et al. 2003). Sin embargo, nuestros resultados muestran un aumento de la capacidad del plasma sanguíneo de inhibir el crecimiento de distintas cepas bacterianas, lo que sugiere que las hembras de estornino dedican más recursos a las defensas contra las bacterias en épocas tardías de reproducción, cuando las probabilidades de infección aumentan (Sorci et al. 1997, Merino et al. 2000), apoyando el papel crucial de los microorganismos en la evolución de las historias vitales de los huéspedes.

Por otra parte, las comunidades bacterianas pueden variar según las características del ambiente que explotan y, por lo tanto, distintas especies de aves entrarán en contacto con distintas comunidades bacterianas según sus hábitos (Bisson et al. 2007, Peralta-Sánchez et al. 2012). Consecuentemente, distintos ambientes implicarían una variación en las presiones selectivas ejercidas por las bacterias sobre sus hospedadores que, tanto a nivel intra- como inter-específico, debería de ir acompañado de una variación en las estrategias antibacterianas, incluida la inversión en defensas. Estudios previos han detectado variación inter-específica en la microbiota de los huevos (Peralta-Sánchez et al. 2018) que se asocia con determinadas características del ciclo vital (Peralta-Sánchez et al. 2012). Por lo tanto, si hay ambientes

bacterianos distintos en nidos de distintas especies, las consecuencias para los pollos, así como las adaptaciones para defenderse de las bacterias, variarán en consonancia.

Una de las estructuras más expuestas a los microorganismos en aves son las plumas (Jacob et al. 2018) y, por tanto, su deterioro y la resistencia a la degradación bacteriana debería de relacionarse con el ambiente bacteriano en el que habitan distintas especies. Para comprobar esta idea, en el capítulo 4 realizamos una comparativa entre la carga bacteriana del nido, la degradación de las plumas, y su susceptibilidad a la degradación por bacterias queratinoliticas, en pollos de 16 especies de aves. Los resultados indicaron que todas las variables diferían significativamente entre especies. En promedio, las plumas no melanizadas estaban más deterioradas que las melanizadas, siendo consistente este resultado con otros trabajos que encontraron que las plumas no melanizadas son más fácilmente degradables (Goldstein et al. 2004, Gunderson et al. 2008, Ruiz-de-Castañeda et al. 2012). Además, la carga bacteriana de los nidos estaba relacionada con el nivel de degradación de las plumas, aunque el signo de la asociación dependía del grupo bacteriano considerado y de la pigmentación de las plumas. También encontramos una asociación positiva entre la susceptibilidad a la degradación de plumas no melanizadas con su deterioro, y con la carga bacteriana del nido. Estos resultados están de acuerdo con la hipótesis de partida, y sugieren que los ambientes bacterianos durante el desarrollo de los pollos determinan la integridad de las plumas, y por lo tanto su resistencia frente a la degradación bacteriana.

2.2 El caso de las plumas ornamentales del macho de estornino negro: Indicador de calidad individual y vinculación con otros caracteres sexuales

Como vimos en la introducción, las plumas ornamentales de la garganta de los machos de estornino están relacionadas con distintos indicadores de calidad de los individuos (Aparicio et al. 2001, Gil and Culver 2011), incluidas sus capacidades antimicrobianas (Ruiz-Rodríguez et al. 2015), y constituyen una señal sexual de gran relevancia en esta especie. Aquí, hemos estudiado de manera correlativa y experimental la relación de este rasgo con la esperanza de vida y el éxito reproductor (capítulo 5), y su influencia en el desarrollo de otra señal como es el color de la base del pico (capítulo 6).

Los principales resultados indican que la longitud de estas plumas se relaciona negativamente con la longitud de los telómeros y su tasa de deterioro. Este resultado puede explicarse por dos causas no excluyentes: (i) Puede ser que los individuos de más edad, con telómeros más cortos, tengan sus plumas de la garganta más largas (Hiraldo and Herrera 1974, Aparicio et al. 2001, Veiga and Polo 2016), o bien que (ii) los costos asociados a la expresión de los caracteres sexuales más desarrollados, como es el aumento del estrés oxidativo (Haussmann and Vleck 2002, Haussmann et al. 2003, Pauliny et al. 2006) y de hormonas sexuales (Gonzalez et al. 2001, Fusani 2008), impliquen un acortamiento desproporcionado de los telómeros. Sin embargo, el acortamiento experimental de las plumas de la garganta de los machos no afectó significativamente a la tasa de deterioro de los telómeros, lo que sugiere que la asociación correlativa no se debió a la longitud de las plumas en sí, sino a variables correlacionadas con la expresión de este carácter. Tampoco podemos descartar que el tiempo transcurrido entre capturas (un año como media) no fuera suficiente para detectar un efecto en la longitud de los telómeros.

En hembras también encontramos una asociación entre la longitud de las plumas de la garganta y la de los telómeros, pero en este caso la relación fue positiva, sugiriendo una dinámica de telómeros en relación al carácter que difiere entre sexos. Además, encontramos una asociación positiva entre la longitud de las plumas de la garganta de los machos y el éxito de la eclosión de las segundas puestas. Una vez más, la manipulación experimental de la longitud de las plumas no influyó en el éxito de la eclosión, lo que sugiere que la asociación detectada no implica una causa-efecto, y las relaciones podrían deberse a otras características fisiológicas y/o morfológicas no manipuladas que covariaron con ellas.

La manipulación de la longitud de las plumas influyó en la expresión de otra señal sexual, la coloración de la base del pico. En concreto, el acortamiento experimental de las plumas de la garganta en los machos tuvo un efecto negativo sobre la intensidad del croma azul un año después de la manipulación. Además, esta coloración de la base del pico de los machos se relacionó positivamente con su condición corporal, y con la longitud de las plumas ornamentales de la garganta. Ambos rasgos (longitud de las plumas de la garganta y coloración del pico) son sexualmente dimórficos, y reflejan la calidad fenotípica de los machos (Aparicio et al. 2001, Navarro et al. 2010). Nuestros resultados demuestran una conexión directa entre estos dos rasgos sugiriendo que pueden funcionar como un todo en un marco de señalización múltiple. La longitud de las plumas de la garganta y la coloración del pico de los estorninos podrían proporcionar información a diferentes escalas temporales, ya que la morfología de las plumas negras y, en concreto su longitud, es relativamente estática desde la muda y proporcionarían información sobre la condición fenotípica y la calidad de los machos en momentos anteriores a la reproducción (Badyaev and Hill 2000, Hebets and Papaj 2005). Además, el deterioro de las plumas proporcionaría información sobre su calidad y la capacidad de los machos para reducir su degradación

(Shawkey et al. 2007, Shawkey et al. 2009, Ruiz-de-Castañeda et al. 2012, Ruiz-Rodríguez et al. 2015). La coloración azulada de la base del pico, sin embargo, sólo se expresa durante la reproducción y puede cambiar en muy cortos períodos de tiempo (obs. pers.). Igual que ocurre para la coloración de otras partes desnudas de distintas especies de aves, la coloración del pico tiene el potencial de cambiar en semanas, días, horas o incluso minutos (Iverson and Karubian 2017), y de ser evaluados continuamente por los receptores como ocurre en otras especies de aves (Velando et al. 2006, Simons and Verhulst 2011, Dey et al. 2015).

Nuestros resultados mostraron una relación positiva entre la coloración del pico y la longitud de las plumas de la garganta, y un efecto negativo del acortamiento de plumas en la intensidad de coloración del pico, por lo que es posible que el papel que juegue la señal más perdurable en el tiempo (las plumas ornamentales) en otro contexto no reproductivo determine posteriormente el desarrollo de la otra señal (base del pico) en la época reproductiva. La longitud de las plumas de la garganta de los machos podría servir para establecer algún tipo de jerarquía social entre los machos del grupo que permitiera una reducción de las interacciones agonísticas entre individuos de diferente estatus (Rohwer 1975, Senar 1999, McGraw and Hill 2000). En algunas especies de aves, la probabilidad de agresión social por parte de sus congéneres está relacionada con las características de las plumas que señalan el estado de las aves (Senar 1990, McGraw et al. 2007, Chaine and Lyon 2008), y sabemos que los individuos que poseen señales de mayor calidad pueden provocar un menor nivel de agresividad en sus congéneres y, por tanto sufran menos agresiones (Lopez-Idiaquez et al. 2016).

