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Plumas, plantas y bacterias en nidos de estornino negro (*Sturnus unicolor*) y sus efectos en la reproducción

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Cristina Ruiz Castellano

Estación Experimental de Zonas Áridas

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Memoria presentada por Cristina Ruiz Castellano para optar al Grado de Doctor en Ciencias Biológicas por la Universidad de Granada.

Esta Tesis ha sido dirigida por Juan José Soler Cruz, Profesor de Investigación, y Gustavo Tomás Gutiérrez, investigador contratado Ramón y Cajal, de la Estación Experimental de Zonas Áridas (EEZA-CSIC). Y tutelada por Carmen Zamora Muñoz profesora titular de la Universidad de Granada

Granada, Octubre de 2016

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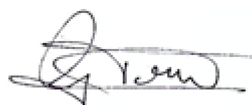

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A los que siempre han estado conmigo

A los que ya no podrán estar

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// Resumen

Los microorganismos patógenos suponen presiones selectivas muy fuertes para las aves durante su estancia en el nido, por lo que éstas han tenido que desarrollar numerosas estrategias defensivas. La utilización de materiales con propiedades antimicrobianas en la construcción de los nidos es una de estas defensas y su estudio constituye el objetivo general de esta Tesis. Uno de los materiales de los nidos más estudiados por sus propiedades antimicrobianas son las plantas verdes aromáticas. Estos materiales se utilizan como parte de la estructura del nido, pero sobre todo como revestimiento del mismo, en contacto directo con huevos y pollos. La actividad antimicrobiana de estas plantas radica en sus compuestos secundarios volátiles que, además, pueden actuar repeliendo o reduciendo el impacto negativo de los ectoparásitos presentes en los nidos. A parte de estos efectos sobre ectoparásitos y microorganismos, también se ha puesto de manifiesto una asociación positiva de la presencia de plantas aromáticas con el crecimiento y el sistema inmunitario en pollos. La mayoría de estos trabajos han sido realizados en pollos de herrerillo (*Cyanistes caeruleus*) y de estornino pinto (*Sturnus vulgaris*), pero poco se sabe del efecto de este material sobre la carga bacteriana de los huevos o en otras especies de aves.

Las plumas son otro de los materiales comúnmente utilizado por las aves para el revestimiento del nido. Éstas también pueden actuar como defensas frente a microorganismos patógenos gracias a las sustancias antimicrobianas producidas por las bacterias capaces de crecer en las plumas. Las bacterias queratinolíticas (i.e., capaces de degradar la queratina, el principal componente de las plumas) son conocidas por su alta capacidad de segregar compuestos antimicrobianos de amplio espectro y que las aves podrían utilizar para reducir la carga parasitaria en sus nidos. Además, sabemos que las bacterias queratinolíticas son capaces de degradar mejor las plumas no pigmentadas debido a la ausencia de pigmentos como la melanina,

con lo que las densidades de bacterias queratinolíticas, y por tanto sus efectos, serán mayores en plumas no pigmentadas. Este efecto antibacteriano de las plumas en los nidos de las aves solo ha sido estudiado con anterioridad en una especie, la golondrina común (*Hirundo rustica*). Los huevos en nidos con plumas no pigmentadas añadidas experimentalmente tuvieron menor carga bacteriana y menor probabilidad de fallo de eclosión que los de nidos con plumas pigmentadas añadidas.

En esta Tesis, nos planteamos estudiar el posible efecto antimicrobiano de las plantas y las plumas de los nidos de estornino negro (*Sturnus unicolor*) durante las etapas de huevos y de pollos. En esta especie, tanto las plumas como las plantas cumplen una función en la comunicación sexual y aquí planteamos que además podrían tener un papel antimicrobiano en los nidos. Para ello manipulamos experimentalmente la composición de plumas y plantas de los nidos de estornino negro durante tres temporadas de reproducción y estimamos el efecto de estos materiales sobre la carga bacteriana (bacterias mesófilas y tres grupos bacterianos que incluyen patógenos para las aves: enterobacterias, estafilococos y enterococos), tanto de la cáscara de los huevos como de la piel de los pollos. Evaluamos asimismo la capacidad antimicrobiana de las colonias bacterianas aisladas de plumas del nido y de la cáscara de los huevos. También estimamos los efectos del experimento sobre diferentes variables relacionadas con el estado de salud de los pollos (peso corporal, éxito de vuelo, sistema inmune y longitud y dinámica de telómeros). Además, utilizamos nidos artificiales con huevos comerciales de codorniz (*Coturnix japonica*) para estudiar el efecto antimicrobiano de estos materiales en los nidos excluyendo la actividad parental (i.e., incubación), ya que esta actividad es capaz de modificar la comunidad bacteriana de los nidos. Finalmente, investigamos si los estorninos muestran alguna preferencia por plumas pigmentadas o no pigmentadas, y por distintas plantas aromáticas o no aromáticas, para construir sus nidos.