Los estorninos mudan las plumas de la garganta varios meses antes de la reproducción, y los machos frecuentemente exhiben estas plumas en lugares muy visibles durante períodos no reproductivos (obs. pers.). Por lo tanto, es posible que los machos de estornino con plumas ornamentales más largas

experimenten menores tasas de agresiones sociales durante el período pre-reproductivo. Sin embargo, el estatus social de los machos experimentales inferido por los receptores evaluando la longitud de las plumas de la garganta, no coincidiría con el que le correspondería a la longitud de estas plumas antes del experimento y, por tanto, los machos experimentales deberían haber sufrido mayores costos sociales que los machos controles. Estos mayores costos sociales podrían explicar el efecto negativo de la manipulación detectado en la coloración del pico como ocurre con distintos caracteres sexuales de otras especies (Møller et al. 2000). Estos resultados son la primera evidencia experimental de una asociación directa entre dos caracteres sexuales que aportan distinto tipo de información a los receptores.

Resumiendo, los resultados de esta tesis en conjunto ponen de manifiesto la importancia de la información que aportan distintas pistas y señales de los individuos, tanto para receptores como para emisores, en escenarios de parasitismo, depredación, y selección sexual. Algunas señales consideradas en esta tesis están relacionadas con el ambiente bacteriano, o el reflejo de la capacidad antimicrobiana de los emisores, lo que apoyaría un papel destacado de los microorganismos en la evolución de estas señales, o de comportamientos que permiten minimizar los efectos negativos de los patógenos. Finalmente, hemos demostrado por primera vez que la expresión de una señal sexual afecta a la expresión de otra señal sexual en un contexto de señales múltiples. Una de estas señales, en estorninos machos, es indicadora de su capacidad antimicrobiana, lo que apoya la importancia de los microorganismos en escenarios de señalizaciones complejas o múltiples.

CONCLUSIONES

1. Las hembras parásitas de cría utilizan el moteado de los huevos, como pista para elegir el nido hospedador. El moteado de los huevos es una pista de la actividad del ectoparasito *Carnus hemapterus* alimentándose del adulto que incuba. Por tanto, al evitar nidos con huevos moteados, las hembras parásitas de cría disminuyen la probabilidad de ectoparasitismo de sus pollos, y evitan parasitar nidos con huevos que ya hubieran iniciado la incubación.

2. La rotura experimental de sacos fecales produjo un aumento en la carga bacteriana del nido, en la probabilidad de depredación, y aunque no significativamente, también en la intensidad de ectoparasitismo. Además, los pollos crecieron menos en nidos experimentales. Estos resultados ponen de manifiesto las ventajas adaptativas asociadas al comportamiento de retirada de los sacos fecales de los nidos por parte de los adultos.

3. Los niveles de defensas antimicrobianas de hembras de estornino mediadas por anticuerpos naturales variaron a lo largo de la temporada de cría. Además, la capacidad antimicrobiana de las hembras se relacionó con el éxito reproductor y con el color de distintas partes del cuerpo (pico, patas, y plumas del dorso y pecho). Estos resultados sugieren un posible papel señalizador de estos caracteres en contextos de selección sexual.

4. El ambiente bacteriano de los nidos varía entre distintas especies de aves, y se relaciona con el estado de degradación de las plumas de los

pollos poco antes de abandonar el nido, y con la resistencia de estas plumas al ataque por bacterias queratinolíticas. Estos resultados sugieren un papel central de las bacterias determinando las características y el estado de las plumas de los pollos poco antes de abandonar el nido. Además, las plumas no melanizadas se degradaron más fácilmente por bacterias, lo que podría explicar, al menos en parte, la enorme variación en la pigmentación de las plumas de distintas especies asociada, por ejemplo, a la explotación de hábitats con distinto riesgo de contaminación bacteriana.

5. La longitud de las plumas de la garganta de los machos de estornino se relaciona negativamente con la longitud y con el acortamiento de los telómeros. Sin embargo, el acortamiento experimental de estas plumas no afectó a las características de los telómeros un año después de la manipulación, por lo que la longitud de las plumas no es la causa de su correlación con telómeros. La edad podría explicar esa relación ya que machos más viejos tendrían telómeros más cortos y plumas de la garganta más largas.

6. El acortamiento experimental de plumas ornamentales antes de la reproducción tuvo un efecto en la coloración de la base del pico de los machos de estornino en la siguiente temporada de cría, la cual se relaciona positivamente con la condición corporal. Estos resultados apoyan por primera vez un vínculo causal entre las expresiones de dos caracteres sexualmente dimórficos que señalizan la calidad del emisor, lo cual resulta esencial para comprender su funcionalidad en el marco de estudio de las señales múltiples.

CONCLUSIONS

1. Brood parasite females use the egg spottiness as a clue to choose conspecific host nests for parasitism. Eggshell spots are produced by the ectoparasite Carnus hemapterus feeding on incubating females. Therefore, by avoiding nests with spotted eggs, brood parasitic females reduce the likelihood of ectoparasitism of their nestlings, and avoid host nests in which the incubation had already started.

2. The experimental breakage of faecal sacs increased bacterial load of nests, the probability of depredation, and, although not significantly, the intensity of ectoparasitism. In addition, the experiment also affected nestling growth negatively. These results highlight the adaptive advantages associated to faecal sacs removal from nests by adults, and suggest a key role of bacteria influencing those effects.

3. Antimicrobial properties of plasma of starling females vary along the breeding season. Moreover, estimates of antimicrobial capacity resulted associated with the colour of different parts of the body (beak, legs, and back and breast feathers), and with reproductive success. These results suggest a possible role of these traits signalling antimicrobial capacity of females in contexts of sexual selection.

4. The nest bacterial environments varies among different bird species, and it is related to wear and resistance to degradation by queratinolytic bacteria of nestling feathers. These results suggest a preponderant role of bacterial environments determining nestling feathers development. In addition, queratinolytic bacteria degraded more easily non-

melanised than melanised feathers, which might suggest that the huge variation in feather pigmentation of different bird species may be due, at least partially, to the risk of bacterial contamination experienced in the habitat where they live.

5. The throat feathers length of starling males resulted negatively related to telomere length and shortening. However, since the experimental reduction of throat feather length did not influence telomeres one year after manipulation, feather length cannot be the cause of the detected associations. Age is a good candidate to explain these relationships, because older males may have shorter telomeres and longer throat feathers.

6. The experimental shortening of ornamental feathers before breeding had an effect on the beak base colouration of starling males in the next breeding season. Moreover, this colouration was positively related to body condition. These results support, for the first time, a causal link between the expressions of two sexually dimorphic traits that provides information on the phenotypic quality of the signaller, which is essential for understanding their functionality within a multiple signals framework.

BIBLIOGRAFÍA

Andersson, M. 1994. *Sexual selection,* Princeton, Princeton University Press.

Aparicio, J. M., Cordero, P. J. and Veiga, J. P. 2001. A test of the hypothesis of mate choice based on heterozygosity in the spotless starling. -*Animal Behaviour,* 62: 1001-1006.

Aragon, P., Massot, M., Gasparini, J. and Clobert, J. 2006. Socially acquired information from chemical cues in the common lizard, Lacerta vivipara. -*Animal Behaviour,* 72: 965-974.

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

Ardia, D. R., Schat, K. A. and Winkler, D. W. 2003. Reproductive effort reduces long-term immune function in breeding tree swallows (*Tachycineta bicolor*). -*Proceedings of the Royal Society of London B, Biological Sciences,* 270: 1679-1683.

Asghar, M., Hasselquist, D., Hansson, B., Zehtindjiev, P., Westerdahl, H. and Bensch, S. 2015. Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. -*Science,* 347: 436-438.

Asghar, M., Yman, V., Homann, M. V., Sonden, K., Hammar, U., Hasselquist, D. and Farnert, A. 2018. Cellular aging dynamics after acute malaria infection: A 12-month longitudinal study. -*Aging Cell,* 17: 8.

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

Azcárate-García, M., Ruiz-Rodriguez, M., Diaz-Lora, S., Ruiz-Castellano, C. and Soler, J. J. 2019. Experimentally broken faecal sacs affect nest bacterial environment, development and survival of spotless starling nestlings. -*Journal of Avian Biology,* 50: 10.

Badas, E. P., Martinez, J., Rivero-De Aguilar, J., Ponce, C., Stevens, M. and Merino, S. 2018. Colour change in a structural ornament is related to individual quality, parasites and mating patterns in the blue tit. -*Science of Nature,* 105: 12.

Badás, E. P., Martínez, J., Rivero De Aguilar Cachafeiro, J., Miranda, F., Figuerola, J. and Merino, S. 2015. Ageing and reproduction: antioxidant supplementation alleviates telomere loss in wild birds. -*Journal of Evolutionary Biology,* 28: 896-905.

Badyaev, A. V. and Hill, G. E. 2000. Evolution of sexual dichromatism: contribution of carotenoid- versus melanin-based coloration. -*Biological Journal of the Linnean Society,* 69: 153-172.

Badyaev, A. V., Hill, G. E. and Weckworth, B. V. 2002. Species divergence in sexually selected traits: Increase in song elaboration is related to

decrease in plumage ornamentation in finches. -*Evolution,* 56: 412-419.

Balmford, A., Rosser, A. M. and Albon, S. D. 1992. Correlates of female choice in resource-defending antelope. -*Behavioral Ecology and Sociobiology,* 31: 107-114.

Bauch, C., Becker, P. H. and Verhulst, S. 2013. Telomere length reflects phenotypic quality and costs of reproduction in a long-lived seabird. -*Proceedings of the Royal Society B-Biological Sciences,* 280.

Bauer, C. M., Graham, J. L., Abolins-Abols, M., Heidinger, B. J., Ketterson, E. D. and Greives, T. J. 2018. Chronological and biological age predict seasonal reproductive timing: an investigation of clutch initiation and telomeres in birds of known age. -*American Naturalist,* 191: 777-782.

Beaulieu, M., Reichert, S., Le Maho, Y., Ancel, A. and Criscuolo, F. 2011. Oxidative status and telomere length in a long-lived bird facing a costly reproductive event. -*Functional Ecology,* 25: 577-585.

Benskin, C. M., Wilson, K., Jones, K. and Hartley, I. R. 2009. Bacterial pathogens in wild birds: a review of the frequency and effects of infection. - *Biological Reviews,* 84: 349-73.

Bisson, I. A., Marra, P. P., Burtt, E. H., Sikaroodi, M. and Gillevet, P. M. 2007. A molecular comparison of plumage and soil bacteria across biogeographic, ecological, and taxonomic scales. -*Microbial Ecology,* 54: 65-81.

Blackburn, E. H. 1991. Structure and function of telomeres. -*Nature,* 350: 569-573.

Blount, J. D., Metcalfe, N. B., Birkhead, T. R. and Surai, P. F. 2003. Carotenoid modulation of immune function and sexual attractiveness in zebra finches. -*Science,* 300: 125-127.

Bonato, M., Evans, M. R., Hasselquist, D. and Cherry, M. I. 2009. Male coloration reveals different components of immunocompetence in ostriches, Struthio camelus. -*Animal Behaviour,* 77: 1033-1039.

Bonser, R. H. C. 1995. Melanin and the abrasion resistance of feathers. -*Condor,* 97: 590-591.

Boonekamp, J. J., Bauch, C., Mulder, E. and Verhulst, S. 2017. Does oxidative stress shorten telomeres? -*Biology letters,* 13: 20170164.

Boonekamp, J. J., Mulder, G. A., Salomons, H. M., Dijkstra, C. and Verhulst, S. 2014. Nestling telomere shortening, but not telomere length, reflects developmental stress and predicts survival in wild birds. -*Proceedings of the Royal Society of London Series B-Biological Sciences,* 281: 20133287.

Bradbury, J. W. and Vehrencamp, S. L. 1998. *Principles of animal communication*, Sinauer Associates, Inc.

Brake, I. 2011. World catalog of the family carnidae (Diptera, Schizophora). -*Myia,* 12: 113-169.

Brandl, H. B., Van Dongen, W. F. D., Darolova, A., Kristofik, J., Majtan, J. and Hoi, H. 2014. Composition of bacterial assemblages in different components of reed warbler nests and a possible role of egg incubation in pathogen regulation. -*Plos One,* 9: 19.

Brittingham, M. C., Temple, S. A. and Duncan, R. M. 1988. A survey of the prevalence of selected bacteria in wild birds. -*Journal of Wildlife Diseases,* 24: 299-307.

Bro-Jorgensen, J. and Dabelsteen, T. 2008. Knee-clicks and visual traits indicate fighting ability in eland antelopes: multiple messages and back-up signals. -*Bmc Biology,* 6: 9.

Bucher, E. H. 1988. Do birds use biological-control against nest parasites. -*Parasitology Today,* 4: 1-3.

Bulluck, L. P., Foster, M. J., Kay, S., Cox, D. E., Viverette, C. and Huber, S. 2017. Feather carotenoid content is correlated with reproductive success and provisioning rate in female Prothonotary Warblers. -*Auk,* 134: 229-239.

Burley, N. 1988. The differential-allocation hypothesis: An experimental test. -*American Naturalist,* 132: 611-628.

Burtt, E. H. 1979. Tips on wings and other things. In: Burtt, E. H. (ed.) *The behavioural significance of color.* Garland Publishing Inc., New York, pp. 75-100.

Burtt, E. H. and Ichida, J. M. 2004. Gloger's rule, feather-degradlng bacteria, and color variation among song sparrows. -*Condor,* 106: 681-686.

Burtt, E. H., Schroeder, M. R., Smith, L. A., Sroka, J. E. and Mcgraw, K. J. 2011. Colourful parrot feathers resist bacterial degradation. -*Biology Letters,* 7: 214-216.

Burtt, E. H. J. and Ichida, J. M. 1999. Occurrence of feather-degrading bacilli in the plumage of birds. -*Auk,* 116: 364-372.

Busula, A. O., Takken, W., De Boer, J. G., Mukabana, W. R. and Verhulst, N. O. 2017. Variation in host preferences of malaria mosquitoes is mediated by skin bacterial volatiles. -*Medical and Veterinary Entomology,* 31: 320-326.

Calvo, J. M., Pascual, J. A., Deceuninck, B. and Peris, S. J. 2000. Intraspecific nest parasitism in the spotless starling Sturnus unicolor. -*Bird Study,* 47: 285-294.

Campisi, J. 2013. Aging, Cellular Senescence, and Cancer. In: Julius, D. (ed.) *Annual Review of Physiology, Vol 75.* Annual Reviews, Palo Alto, pp. 685-705.

Candolin, U. 2003. The use of multiple cues in mate choice. -*Biological Reviews,* 78: 575-595.

Chaine, A. S. and Lyon, B. E. 2008. Intrasexual selection on multiple plumage ornaments in the lark bunting. -*Animal Behaviour,* 76: 657-667.

Chaine, A. S. and Lyon, B. E. 2015. Signal architecture: temporal variability and individual consistency of multiple sexually selected signals. -*Functional Ecology*, 29: 1178-1188.

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

Costopoulos, K., Kovacs, J. L., Kamins, A. and Gerardo, N. M. 2014. Aphid facultative symbionts reduce survival of the predatory lady beetle Hippodamia convergens. -*Bmc Ecology,* 14.

Cramp, S. 1998. *Cramp's the complete birds of the Western Palearctic,* Oxford, Optimedia, Oxford University Press.

Criscuolo, F., Bize, P., Nasir, L., Metcalfe, N. B., Foote, C. G., Griffiths, K., Gault, E. A. and Monaghan, P. 2009. Real-time quantitative PCR assay for measurement of avian telomeres. -*Journal of Avian Biology,* 40: 342-347.

Danchin, E., Giraldeau, L. A., Valone, T. J. and Wagner, R. H. 2004. Public information: from nosy neighbors to cultural evolution. -*Science,* 305: 487-491.

Danchin, E., Heg, D. and Doligez, B. 2001. Public information and breeding habitat selection. In: Clobert, J., Danchin, E., Dhondt, A. A. and Nichols, J. D. (eds.) *Dispersal.* Oxford University Press, Oxford, UK, pp. 243-258.

Darwin, C. R. 1871. *The descent of man and selection in relation to sex,* London, John Murray.

Dawson, A., Hinsley, S. A., Ferns, P. N., Bonser, R. H. C. and Eccleston, L. 2000. Rate of moult affects feather quality: a mechanism linking current reproductive effort to future survival. -*Proceedings of the Royal Society B-Biological Sciences,* 267: 2093-2098.

Dey, C. J., Valcu, M., Kempenaers, B. and Dale, J. 2015. Carotenoid-based bill coloration functions as a social, not sexual, signal in songbirds (Aves: Passeriformes). -*Journal of Evolutionary Biology,* 28: 250-258.