La adición de plumas experimentales a los nidos, independientemente de su color, redujo la carga bacteriana tanto de la cáscara de los huevos como de la piel de los pollos. Sin embargo, no encontramos diferencias consistentes entre tratamientos de plumas pigmentadas y plumas no pigmentadas. Durante las fases de huevos y pollos en nidos de estornino, los tratamientos con plumas pigmentadas redujeron la carga bacteriana más que los tratamientos con plumas no pigmentadas. Sin embargo, este efecto dependió del grupo bacteriano (enterococos y bacterias mesófilas en huevos y estafilococos en pollos) y sólo se puso de manifiesto en nidos naturales en uno de los dos años de estudio. Por otra parte, no hubo diferencias entre los tratamientos experimentales de ambos tipos de plumas sobre la carga bacteriana de los huevos de nidos artificiales o en el grupo de bacterias mesófilas de la piel de los pollos. Estos resultados sugieren que el efecto antibacteriano de las plumas y su acción diferencial en función de su pigmentación dependen de características ambientales de los nidos y/o pueden ser moduladas por la actividad parental.

El efecto antibacteriano de las plantas verdes en los nidos no fue tan claro como el de las plumas. No encontramos un efecto de las plantas sobre la carga bacteriana de la cáscara de los huevos o de la piel de los pollos en nidos naturales, pero sí en la cáscara de los huevos de nidos artificiales (i.e., sin incubación). Además, detectamos un efecto de este material en la interacción con el tratamiento de plumas. La prevalencia de estafilococos en la piel de los pollos fue menor en nidos con plantas y plumas añadidas experimentalmente. Estos resultados, por tanto, ponen de manifiesto un efecto antibacteriano de las plantas que depende de la composición de plumas que haya en los nidos.

Además, encontramos que tanto las plumas como las plantas tuvieron un efecto positivo en los pollos en términos de peso corporal, respuesta inmune, longitud y dinámica de los telómeros, y éxito de vuelo de los pollos. Detectamos asimismo relaciones negativas entre la carga bacteriana en la piel

de los pollos y las diferentes variables relacionadas con su estado de salud y calidad fenotípica, lo que sugiere que el efecto de los materiales del nido sobre la salud y calidad de los pollos está mediado por sus efectos sobre la comunidad microbiana.

La hipótesis de una función antimicrobiana de las plumas asume que las bacterias que crecen en ellas tienen capacidades antimicrobianas relativamente altas. Además, la actividad parental en los nidos podría influir en las características y capacidad de segregación de compuestos antimicrobianos de estas bacterias. Por tanto, abordamos la hipótesis de una mayor capacidad antimicrobiana de las bacterias procedentes de plumas comparándola con la de bacterias procedentes de la superficie de los huevos en nidos naturales de estornino y en nidos artificiales (i.e., sin incubación) donde se habían añadido plumas experimentales y huevos de codorniz. Encontramos que la densidad de bacterias queratinolíticas en huevos de nidos naturales fue mayor que en huevos de nidos artificiales. De acuerdo con la hipótesis pusimos de manifiesto que la capacidad antimicrobiana de las colonias aisladas de plumas del nido era mayor que la de las colonias aisladas de la cáscara de los huevos y que, en general, las bacterias procedentes de nidos naturales presentaron mayor actividad antimicrobiana que las de nidos artificiales. Estos resultados sugieren que la actividad de incubación podría favorecer el establecimiento de bacterias queratinolíticas con mayor capacidad antimicrobiana que ayuden a moldear el ambiente bacteriano en los nidos de las aves.

Por último, encontramos que los estorninos prefirieron claramente plumas no pigmentadas para construir sus nidos, tanto antes como durante la puesta de los huevos. Sin embargo, la selección de las plantas varió entre las dos fases reproductivas. Antes de la puesta, los estorninos no mostraron una selección diferencial entre plantas aromáticas y no aromáticas, lo que sugiere una función predominantemente sexual de las plantas verdes en esta fase.

Durante la puesta de los huevos, la selección fue claramente hacia plantas aromáticas, lo que apoya una función de automedicación dadas las conocidas propiedades antibacterianas de estas plantas.

En conjunto, nuestros resultados apoyan una función antimicrobiana de las plumas y las plantas en el nido de las aves, reduciendo la comunidad bacteriana de la cáscara de los huevos y de la piel de los pollos y mejorando el desarrollo de la descendencia. Estos efectos parecen estar sujetos a importantes variaciones, debidas por ejemplo a las condiciones ambientales o a comportamientos parentales como la incubación. Estas propiedades antimicrobianas de las plumas y las plantas pueden ser las que determinen la selección activa de estos materiales por parte de los adultos a la hora de construir sus nidos.