Dugdale, H. L. and Richardson, D. S. 2018. Heritability of telomere variation: it is all about the environment! -*Philosophical Transactions of the Royal Society B-Biological Sciences,* 373: 14.

Eisenberg, D. T. A., Borja, J. B., Hayes, M. G. and Kuzawa, C. W. 2017. Early life infection, but not breastfeeding, predicts adult blood telomere lengths in the Philippines. -*American Journal of Human Biology,* 29: 11.

Endler, J. A. and Houde, A. E. 1995. Geographic-variation in female preferences for male traits in poecilia-reticulata. -*Evolution,* 49: 456-468.

Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M. and Xavier, J. B. 2012. Animal behavior and the microbiome. -*Science,* 338: 198-199.

Ezenwa, V. O. and Williams, A. E. 2014. Microbes and animal olfactory communication: Where do we go from here? -*Bioessays,* 36: 847-854.

Fisher, R. A. 1930. *The genetical theory of natural selection,* Oxford, Oxford University Press.

Fogarty, D. T., Elmore, R. D., Fuhlendorf, S. D. and Loss, S. R. 2017. Influence of olfactory and visual cover on nest site selection and nest success for grassland-nesting birds. -*Ecology and Evolution,* 7: 6247-6258.

Folstad, I. and Karter, A. J. 1992. Parasites, bright males, and the immunocompetence handicap. -*American Naturalist,* 139: 603-622.

Francis, C. M. and Wood, D. S. 1989. Effects of age and wear on wing length of wood warblers. -*Journal of Field Ornithology,* 60: 495-503.

Fülöp, A., Czirják, G. Á., Pap, P. L. and Vágási, C. I. 2015. Feather-degrading bacteria, uropygial gland size and feather quality in House Sparrows *Passer domesticus*. -*Ibis*, 158: 362-370.

Fusani, L. 2008. Testosterone control of male courtship in birds. -*Hormones and Behavior,* 54: 227-233.

Galván, I. and Alonso-Alvarez, C. 2009. The expression of melanin-based plumage is separately modulated by exogenous oxidative stress and a melanocortin. -*Proceedings of the Royal Society B: Biological Sciences, 276: 3089-3097.*

Gil, D. and Culver, R. 2011. Male ornament size in a passerine predicts the inhibitory effect of testosterone on macrophage phagocytosis. -*Functional Ecology,* 25: 1278-1283.

Girard, M. B., Elias, D. O. and Kasumovic, M. M. 2015. Female preference for multi-modal courtship: multiple signals are important for male mating success in peacock spiders. -*Proceedings of the Royal Society B-Biological Sciences,* 282: 10.

Goldstein, G., Flory, K. R., Browne, B. A., Majid, S., Ichida, J. M. and Burtt, E. H. 2004. Bacterial degradation of black and white feathers. -*Auk,* 121: 656-659.

Gomez, D. 2006. *AVICOL, a program to analyse spectrometric data, free program available from the author at dodogomez@yahoo.fr*.

González-Braojos, S., Sanz, J. J. and Moreno, J. 2017. Decline of a montane Mediterranean pied flycatcher *Ficedula hypoleuca* population in relation to climate. -*Journal of Avian Biology,* 48: 1383-1393.

González-Braojos, S., Vela, A. I., Ruiz-De-Castañeda, R., Briones, V. and Moreno, J. 2012. Age-related changes in abundance of enterococci and Enterobacteriaceae in Pied Flycatcher (*Ficedula hypoleuca*) nestlings and their association with growth. -*Journal of Ornithology,* 153: 181-188.

González, G., Sorci, G., Smith, L. C. and De Lope, F. 2001. Testosterone and sexual signalling in male house sparrows (*Passer domesticus*). -*Behavioral Ecology and Sociobiology,* 50: 557-562.

Gow, E. A., Wiebe, K. L. and Musgrove, A. 2015. Nest sanitation in response to short- and long-term changes of brood size: males clean more in a sex-role-reversed species. -*Animal Behaviour,* 104: 137-143.

Grimaldi, D. A. 1997. *The bird flies, genus Carnus : species revision, generic relationships, and a fossil Meoneura in amber (Diptera, Carnidae),* New York, NY., American Museum of Natural History.

Guallar, S. and Figuerola, J. 2016. Factors influencing the evolution of moult in the non-breeding season: insights from the family Motacillidae. -*Biological Journal of the Linnean Society,* 118: 774-785.

Guigueno, M. F. and Sealy, S. G. 2012. Nest sanitation in passerine birds: implications for egg rejection in hosts of brood parasites. -*Journal of Ornithology,* 153: 35-52.

Gunderson, A. R. 2008. Feather degrading bacteria: a new frontier in avian and host-parasite research? -*Auk,* 125: 972-979.

Gunderson, A. R., Frame, A. M., Swaddle, J. P. and Forsyth, M. H. 2008. Resistance of melanized feathers to bacterial degradation: is it really so black and white? -*Journal of Avian Biology,* 39: 539-545.

Gwinner, H. and Berger, S. 2005. European starlings: nestling condition, parasites and green nest material during the breeding season. -*Journal of Ornithology,* 146: 365-371.

Hamilton, W. D. 1990. Mate choice near or far. -*American Zoologist,* 30: 341-352.

Hamilton, W. D. and Zuk, M. 1982. Heritable true fitness and bright birds: A role for parasites. -*Science,* 218: 384-387.

Hanson, T. 2011. *Feathers: the evolution of a natural miracle,* New York, Basic Books.

Hart, B. 1997. Behavioural defence. -*Host-parasite evolution: general principles and avian models. Oxford University Press, Oxford*: 59-77.

Haussmann, M. F. and Vleck, C. M. 2002. Telomere length provides a new technique for aging animals. -*Oecologia,* 130: 325-328.

Haussmann, M. F., Winkler, D. W., O'reilly, K. M., Huntington, C. E., Nisbet, I. C. T. and Vleck, C. M. 2003. Telomeres shorten more slowly in long-lived birds and mammals than in short-lived ones. -*Proceedings of the Royal Society B-Biological Sciences,* 270: 1387-1392.

Hebets, E. A. and Papaj, D. R. 2005. Complex signal function: developing a framework of testable hypotheses. -*Behavioral Ecology and Sociobiology,* 57: 197-214.

Herborn, K. A., Heidinger, B. J., Boner, W., Noguera, J. C., Adam, A., Daunt, F. and Monaghan, P. 2014. Stress exposure in early post-natal life reduces telomere length: an experimental demonstration in a long-lived seabird. -*Proceedings of the Royal Society B-Biological Sciences,* 281: 10.1098/rspb.2013.3151.

Herrick, F. H. 1900. Care of nest and young. -*The Auk,* 17: 100-103.

Hill, G. E. 2000. Energetic constraints on expression of carotenoid-based plumage coloration. -*Journal of Avian Biology,* 31: 559-566.

Hill, G. E., Hill, G. E. and Mcgraw, K. J. 2006a. *Bird coloration: function and evolution*, Harvard University Press.

Hill, G. E., Hill, G. E. and Mcgraw, K. J. 2006b. *Bird coloration: mechanisms and measurements*, Harvard University Press.

Hill, G. E. and Montgomerie, R. 1994. Plumage color signals nutritional condition in the house finch. -*Proceedings of the Royal Society B-Biological Sciences,* 258: 47-52.

Hiraldo, F. and Herrera, C. M. 1974. Dimorfismo sexual y diferenciación de edades en *Sturnus unicolor* Temm. -*Doñana, Acta Vert,* 1: 149-170.

Hoi, H., Darolova, A., Kristofik, J. and Hoi, C. 2018. The effect of the ectoparasite *Carnus hemapterus* on immune defence, condition, and health of nestling European Bee-eaters. -*Journal of Ornithology,* 159: 291-302.

Hoi, H., Kristofik, J., Darolova, A. and Hoi, C. 2010. Are parasite intensity and related costs of the milichiid fly *Carnus hemapterus* related to host sociality? -*Journal of Ornithology,* 151: 907-913.

Horrocks, N. P. C., Matson, K. D., Shobrak, M., Tinbergen, J. M. and Tieleman, B. I. 2012. Seasonal patterns in immune indices reflect microbial loads on birds but not microbes in the wider environment. -*Ecosphere,* 3.

Ibáñez-Álamo, J. D., Rubio, E. and Soler, J. J. 2017. Evolution of nestling faeces removal in avian phylogeny. -*Animal Behaviour,* 124: 1-5.