Summary

Selective pressures imposed to birds by pathogenic microorganisms are very strong during the nesting period, so birds have evolved numerous defensive strategies to counteract its effects. The use of nest materials with antimicrobial properties is one of the birds' defenses and its research constitutes the main aim of this Thesis. Aromatic green plants are one of the most studied nest materials, due to their antimicrobial properties. This material can be used in the structural layer of nests, but also as a nest lining material, in direct contact with the eggs and nestlings. The antimicrobial characteristics of aromatic plants come from their secondary metabolites, which can repel, or reduce the negative effects of nest ectoparasites. Besides the effects against ectoparasites and microorganisms, the presence of aromatic plants is positively related with nestling growth and immune system of nestlings. Most of these studies have been conducted on blue tit (*Cyanistes caeruleus*) and common starling (*Sturnus vulgaris*) nestlings, but little is known about the effect of aromatic plants on egg bacterial load and in other avian species.

Feathers are another common nest lining material employed by birds. Feathers can also act as antimicrobial material due to the compounds produced by bacteria that grow in feathers. Keratinolytic bacteria (i.e., those able to degrade keratin, the main feather constituent) are known by their high capability to segregate antimicrobial compounds, which birds can use to reduce the parasitic load in their nests. In addition, we know that keratinolytic bacteria can degrade unpigmented feathers better that pigmented ones, due to the absence of pigments, like melanin. Thus, keratinolytic bacterial load, and their effects, could be higher in unpigmented feathers. This antibacterial effect of feathers in birds' nests was only studied in one species, the barn swallow (*Hirundo rustica*): eggs in nests with unpigmented feathers experimentally added had lower bacterial load and lower probability of hatching failure than those in nests with pigmented feathers added.

In the present Thesis, we aim to study the possible antimicrobial effect of plants and feathers in spotless starling (*Sturnus unicolor*) nests during the egg and nestling stages. In this species, feathers and plants have a function in sexual communication between mates and we suggest that they may have an additional antibacterial role in nests. For this aim, we experimentally manipulated feathers and green plants in spotless starling nests during three reproductive seasons and we estimated the effect of these materials on bacterial load (mesophilic bacteria and three bacterial groups that include bird pathogens: enterobacteria, staphylococci and enterococci) on eggshells and nestling skin. We also assessed the antimicrobial capability of isolated colonies from feathers and eggshells. Moreover, we estimated the experimental effects of feathers and plants in nests on several variables related with nestling health and phenotypic quality (body mass, fledging success, immune response and telomere length and dynamics). We also used artificial nests deployed with quail (*Coturnix japonica*) eggs to study the antimicrobial effects of feathers and plants excluding the effects of parental activity (i.e., incubation), because this activity is known to modify the nest bacterial community. Finally, we investigated the starling preference for unpigmented or pigmented feathers and for aromatic or non-aromatic plants to building their nests.

Experimental addition of feathers to the nests, irrespective of their colour, reduced bacterial load on eggshells and nestlings. However, we did not find consistent differences between unpigmented and pigmented feather treatments. During the egg and nestling stage, pigmented feather treatment reduced bacterial load more that unpigmented feather treatment. However, this effect was dependent on bacterial group (enterococci and mesophilic bacteria in eggs and staphylococci in nestlings) and was only found in natural nests in one study year. On the other hand, there were not differences between feather pigmentation treatments on eggshell bacterial load in artificial nests or

in mesophilic bacterial load on nestling skin. These results suggest that the antibacterial effect of feathers and of feather pigmentation depends on environmental conditions and/or can be modulated by parental activity.

The antimicrobial effect of green plants was not as clear as for feathers. We did not find an effect of green plants on bacterial load of eggshells or nestling skin in natural nests, but there was an effect on eggshell bacterial loads in artificial nests (i.e., without incubation). In addition, we detected an effect of green plants in interaction with feather treatments. Staphylococci prevalence on nestling skin was lower in nests with aromatic plants and feathers experimentally added. These results suggest that the antibacterial effect of aromatic plants could depend on nest feather composition.

In addition, we found that feathers and aromatic plants in nests had positive effects in nestlings in terms of body mass, immune response, telomere length and dynamics, and in fledging success of nestlings. We also detected negative relationships between bacterial loads and these variables related with nestling health and phenotypic quality, which suggest that the effects of nest materials on nestling health and quality is driven by their effects on the nest bacterial community.