Ibáñez-Álamo, J. D., Ruiz-Raya, F., Rodríguez, L. and Soler, M. 2016. Fecal sacs attract insects to the nest and provoke an activation of the immune system of nestlings. -*Frontiers in Zoology,* 13: 9.

Ibáñez-Álamo, J. D., Ruiz-Rodríguez, M. and Soler, J. J. 2014. The mucous covering of fecal sacs prevents birds from infection with enteric bacteria. -*Journal of Avian Biology,* 45: 354-358.

Iverson, E. N. K. and Karubian, J. 2017. The role of bare parts in avian signaling. -*Auk,* 134: 587-611.

Jacob, J. and Ziswiler, V. 1982. The uropygial gland. In: Farner, D. S., King, J. R. and Parkes, K. C. (eds.) *Avian Biology.* Academic Press, London, pp. 359-362.

Jacob, S., Immer, A., Leclaire, S., Parthuisot, N., Ducamp, C., Espinasse, G. and Heeb, P. 2014. Uropygial gland size and composition varies according to experimentally modified microbiome in Great tits. -*BMC Evolutionary Biology,* 14.

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

Jacob, S., Salle, L., Zinger, L., Chaine, A. S., Ducamp, C., Boutault, L., Russell, A. F. and Heeb, P. 2018. Chemical regulation of body feather microbiota in a wild bird. -*Molecular Ecology,* 27: 1727-1738.

Johnson, S. and Candolin, U. 2017. Predation cost of a sexual signal in the threespine stickleback. -*Behavioral Ecology,* 28: 1160-1165.

Johnstone, R. A. 1996. Multiple displays in animal communication: 'Backup signals' and 'multiple messages'. -*Philosophical Transactions of the Royal Society B-Biological Sciences,* 351: 329-338.

Jones, A. G. and Ratterman, N. L. 2009. Mate choice and sexual selection: What have we learned since Darwin? -*Proceedings of the National Academy of Sciences of the United States of America,* 106: 10001-10008.

Jovani, R. and Rohwer, S. 2017. Fault bars in bird feathers: mechanisms, and ecological and evolutionary causes and consequences. -*Biological Reviews,* 92: 1113-1127.

Kattan, G. H. 1997. Shiny cowbirds follow the shotgun strategy of brood parasitism. -*Animal Behaviour,* 53: 647-654.

Kim, S. Y. and Velando, A. 2015. Antioxidants safeguard telomeres in bold chicks. -*Biology Letters,* 11.

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

Kotrschal, A., Ilmonen, P. and Penn, D. J. 2007. Stress impacts telomere dynamics. -*Biology Letters,* 3: 128-130.

Lang, J. D., Straight, C. A. and Gowaty, P. A. 2002. Observations of fecal sac disposal by Eastern Bluebirds. -*The Condor,* 104: 205-207.

Leclaire, S., Jacob, S., Greene, L. K., Dubay, G. R. and Drea, C. M. 2017. Social odours covary with bacterial community in the anal secretions of wild meerkats. -*Scientific Reports,* 7: 13.

Lezana, L., Miranda, R., Campos, F. and Peris, S. J. 2000. Sex differentiation in the spotless starling (*Sturnus unicolor*, Temminck 1820). -*Belgian Journal of Zoology,* 130: 139-142.

Lombardo, M. P., Thorpe, P. A., Cichewicz, R., Henshaw, M., Millard, C., Steen, C. and Zeller, T. K. 1996. Communities of cloacal bacteria in Tree Swallow families. -*Condor,* 98: 167-172.

Lopez-Idiaquez, D., Vergara, P., Fargallo, J. A. and Martinez-Padilla, J. 2016. Female plumage coloration signals status to conspecifics. -*Animal Behaviour,* 121: 101-106.

López-Rull, I., Celis, P. and Gil, D. 2007a. Egg colour covaries with female expression of a male ornament in the spotless starling (*Sturnus unicolor*). -*Ethology,* 113: 926-933.

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

Lyon, B. E. and Eadie, J. M. 2017. Why do birds lay eggs in conspecifcs' nests? In: Soler M (ed) *Avian brood parasitism: behavior, ecology, evolution and coevolution*. Springer, Cham, pp 105–123.

Macías-García, C. and Ramírez, E. 2005. Evidence that sensory traps can evolve into honest signals. -*Nature,* 434: 501-505.

Martín-Vivaldi, M., Peña, A., Peralta-Sánchez, J. M., Sánchez, L., Ananou, S., Ruiz-Rodríguez, M. and Soler, J. J. 2010. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. -*Proceedings of the Royal Society of London B, Biological Sciences,* 277: 123-130.

Martín-Vivaldi, M., Ruiz-Rodríguez, M., Mendez, M. and Soler, J. J. 2006. Relative importance of factors affecting nestling immune response differs between junior and senior nestlings within broods of hoopoes *Upupa epops*. -*Journal of Avian Biology,* 37: 467-476.

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

Martín-Vivaldi, M., Soler, J. J., Peralta-Sánchez, J. M., Arco, L., Martín-Platero, A. M., Martínez-Bueno, M., Ruiz-Rodríguez, M. and Valdivia, E. 2014. Special structures of hoopoe eggshells enhance the adhesion of symbiont-carrying uropygial secretion that increase hatching success. - *Journal of Animal Ecology,* 83: 1289-301.

Martín, J. and López, P. 2009. Multiple color signals may reveal multiple messages in male Schreiber's green lizards, Lacerta schreiberi. -*Behavioral Ecology and Sociobiology,* 63: 1743-1755.

Mason, N. A., Shultz, A. J. and Burns, K. J. 2014. Elaborate visual and acoustic signals evolve independently in a large, phenotypically diverse radiation of songbirds. -*Proceedings of the Royal Society B-Biological Sciences,* 281: 9.

Matson, K. D., Ricklefs, R. E. and Klasing, K. C. 2005. A hemolysis-hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. -*Developmental and Comparative Immunology,* 29: 275-286.

Maynard-Smith, J. and Harper, D. 2003. *Animal Signals,* Oxford, Oxford University Press.

Mcfall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Loso, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E.,

Rawls, J. F., Reid, A., Ruby, E. G., Rumpho, M., Sanders, J. G., Tautz, D. and Wernegreen, J. J. 2013. Animals in a bacterial world, a new imperative for the life sciences. -*Proceedings of the National Academy of Sciences of the USA,* 110: 3229-3236.

Mcgraw, K. J. and Hill, G. E. 2000. Carotenoid-based ornamentation and status signaling in the house finch. -*Behavioral Ecology,* 11: 520-527.

Mcgraw, K. J., Medina-Jerez, W. and Adams, H. 2007. Carotenoid-based plumage coloration and aggression during molt in male house finches. -*Behaviour,* 144: 165-178.

Mennerat, A., Mirleau, P., Blondel, J., Perret, P., Lambrechts, M. M. and Heeb, P. 2009a. Aromatic plants in nests of the blue tit *Cyanistes caeruleus* protect chicks from bacteria. -*Oecologia,* 161: 849-55.

Mennerat, A., Perret, P., Bourgault, P., Blondel, J., Gimenez, O., Thomas, D. W., Heeb, P. and Lambrechts, M. M. 2009b. Aromatic plants in nests of blue tits: positive effects on nestlings. -*Animal Behaviour,* 77: 569-574.

Merino, S., Møller, A. P. and De Lope, F. 2000. Seasonal changes in cell-mediated immunocompetence and mass gain in nestling barn swallows: a parasite-mediated effect? -*Oikos,* 90: 327-332.

Merrill, L., Naylor, M. F. and Grindstaff, J. L. 2016. Imperfect past and present progressive: beak color reflects early-life and adult exposure to antigen. -*Behavioral Ecology,* 27: 1320-1330.

Mihailova, M., Berg, M. L., Buchanan, K. L. and Bennett, A. T. D. 2018. Olfactory eavesdropping: The odor of feathers is detectable to mammalian predators and competitors. -*Ethology,* 124: 14-24.

Mills, T. K., Lombardo, M. P. and Thorpe, P. A. 1999. Microbial colonization of the cloacae of nestling tree swallows. -*Auk,* 116: 947-956.

Møller, A. P. 1994. *Sexual selection and the barn swallow,* Oxford, UK, Oxford University Press.