The hypothesis of the antimicrobial effects of feathers assumes that bacteria growing in feathers have high antimicrobial capabilities. In addition, parental activity in the nests could have an influence on the characteristics and segregation of antimicrobial compounds by these bacteria. Thus, we explored the hypothetical higher antimicrobial activity of bacteria from feathers than that of bacteria from eggshells in starling nests and in artificial nests (i.e., without incubation) following experimental addition of feathers. We found that keratinolytic bacterial density on eggshells was higher in starling nests than in artificial nests. In accordance with the hypothesis we show that

antimicrobial capabilities were higher in bacterial colonies isolated from feathers than from eggshells and, overall, bacterial colonies from starling nests showed higher antimicrobial activity than bacterial colonies from artificial nests. These results suggest that incubation activity may favour growth of keratinolytic bacteria with higher antimicrobial capabilities that contributes to shape the nest bacterial environment.

Finally, we found that starlings clearly selected unpigmented over pigmented feathers to build their nests, both before and during the egg laying period. However, plant selection was different between both reproductive stages. Before egg laying, starlings did not select between aromatic or non-aromatic plants, which suggest a primary sexual function of green plants in this stage. Conversely, starlings clearly selected aromatic over non-aromatic plants during egg laying, which supports a self-medication function given the known antimicrobial properties of aromatic plants.

Overall, all these results support an antimicrobial function of feathers and plants in avian nests, reducing bacterial load on eggshells and nestling skin, and improving offspring development. These effects appear to be subjected to important variations, driven for example by environmental conditions and/or parental activities such as incubation. These antimicrobial properties of feathers and plants could be the characteristic that determines the observed active selection of these materials by birds to build their nests.

Introducción General

El nido es una estructura esencial para el desarrollo de la descendencia en la mayoría de las especies de aves (Hansell 2000). Existe una gran variabilidad en los nidos de las diferentes especies, tanto en la composición material como en su localización o complejidad (Hansell 2000). Algunas especies de aves depositan los huevos directamente sobre el suelo, como puede ser el caso de la alondra común (*Alauda arvensis*) o el chorlitejo patinegro (*Charadrius alexandrinus*) (Amat 2016; Pérez-Granados et al. 2016), mientras que otras estructuran sus nidos de diferentes formas, desde lo más sencillo como el de la paloma torcaz (*Columba palumbus*) que hace una plataforma con ramas y palitos, a los que hacen nidos con forma de cuenco o los totalmente cerrados como podría ser el caso de las aves tejedoras (familia *Ploceidae*) (Hansell 2000). Además pueden localizarse en diferentes sitios, en las ramas de los árboles, en cavidades de árboles o paredes, acantilados o incluso sobre el agua, hechos con materiales que les permitan flotar (Hansell 2000). Pero en casi todos ellos se pueden diferenciar dos partes, una parte estructural, de soporte, que no entra en contacto directo con los huevos, pollos o los adultos, y otra parte de revestimiento que está directamente en contacto con el contenido del nido, y para la que muchas especies utilizan materiales especiales como plumas y plantas verdes que ayudan a crear un microambiente adecuado para el crecimiento y desarrollo de la descendencia (Hansell 2000; Mainwaring et al. 2014).

La construcción de un tipo u otro de nido tiene un fuerte componente filogenético en las aves (Clayton & Harvey 1993; Winkler & Sheldon 1993; Hansell 2000; Hall et al. 2015). Las características de los nidos en general varían dependiendo de factores abióticos (i.e., temperatura, humedad, viento, insolación, etc.) y bióticos (i.e., depredación, parasitismo, etc.) específicos de los lugares de nidificación (Hansell 2000). Por tanto, los nidos tienen una función de soporte y otras relacionadas con aquellas que faciliten y aseguren

la crianza de la descendencia como son las de aislamiento, termorregulación, anti-depredadora, etc. Muchas de estas funciones dependen directamente de la cantidad y tipo de materiales que utilicen las diferentes especies.

La construcción del nido es un proceso costoso (Collias & Collias 1984; Hansell 2000; Mainwaring & Hartley 2013). Buscar los materiales para la construcción, y transportarlos al nido (Bailey et al. 2016), además de la propia construcción, suponen un gasto en tiempo y energía que las aves deben ajustar para optimizar el balance entre los costos y los beneficios (Hansell 2000). La construcción del nido también implica costos de depredación ya que durante este periodo las aves son más previsibles y detectables por depredadores (Collias & Collias 1984; Lima 2009). También pueden existir restricciones relacionados con el parasitismo (Hansell 2000) ya que las condiciones de temperatura y humedad de los nidos de las aves proporcionan un ambiente propicio para el establecimiento de multitud de ectoparásitos (moscas, pulgas, ácaros, etc.) (Loye & Zuk 1991; Clayton & Moore 1997; López-Rull & Macías-García 2015), y para el crecimiento de microorganismos potencialmente patógenos (i.e., hongos y bacterias) (Benskin et al. 2009; Soler et al. 2010).

Debido a los costos asociados, el comportamiento de construcción del nido por parte del macho y de la hembra, además del nido en sí, se ha estudiado en contextos de señalización sexual. Tanto los materiales utilizados para la construcción del nido, como el tamaño del nido informarían de la calidad de los constructores y, por tanto, de la calidad fenotípica de los individuos (Dawkins 1982; Collias & Collias 1984; Schaedelin & Taborsky 2009; Moreno 2012; Mainwaring et al. 2014). Gracias a la información que los nidos transmiten sobre las características de sus constructores, se consideran un carácter típico de fenotipo extendido (sensu Dawkins 1982) sobre el que puede actuar la selección sexual. El nido juega un papel importante en los procesos de emparejamiento en muchas especies de aves en

las que, por ejemplo, las hembras eligen pareja en función de las características del nido (ver ejemplos en Friedl & Klump 1999; Veiga et al. 2006). En contextos sexuales post-emparejamiento, uno o los dos miembros de la pareja invertirá en la reproducción, dependiendo de las características del nido y/o del esfuerzo del otro en la construcción (Burley 1986; 1988; Soler et al. 1998). Una inversión parental diferencial mejoraría el éxito reproductor del constructor, por lo que los nidos se pueden considerar caracteres sexuales secundarios (Soler et al. 1998; Mainwaring et al. 2014). Aunque existen bastantes evidencias apoyando esta idea (Moreno et al. 2012), destacamos aquí como ejemplo los resultados obtenidos en herrerillos (*Cyanistes caeruleus*), una especie en la que son las hembras las que principalmente construyen los nidos. Distintos estudios pusieron de manifiesto que (i) el tamaño del nido es un indicador de la calidad fenotípica de la hembra (Tomás et al. 2006), (ii) tanto el tamaño del nido como la presencia de plantas verdes en el nido incrementan la inversión parental del macho (Tomás et al. 2013), y (iii) el acarreo de plumas por parte del macho induce un aumento en el tamaño de puesta (Sanz & García-Navas 2011).

El ejemplo anterior del herrerillo es especialmente adecuado en el contexto de la presente Tesis Doctoral ya que nos permite enlazar la actividad constructora del nido con el uso de plantas verdes y de plumas, materiales utilizados por muchas especies de aves y de los que se presumen unos efectos antiparasitarios y/o antimicrobianos (Peralta-Sánchez et al. 2010; Dubiec et al. 2013). Los nidos, por tanto, también podrían jugar un papel importante como defensas frente a infecciones de huevos y de pollos por parte de diferentes patógenos. En los siguientes apartados introduciré las principales defensas con las que cuentas las aves para disminuir el riesgo de contagio, para después explicar detalladamente el efecto de los materiales del nido, más específicamente de las plumas y las plantas, y del comportamiento de incubación, sobre la carga bacteriana de los nidos.

Defensas antiparasitarias en los nidos de las aves

Debido a la gran cantidad de barreras físicas y químicas antibacterianas, los huevos son definidos en la literatura sobre aves de corral como “alimento empaquetado, compartimentado y aséptico” (Board et al. 1994). Sin embargo, algunas bacterias son capaces de traspasar la cáscara a través de los poros, resistir a las defensas químicas de los huevos, e infectar al embrión (Board 1966; Bruce & Drysdale 1994). De hecho, la infección bacteriana es una de las principales causas de fallo en el desarrollo y/o viabilidad de los huevos (Pinowski et al. 1994; Cook et al. 2003; 2005b; Godard et al. 2007; Hansen et al. 2015). Las infecciones bacterianas son también una importante fuente de enfermedades durante el crecimiento de los pollos (Pinowski et al. 1994; González-Braojos et al. 2012b; Jacob et al. 2015) y, por tanto, son fuerzas selectivas que favorecen la evolución de diferentes mecanismos defensivos antibacterianos que actúan durante el desarrollo de los embriones y pollos en los nidos.