Møller, A. P., Barbosa, A., Cuervo, J. J., De Lope, F., Merino, S. and Saino, N. 1998. Sexual selection and tail streamers in the barn swallow. -*Proceedings of the Royal Society of London B, Biological Sciences,* 265: 409-414.

Møller, A. P., Biard, C., Blount, J. D., Houston, D. C., Ninni, P., Saino, N. and Surai, P. F. 2000. Carotenoid-dependent signals: Indicators of foraging efficiency, immunocompetence or detoxification ability? -*Avian and Poultry Biology Reviews,* 11: 137-159.

Møller, A. P., Christe, P. and Lux, E. 1999. Parasitism, host immune function, and sexual selection. -*Quarterly Review of Biology,* 74: 3-20.

Møller, A. P., Czirjak, G. A. and Heeb, P. 2009. Feather micro-organisms and uropygial antimicrobial defences in a colonial passerine bird. -*Functional Ecology,* 23: 1097-1102.

Møller, A. P. and Jennions, M. D. 2001. How important are direct fitness benefits of sexual selection? -*Naturwissenschaften,* 88: 401-415.

Møller, A. P., Peralta-Sánchez, J. M., Nielsen, J. T., López-Hernández, E. and Soler, J. J. 2012. Goshawk prey have more bacteria than non-prey. -*Journal of Animal Ecology,* 81: 403-410.

Møller, A. P. and Pomiankowski, A. 1993. Why have birds got multiple sexual ornaments. -*Behavioral Ecology and Sociobiology,* 32: 167-176.

Monaghan, P. and Haussmann, M. F. 2006. Do telomere dynamics link lifestyle and lifespan? -*Trends in Ecology & Evolution,* 21: 47-53.

Morales, J. and Velando, A. 2018. Coloration of chicks modulates costly interactions among family members. -*Behavioral Ecology,* 29: 894-903.

Moreno-Rueda, G. 2017. Preen oil and bird fitness: a critical review of the evidence. -*Biological Reviews*, 92: 2131-2143.

Naguib, M. 2005. Singing interactions in songbirds: implications for social relations and territorial settlement. -*Animal communication networks*: 300-319.

Narushin VG. 2005. Production, modeling, and education: Egg geometry calculation using the measurements of length and breadth. -*Poultry Science*, 84: 482-4

Navarro, C., Pérez-Contreras, T., Avilés, J. M., Mcgraw, K. J. and Soler, J. J. 2010. Beak colour reflects circulating carotenoid and vitamin A levels in spotless starlings (*Sturnus unicolor*). -*Behavioral Ecology and Sociobiology,* 64: 1057-1067.

Otte, D. 1974. Effects and functions in the evolution of signaling systems. -*Annual Review of Ecology and Systematics,* 5: 385-417.

Paasivaara, A., Rutila, J., Pöysä, H. and Runko, P. 2010. Do parasitic common goldeneye *Bucephala clangula* females choose nests on the basis of host traits or nest site traits? -*Journal of Avian Biology,* 41: 662-671.

Pardal, S., Alves, J. A., Mota, P. G. and Ramos, J. A. 2018. Dressed to impress: breeding plumage as a reliable signal of innate immunity. -*Journal of Avian Biology,* 49: 13.

Parejo, D., Avilés, J. M. and Rodríguez, J. 2012. Alarm calls modulate the spatial structure of a breeding owl community. -*Proceedings of the Royal Society B: Biological Sciences,* 279: 2135-2141.

Parolini, M., Romano, A., Costanzo, A., Khoriauli, L., Santagostino, M., Nergadze, S. G., Canova, L., Rubolini, D., Giulotto, E. and Saino, N. 2017. Telomere length is reflected by plumage coloration and predicts seasonal reproductive success in the barn swallow. -*Molecular Ecology,* 26: 6100-6109.

Pauliny, A., Wagner, R. H., Augustin, J., Szép, T. and Blomqvist, D. 2006. Age-independent telomere length predicts fitness in two bird Species. -*Molecular Ecology,* 15: 1681-1687.

Peralta-Sánchez, J. M., Martín-Platero, A. M., Wegener-Parfrey, L., Martínez-Bueno, M., Rodríguez-Ruano, S., Navas-Molina, J. A., Vazquez-Baeza, Y., Martín-Galvez, D., Martín-Vivaldi, M., Ibánez-Álamo, J.

D., Knight, R. and Soler, J. J. 2018. Bacterial density rather than diversity correlates with hatching success across different avian species. -*FEMS Microbiology Ecology,* 94: 13.

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

Peralta-Sánchez, J. M., Møller, A. P., Martín-Platero, A. M. and Soler, J. J. 2010. Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: an experimental study. -*Functional Ecology,* 24: 426-433.

Pérez-Contreras, T., Soler, J. J. and Soler, M. 2008. Needle asymmetry, pine vigour and pine selection by the processionary moth *Thaumetopoea pityocampa*. -*Acta Oecologica,* 33: 213-221.

Perrier, C., De Lope, F., Møller, A. P. and Ninni, P. 2002. Structural coloration and sexual selection in the barn swallow *Hirundo rustica*. -*Behavioral Ecology,* 13: 728-736.

Petit, K. E., Petit, L. J. and Petit, D. R. 1989. Fecal sac removal: Do the pattern and distance of dispersal affect the chance of nest predation? -*The Condor,* 91: 479-482.

Pinowski, J., Barkowska, M., Kruszewicz, A. H. and Kruszewicz, A. G. 1994. The causes of the mortality of eggs and nestlings of *Passer* Spp. -*Journal of Biosciences,* 19: 441-451.

Playfair, J. and Bancroft, G. 2004. *Infection and immunity,* Oxford, Oxford University Press.

Polin, S., Le Gallic, J. F., Simon, J. C., Tsuchida, T. and Outreman, Y. 2015. Conditional reduction of predation risk associated with a facultative symbiont in an insect. -*PLoS One,* 10: e0143728.

Potti, J., Moreno, J., Yorio, P., Briones, V., García-Borboroglu, P., Villar, S. and Ballesteros, C. 2002. Bacteria divert resources from growth for magellanic penguin chicks. -*Ecology Letters,* 5: 709-714.

Quan, R. C., Li, H., Wang, B. and Goodale, E. 2015. The relationship between defecation and feeding in nestling birds: observational and experimental evidence. -*Frontiers in Zoology,* 12: 7.

R Core Development Team. 2016. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rayner, J. M. V. 1988. Form and function in avian flight. In: JOHNSTON, R. F. (ed.) *Current Ornithology.* Springer US, Boston, MA, pp. 1-66.

Rohwer, S. 1975. Social significance of avian winter plumage variability. -*Evolution,* 29: 593-610.

Roulin, A. 2016. Condition-dependence, pleiotropy and the handicap principle of sexual selection in melanin-based colouration. -*Biological Reviews,* 91: 328-348.

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

Ruiz-De-Castañeda, R., Burtt Jr, E., González--Braojos, S. and Moreno, J. 2012. Bacterial degradability of an intrafeather unmelanized ornament: a role for feather-degrading bacteria in sexual selection? -*Biological Journal of the Linnean Society,* 105: 409-419.

Ruiz-Rodríguez, M., Møller, A. P., Mousseau, T. A. and Soler, J. J. 2016. Defenses against keratinolytic bacteria in birds living in radioactively contaminated areas. -*The Science of Nature,* 103: 1-8.

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

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

Schull, Q., Dobson, F. S., Stier, A., Robin, J. P., Bize, P. and Viblanc, V. A. 2016. Beak color dynamically signals changes in fasting status and parasite loads in king penguins. -*Behavioral Ecology,* 27: 1684-1693.

Schulte, B. A., Goodwin, T. E. and Ferkin, M. H. 2016. *Chemical Signals in Vertebrates 13*, Springer.

Searcy, W. A. and Nowicki, S. 2005. *The evolution of animal communication. Reliability and deception in signaling systems,* Princeton (NJ), Princeton University Press.

Senar, J. 1990. Agonistic communication in social species: what is communicated? -*Behaviour,* 112: 270-283.

Senar, J. 1999. Plumage coloration as a signal of social status. *Proceedings of the International Ornithological Congress, 1999.* BirdLife South Africa, pp. 1669-1686.

Senar, J. 2006. Color displays as intrasexual signal of agression and dominance In: HILL, G. E. and MCGRAW, K. J. (eds.) *Bird coloration: Function and evolution.* Harvard University Press, Cambridge, Massachusetts, pp. 87-136.