En los huevos, la primera barrera frente a los microrganismos es la cáscara, la cual protege física y químicamente al embrión del traspaso de las bacterias (Board & Fuller 1994; Mine et al. 2003; Wellman-Labadie et al. 2008b; D'Alba & Shawkey 2015; D'Alba et al. 2016). Además, en el interior de los huevos existen barreras químicas, como es la presencia de ovotransferinas y lisozimas (Wellman-Labadie et al. 2007; 2008c; Grizard et al. 2015), depositadas por la madre durante la formación de los huevos (Shawkey et al. 2008; Bonisoli-Alquati et al. 2010), que actúan no solo durante la fase de huevo, sino que incluso protegen a los pollos durante sus primeros días de vida (Saino et al. 2002). Durante la etapa de pollos, el sistema inmune es la principal barrera frente a los parásitos (Sheldon & Verhulst 1996; Norris & Evans 2000; Merino 2010). Otro tipo de defensas antiparasitarias son las relacionadas con el comportamiento de los adultos, entre las que se incluyen distintos tipos de cuidados parentales. En algunas

especies, los progenitores optan por construir un nuevo nido en cada estación reproductora, o incluso en cada intento reproductor dentro de una misma estación, asegurándose de esta forma la eliminación de parásitos que alcanzaron el nido en eventos reproductivos anteriores y, por tanto, una menor carga parasitaria y bacteriana en el nido (Pacejka & Thompson 1996; Hansell 2000; Mazgajski 2007; González-Braojos et al. 2012a). Además, los adultos pueden realizar activamente tareas de limpieza de los nidos, eliminando elementos que puedan aumentar la carga parasitaria en el nido, como pueden ser las heces, pollos muertos, restos de las cáscaras de huevo, etc. (Weatherhead 1984; Guigueno & Sealy 2012; Bolopo et al. 2015). También pueden utilizar su secreción uropigial con propiedades antimicrobianas para impregnar la superficie de los huevos, como es el caso de la abubilla (*Upupa epops*) (Soler et al. 2008a; 2012). Los padres también pueden disminuir directamente la probabilidad de infección de los embriones mediante la incubación, ya que reduce el grado de humedad que favorece la infección de los huevos (D'Alba et al. 2010; Ruiz-De-Castañeda et al. 2011). Otro de los mecanismos defensivos asociados a una reducción de la carga bacteriana en los nidos es la utilización de sustancias producidas por otros organismos para protegerse contra los parásitos, y que se interpreta como una forma de automedicación (Clayton & Wolfe 1993; De Roode et al. 2013). Estos dos últimos tipos de comportamiento asociados con la reducción de los patógenos presentes en los nidos, la incubación y la automedicación, son en los que nos centramos en esta Tesis y explico con más detalle a continuación.

Automedicación

La automedicación se define como la utilización de sustancias producidas por otros animales o plantas para protegerse contra los patógenos (Clayton & Wolfe 1993; De Roode et al. 2013). Esta puede clasificarse según el modo de aplicación (por ingestión, absorción, tópica o por proximidad) (Clayton & Wolfe 1993), o según la acción ante el parásito o su efecto (profiláctica o

terapéutica) (De Roode et al. 2013). La utilización de materiales con propiedades antiparasitarias es un comportamiento muy extendido en la naturaleza, habiéndose descrito en insectos, aves y mamíferos (De Roode et al. 2013). En el caso de las aves, es muy común la utilización de materiales con propiedades antiparasitarias y antimicrobianas en los nidos, siendo los materiales más estudiados las plantas verdes y las plumas.

1. Plantas

El material más común que podemos encontrar en el nido de las aves es de origen vegetal (Hansell 2000; Dubiec et al. 2013). Este material puede formar parte tanto de la estructura del nido, como de los materiales que lo revisten (Hansell 2000). Además del material vegetal seco que suele ser el más habitual, numerosas especies de aves incorporan fragmentos de plantas verdes, frescas, usualmente aromáticas, en sus nidos. Algunos autores han observado que estas plantas frescas no son introducidas de forma aleatoria en los nidos, ya que determinadas plantas se encuentran en una proporción mucho mayor a la que se esperaría por azar o por la abundancia en el medio (Clark & Mason 1985; Pires et al. 2012). Existen evidencias de que esta selección la hacen a través del olfato, detectando los compuestos volátiles de la plantas y manteniendo así los nidos con plantas frescas (Petit et al. 2002). Por tanto, es posible que esta selección la realicen según las propiedades que las plantas frescas y aromáticas puedan ofrecerles.

Aunque a las plantas verdes se les han atribuido numerosas funciones en los nidos, como la de reducir la pérdida de agua (Taverner 1933; Sengupta 1968) o mimetizar el nido (Skutch 1976), las más estudiadas han sido la de cortejo (selección sexual) y la de protección de huevos y pollos frente a infecciones (Dubiec et al. 2013; Mainwaring et al. 2014; Scott-Baumann & Morgan 2015). La función sexual de las plantas en los nidos de las aves se ha estudiado en numerosas especies. Por ejemplo, en el estornino pinto (*Sturnus*

vulgaris) los machos con más plantas en sus nidos son los que tienen mayor éxito a la hora de atraer a las hembras (Brouwer & Komdeur 2004), mientras que en el estornino negro (*Sturnus unicolor*) los machos que llevan una mayor cantidad de plantas son los que controlan un mayor número de oquedades aptas para la reproducción (i.e., cajas-nido) y que atraen a más hembras (Veiga et al. 2006). Además, este comportamiento del macho es respondido por la hembra llevando plumas al nido (Polo & Veiga 2006), lo que sugiere que tanto las plantas como las plumas forman parte del cortejo del estornino negro. En estos contextos sexuales la presencia de plantas verdes induce una inversión parental diferencial en términos de esfuerzo en cuidados de la descendencia (Polo et al. 2004; Tomás et al. 2013) o de tamaño de puesta (López-Rull & Gil 2009). Estos efectos seguramente están mediados hormonalmente (Polo et al. 2010) y explican la relación encontrada entre comportamientos de construcción del nido y éxito reproductor o de reclutamiento de los pollos (Polo et al. 2015).

La función antiparasitaria de las plantas que las aves llevan a los nidos también ha sido ampliamente estudiada (Dubiec et al. 2013; Scott-Baumann & Morgan 2015). Los efectos antiparasitarios son debidos a compuestos secundarios volátiles que segregan algunas plantas, que actúan como repelentes o fumigantes de muchos parásitos incluidos las bacterias y que, por tanto, proporcionan protección al nido (Clark 1991; Dubiec et al. 2013). Estos efectos parecen generales ya que, por ejemplo, sabemos que varias especies de aves que utilizan plantas verdes en sus nidos tienen una menor prevalencia y abundancia de moscas parásitas del género *Philornis* (Dubiec et al. 2013). Además, debido a estos efectos preventivos sobre el parasitismo (profilaxis), o a efectos farmacológicos directos, las plantas favorecen un mejor crecimiento de los pollos (Dubiec et al. 2013).

Sin duda, las dos especies de aves donde más se ha investigado la función de las plantas verdes en los nidos son el herrerillo común y el

estornino pinto (Dubiec et al. 2013). En ambas especies hay evidencias de que las plantas llevadas al nido pueden reducir la carga parasitaria, tanto de ectoparásitos (Clark & Mason 1985; Lafuma et al. 2001) como de bacterias (Gwinner & Berger 2005; Mennerat et al. 2009a), apoyando por tanto la hipótesis de protección del nido. No obstante, también hay evidencias a favor de un efecto estimulador de las plantas sobre los pollos (Gwinner et al. 2000; Mennerat et al. 2009b), ya que éstas mejoraron el estado de salud de los pollos de herrerillo y de estornino pinto, aumentando su peso, el valor del hematocrito o estimulando componentes concretos de su sistema inmune, como un aumento en el número de basófilos (Gwinner et al. 2000; Mennerat et al. 2009b). Los efectos de las plantas sobre la carga bacteriana y sobre el estado físico de los pollos, han sido estudiados principalmente en el estornino pinto y el herrerillo, y hasta lo que sabemos, únicamente en pollos. En esta Tesis nos planteamos estudiar el efecto de las plantas verdes en los nidos de estornino negro, una especie cercana al estornino pinto, prestando especial atención en su hipotética función antimicrobiana, no solo en pollos, sino también en huevos.

2. *Plumas*

Otro de los materiales más usados por las aves, sobre todo en el revestimiento de los nidos, son las plumas (Hansell 2000). Tradicionalmente se han estudiado las plumas por su función termorreguladora, protegiendo a los huevos y pollos de las condiciones ambientales (Hilton et al. 2004; Windsor et al. 2013). Otra de las funciones estudiadas en las plumas, al igual que en las plantas, es la función sexual, señalando el estatus o calidad de los individuos (Polo & Veiga 2006; Sanz & García-Navas 2011; García-Navas et al. 2015), e influyendo en la inversión parental de la pareja (Sanz & García-Navas 2011; García-López de Hierro et al. 2013).

Más recientemente se ha propuesto una función antibacteriana para las plumas que recubren los nidos de las aves (Soler et al. 2010). El principal componente de las plumas es la queratina, una proteína que puede ser degradada por las llamadas bacterias queratinolíticas. Una de las más comunes y conocidas es el *Bacillus licheniformis* (Burtt Jr & Ichida 1999; Whitaker et al. 2005), pero existen otras muchas del género *Bacillus* y de otros géneros como *Pseudomonas* (Shawkey et al. 2005; Dille et al. 2016). Todas estas bacterias son capaces de segregar diferentes metabolitos secundarios, capaces de inhibir el crecimiento de otras bacterias (Callow & Work 1952; Riley & Wertz 2002; Ghequire & De Mot 2014; Lee et al. 2014a) que compiten con ellas por el substrato y el espacio, en lo que se conoce como fenómeno de interferencia bacteriana (Riley & Wertz 2002). La idea es que las aves pueden obtener beneficios de las sustancias segregadas por las bacterias queratinolíticas que crecen en las plumas que incorporan a sus nidos (Soler et al. 2010).