Senar, J. C. and Camerino, M. 1998. Status signalling and the ability to recognize dominants: an experiment with siskins (*Carduelis spinus)*. -*Proceedings of the Royal Society of London B, Biological Sciences,* 265: 1515-1520.

Senar, J. C., Polo, V., Uribe, F. and Camerino, M. 2000. Status signalling, metabolic rate and body mass in the siskin: the cost of being a subordinate. -*Animal Behaviour,* 59: 103-110.

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

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

Shawkey, M. D., Pillai, S. R., Hill, G. E., Siefferman, L. M. and Roberts, S. R. 2007. Bacteria as an agent for change in structural plumage color: Correlational and experimental evidence. -*American Naturalist,* 169: S112-S121.

Sheldon, B. C. 2000. Differential allocation: tests, mechanisms and implications. -*Trends in Ecology & Evolution,* 15: 397-402.

Simons, M. J. P. and Verhulst, S. 2011. Zebra finch females prefer males with redder bills independent of song rate-a meta-analysis. -*Behavioral Ecology,* 22: 755-762.

Smith, J. W., Benkman, C. W. and Coffey, K. 1999. The use and misuse of public information by foraging red crossbills. -*Behavioral Ecology,* 10: 54-62.

Soler, J. J., Martín-Vivaldi, M., Peralta-Sánchez, J. M. and Ruiz-Rodríguez, M. 2010. Antibiotic-producing bacteria as a possible defence of birds against pathogenic microorganisms. -*Open Ornithology Journal,* 3: 93-100.

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

Soler, J. J., Møller, A. P., Soler, M. and Martínez, J. G. 1999. Interactions between a brood parasite and its host in relation to parasitism and immune defence. -*Evolutionary Ecology Research,* 1: 189-210.

Soler, J. J., Navarro, C., Pérez-Contreras, T., Avilés, J. M. and Cuervo, J. J. 2008b. Sexually selected egg coloration in spotless starlings. -*American Naturalist,* 171: 183-194.

Soler, J. J., Peralta-Sánchez, J. M., Flensted-Jensen, E., Martín-Platero, A. M. and Møller, A. P. 2011. Innate humoural immunity is related to eggshell bacterial load of European birds: a comparative analysis. -*Naturwissenschaften,* 98: 807-813.

Soler, J. J., Peralta-Sánchez, J. M., Martín-Platero, A. M., Martín-Vivaldi, M., Martínez-Bueno, M. and Møller, A. P. 2012a. The evolution of size of the uropygial gland: mutualistic feather mites and uropygial secretion reduce bacterial loads of eggshells and hatching failures of European birds. -*Journal of Evolutionary Biology,* 25: 1779-1791.

Soler, J. J., Peralta-Sánchez, J. M., Martín-Vivaldi, M., Martín-Platero, A. M., Flensted-Jensen, E. and Møller, A. P. 2012b. Cognitive skills and

bacterial load: comparative evidence of costs of cognitive proficiency in birds. -*Naturwissenschaften,* 99: 111-122.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martin-Vivaldi, M., Martinez-De La Puente, J., Ruiz-Rodriguez, M. and Tomas, G. 2017. Telomere length and dynamics of spotless starling nestlings depend on nest-building materials used by parents. -*Animal Behaviour,* 126: 89-100.

Soler, J. J., Ruiz-Castellano, C., Figuerola, J., Martinez-De La Puente, J., Ruiz-Rodriguez, M. and Tomas, G. 2018. Egg colouration predicts brood size, telomere length and body condition of spotless starling fledglings. -*Journal of Avian Biology,* 49: 12.

Soler, J. J., Ruiz Castellano, C., Martínez-De La Puente, J., Tomás, G., Ruiz-Rodríguez, M. and Figuerola, J. 2015a. Telomere dynamics in parasitic great spotted cuckoos and their magpie hosts. -*Journal of Evolutionary Biology,* 28: 1610-1617.

Soler M (ed.). 2017 *Avian brood parasitism: behaviour, ecology, evolution and coevolution.* Berlin, Germany: Springer International Publishing

Soler, M., Perez-Contreras, T. and Soler, J. J. 2015b. Synchronization of laying by great spotted cuckoos and recognition ability of magpies. -*Journal of Avian Biology,* 46: 608-615.

Sorci, G., Soler, J. J. and Møller, A. P. 1997. Reduced immunocompetence of nestlings in replacement clutches of the european magpie (*Pica pica*). -*Proceedings of the Royal Society of London B, Biological Sciences,* 264: 1593-1598.

Stettenheim, P. R. 2000. The integumentary morphology of modern birds — An Overview. -*American Zoologist,* 40: 461-477.

Stoddard, M. C. and Prum, R. O. 2011. How colorful are birds? Evolution of the avian plumage color gamut. -*Behavioral Ecology,* 22: 1042-1052.

Stuart-Fox, D. M., Moussalli, A., Marshall, N. J. and Owens, I. P. F. 2003. Conspicuous males suffer higher predation risk: visual modelling and experimental evidence from lizards. -*Animal Behaviour,* 66: 541-550.

Suram, A., Kaplunov, J., Patel, P. L., Ruan, H. H., Cerutti, A., Boccardi, V., Fumagalli, M., Di Micco, R., Mirani, N., Gurung, R. L., Hande, M. P., Di Fagagna, F. D. and Herbig, U. 2012. Oncogene-induced telomere dysfunction enforces cellular senescence in human cancer precursor lesions. -*Embo Journal,* 31: 2839-2851.

Swaddle, J. P., Witter, M. S., Cuthill, I. C., Budden, A. and Mccowen, P. 1996. Plumage condition affects flight performance in common starlings: Implications for developmental homeostasis, abrasion and moult. -*Journal of Avian Biology,* 27: 103-111.

Taff, C. C. and Freeman-Gallant, C. R. 2017. Sexual signals reflect telomere dynamics in a wild bird. -*Ecology and Evolution,* 7: 3436-3442.

Templeton, C. N. and Greene, E. 2007. Nuthatches eavesdrop on variations in heterospecific chickadee mobbing alarm calls. -*Proceedings of the*

National Academy of Sciences of the United States of America, 104: 5479-5482.

Theis, K. R., Venkataraman, A., Dycus, J. A., Koonter, K. D., Schmitt-Matzen, E. N., Wagner, A. P., Holekamp, K. E. and Schmidt, T. M. 2013. Symbiotic bacteria appear to mediate hyena social odors. -*Proceedings of the National Academy of Sciences of the United States of America,* 110: 19832-19837.

Tibbetts, E. A. and Dale, J. 2004. A socially enforced signal of quality in a paper wasp. -*Nature,* 432: 218-222.

Tomás, G., Martín-Gálvez, D., Ruiz-Castellano, C., Ruiz-Rodríguez, M., Peralta-Sánchez, J. M., Martín-Vivaldi, M. and Soler, J. J. 2018. Ectoparasite activity during incubation increases microbial growth on avian eggs. -*Microbial Ecology,* 76: 555-564.

Tomás, G., Martín-Gálvez, D., Ruiz-Rodríguez, M. and Soler, J. 2017. Intraspecific avian brood parasites avoid host nests infested by ectoparasites. -*Journal of Ornithology,* 158: 561-567.

Tomás, G. and Soler, J. J. 2016. Begging and ectoparasite attraction. -*Animal Behaviour,* 113: 93-98.

Torres, R. and Velando, A. 2005. Male preference for female foot colour in the socially monogamous blue-footed booby, Sula nebouxii. -*Animal Behaviour,* 69: 59-65.

Valone, T. J. 1989. Group foraging, public information, and patch estimation. -*Oikos,* 56: 357-363.

Valone, T. J. and Templeton, J. J. 2002. Public information for the assessment of quality: a widespread social phenomenon. -*Philosophical Transactions of the Royal Society of London Series B-Biological Sciences,* 357: 1549-1557.

Van Lieshout, S. H., Bretman, A., Newman, C., Buesching, C. D., Macdonald, D. W. and Dugdale, H. L. 2019. Individual variation in early-life telomere length and survival in a wild mammal. -*Molecular ecology*.

Veiga, J. and Polo, P. 2016. Estornino Negro–Sturnus unicolor. In: *Enciclopedia Virtual de los Vertebrados Españoles*. (eds. A. Salvador and M. B. Morales). Madrid: Museo Nacional de Ciencias Naturales.

Velando, A., Beamonte-Barrientos, R. and Torres, R. 2006. Pigment-based skin colour in the blue-footed booby: an honest signal of current condition used by females to adjust reproductive investment. -*Oecologia,* 149: 535-542.

Velando, A., Torres, R. and Espinosa, I. 2005. Male coloration and chick condition in blue-footed booby: a cross-fostering experiment. -*Behavioral Ecology and Sociobiology,* 58: 175-180.

Verea, C., Vitelli-Flores, J., Isturiz, T., Rodriguez-Lemoine, V. and Bosque, C. 2017. The effect of uropygial gland secretions of Spectacled Thrushes (Turdus nudigenis) on feather degradation and bacterial growth in vitro. -*Journal of Ornithology,* 158: 1035-1043.

Verhulst, N. O., Qiu, Y. T., Beijleveld, H., Maliepaard, C., Knights, D., Schulz, S., Berg-Lyons, D., Lauber, C. L., Verduijn, W., Haasnoot, G. W., Mumm, R., Bouwmeester, H. J., Claas, F. H. J., Dicke, M., Van Loon, J. J. A., Takken, W., Knight, R. and Smallegange, R. C. 2011. Composition of human skin microbiota affects attractiveness to malaria mosquitoes. -*Plos One,* 6.

Webb, W. H., Brunton, D. H., Aguirre, J. D., Thomas, D. B., Valcu, M. and Dale, J. 2016. Female song occurs in songbirds with more elaborate female coloration and reduced sexual dichromatism. -*Frontiers in Ecology and Evolution,* 4: 22.

Webster, M. S., Ligon, R. A. and Leighton, G. M. 2018. Social costs are an underappreciated force for honest signalling in animal aggregations. -*Animal Behaviour,* 143: 167-176.

Westneat, D. F. and Rambo, T. B. 2000. Copulation exposes female Red-winged Blackbirds to bacteria in male semen. -*Journal of Avian Biology,* 31: 1-7.

Wiley, J. W. 1988. Host selection by the shiny cowbird. -*Condor,* 90: 289-303.

Zahavi, A. 1975. Mate selection--A selection for a handicap. -*Journal of Theoretical Biology,* 53: 205-214.

Zahavi, A. 1977. Cost of honesty - (further remarks on handicap principle). -*Journal of Theoretical Biology,* 67: 603-605.

Zuk, M. 1992. The role of parasites in sexual selection: current evidence and future directions. -*Advances in the Study of Behavior,* 21: 39-68.

Zuk, M. and Johnsen, T. S. 2000. Social environment and immunity in male red jungle fowl. - *Behavioral Ecology,* 11: 146-153.

AGRADECIMIENTOS

Bueno, pues llego el momento de los agradecimientos. Ahora toca echar la vista atrás y recordar a todos los que de una manera u otra forman parte de esta tesis y a los que nunca podré agradecer lo suficiente todo el apoyo (logístico y anímico) que me han brindado. También quisiera disculparme por adelantado si se me olvida alguien, han sido unos años tan intensos que al intentar rememorar todo de golpe seguro que se me pasa alguien importantísimo que se merecería aparecer en este apartado, espero que me disculpeis.

Es de justicia que el que abra esta sección sea Juan, ya que sin él nada de esto hubiera sido posible. ¡Gracias por todo Juan! Tu paciencia (la de veces que has tenido que sacarme de los árboles para permitirme ver el bosque), tu disponibilidad para todo (desde ayudarme en el campo hasta resolverme cualquier duda), tus consejos y tantas otras cosas… Siempre te agradeceré el que me dieras esta oportunidad. De la misma manera quiero agradecer a Magda que aceptara dirigirme también la tesis. Te agradezco de verdad todo lo que has hecho por mi durante todos estos años, desde los consejos siempre certeros, tu valiosa ayuda en el campo y en el laboratorio y hasta asegurarte que en mis estancias en Francia me sintiera como en casa. Muchas gracias a los dos, ¡no podría haber tenido unos mejores directores!

También quiero agradecer a László Zsolt Garamszegi, al que considero mi padrino en el mundillo científico, la oportunidad que me dió de trabajar con su grupo e iniciarme en el campo en la época del máster de etología. Siempre atento y dispuesto a echarme una mano. ¡Tenemos pendientes unas cervezas!

Por supuesto también me vienen a la cabeza y de manera muy especial mis chumberas y chumberos: Cristina (mi compañera estornina y la que tantas veces ha venido a mi rescate al campo contra las legiones de pollos), Bea, Loli, Mónica, Juan, Ari, Pilu, Iñaqui, Raúl, Gustavo, Ángel, María Jesús, Mónica Cantabria, Teresa, Jorge, Carme, Jorge (portugués), Angela, Paco y tantos otros/as. Habéis sido mi familia allí en Almería y no sé qué hubiera hecho sin vosotros/as y todas esas comidas, cafés, disfraces, películas, partidos de basket, rutas por el campo (de las que en algunos casos sobrevivimos de milagro), etc. y sobre todo risas, muchas risas de las que dan la vida, ¡sois los mejores!

A todo el personal de la EEZA que hacia las cosas más fáciles en tu día a día. A Estefanía por todo lo que me ha ayudado en el laboratorio (¿qué haríamos sin ti?), a Pilar siempre dispuesta y eficiente, a Olga por ayudarme con tantas cosas en el laboratorio, al resto de personal técnico, investigadores, administración, mantenimiento, limpieza, etc… Ha sido un placer trabajar con vosotros.

A Carmen Zamora por aguantar pacientemente todas mis dudas y papeleos, a Paqui por su tutoría y su ayuda en las prácticas (¡muchas gracias por ayudarme a ajustar mis horarios!) y a Manuel Martin-Vivaldi por sus consejos y correcciones. Al profesorado y personal del Departamento de Zoología de la UGR por toda la ayuda en el ámbito docente y académico y por darme la oportunidad de dar las prácticas que tanto he disfrutado. También gracias al Departamento de Microbiología y en especial a Pirri y Platero por permitirme trabajar en los laboratorios del departamento y tutorizarme.

A Silvia, con la que empecé a la par esta aventura y con la que he ido hombro con hombro en mil y una ocasiones (campo, laboratorio, docencia, congresos, ¡que de cosas que hemos pasado!). A Natalia, por las grandes conversaciones que teníamos en la época de campo. A Laura y Juanma por sacar siempre un hueco para un café cuando iba de visita a Granada. A Virginia y Álvaro por acogerme siempre en su casa (¡hasta la friolera de casi un mes en una ocasión!) y hacerme sentir como en la mía. También a Dani por la portada tan chula que podéis ver en esta tesis.

A los miembros de la unidad de "Ecology Systematics Evolution" de la universidad de Paris-Sud Purificación y Anders, gracias por darme dos veces la oportunidad de ir a Francia y aprender tanto, siendo tan atentos y ayudándome en todo lo que necesitaba. A Raquel, Guifré, Rafa, Irene, Laura y Adriana por hacerme sentir tan a gusto en París. A Jordi Figuerola por dejarme trabajar con su grupo en la EBD, me encanto compartir trabajo y experiencias con gente tan estupenda como Josué, Nene, Alazne, Rafa, Martina o Fran.

A mis padres Manolo y Matilde, a mis hermanos y hermana Blanca, Tomas y José Luis y a mi abuela Matilde, por apoyarme en todo contra viento y marea, siempre puedo contar con vosotros, os quiero! A mi familia Sanluqueña: mis suegros Manolo (¡gracias por la revisión ortografica!) y Loli y mi cuñado Manolo por estar siempre ahí, sobre todo en los momentos más difíciles. También a mis amigos de toda la vida Marco, Donato, Fran, Carlos Avila y Carlos Cruz, gracias por las, aunque escasas por las circustancias de cada uno, revigorizantes reuniones que seguimos haciendo después de tantos años (y las que nos quedan!).

Y, por último, pero por supuesto no menos importantes (más bien todo lo contrario) gracias al amor de mi vida y a nuestro pequeño: Mónica y Manuel. Gracias a ti Mónica por toda la paciencia y sacrificio que has tenido durante todos estos años. Sin tu apoyo incondicional no hubiera llegado hasta aquí, gran parte de esta tesis es también tuya. Y gracias a mi pequeño Manuel, el mejor de mis capítulos, por toda la alegría que nos das (y algún que otro dolor de cabeza, jajaja, pero que enseguida se quita al escuchar un "Papá, ven") y por enseñarnos hasta donde se puede querer a otro ser humano.

A todos/as, ¡gracias!

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