Debido a la presencia de pigmentos como la melanina, el crecimiento de bacterias queratinolíticas es menor en plumas pigmentadas que en las no pigmentadas (Goldstein et al. 2004; Gunderson et al. 2008). Esto puede implicar que la capacidad antimicrobiana asociada a las comunidades de plumas pigmentadas y no pigmentadas también difiera. Es incluso posible que la capacidad antagónica dependa de la composición de plumas pigmentadas y no pigmentadas del nido (Peralta-Sánchez et al. 2014). En cualquier caso, la segregación de estos compuestos antimicrobianos depende además de otros muchos factores ambientales, como pueden ser el pH, la temperatura, humedad, disponibilidad de nutrientes, etc. (Biswas et al. 1991; Mataragas et al. 2003; Todorov & Dicks 2006), por lo que los factores ambientales en los nidos, o los comportamientos de los adultos, podrían estar influyendo en la actividad antimicrobiana de las comunidades bacterianas presentes en los nidos.

Los estudios que han demostrado la capacidad antibacteriana de las plumas en los nidos han sido realizados con golondrinas comunes (*Hirundo rustica*). En ellos se puso de manifiesto una preferencia de los adultos por revestir sus nidos con plumas no pigmentadas (Peralta-Sánchez et al. 2010) y una reducción en la carga bacteriana (Peralta-Sánchez et al. 2010) y en el riesgo de fallos de eclosión en huevos incubados en nidos experimentales a los que se les había proporcionado plumas no pigmentadas y retirado las pigmentadas (Peralta-Sánchez et al. 2011). Además, también se detectó que las colonias bacterianas aisladas de plumas no pigmentadas, tenían una mayor capacidad antimicrobiana que las aisladas de plumas pigmentadas, sobre todo de aquellas que procedían de nidos experimentales de plumas pigmentadas (Peralta-Sánchez et al. 2014). En esta Tesis nos planteamos comprobar predicciones de esta hipótesis en el estornino negro, una especie que, como ya hemos mencionado, utiliza las plumas en sus nidos como señal sexual de la hembra hacia el macho, pero que además podrían tener una función antimicrobiana como ocurre en la golondrina. Además, el estornino negro también utiliza plantas verdes en sus nidos, por lo que podría haber un efecto conjunto de plantas verdes y de plumas. Además, el efecto de las plumas en los nidos solo ha sido investigado sobre la carga bacteriana de los huevos de golondrina, con lo que un estudio de los efectos de las plumas sobre la carga bacteriana de la piel de los pollos sería también necesario para comprender mejor el significado funcional de las plumas en los nidos de las aves.

Incubación

La incubación de los huevos es una actividad fundamental en la reproducción de la mayoría de las especies de aves. Pone en marcha el desarrollo embrionario, y proporciona al huevo un ambiente estable de temperatura y humedad, necesario para el apropiado crecimiento del embrión (Deeming 2002; Nord & Nilsson 2011; Hepp & Kennamer 2012; DuRant et al. 2012; Berntsen & Bech 2016). Más recientemente, se ha sugerido que la incubación

juega un papel importante disminuyendo la probabilidad de infección bacteriana de los embriones. Varios estudios han comprobado que la incubación afecta negativamente a la carga bacteriana y/o a la diversidad de comunidades de la cáscara de los huevos (Cook et al. 2005a; Shawkey et al. 2009; Grizard et al. 2014). Este efecto, parece estar mediado por la reducción de la humedad en la cáscara de los huevos que provoca la incubación (D'Alba et al. 2010; Ruiz-De-Castañeda et al. 2011). Estos resultados se han encontrado sobre todo en ambientes tropicales (Cook et al. 2003; 2005a), mientras que en otras latitudes no se ha detectado ese efecto (Wang et al. 2011), o incluso, se detecta el opuesto (Giraudeau et al. 2014). Estas diferencias quizás puedan ser debidas a las diferentes temperaturas y humedades asociadas a los diferentes climas que, a su vez, afectan al ambiente bacteriano del nido (Board & Fuller 1994). Otra explicación es que se deban a los diferentes efectos que la incubación tiene sobre distintos tipos de bacterias. En algunos trabajos han puesto de manifiesto una reducción en la prevalencia de bacterias potencialmente patógenas (Cook et al. 2005a; Brandl et al. 2014), mientras que otros han encontrado evidencias de que la incubación puede estar favoreciendo el crecimiento de determinados grupos bacterianos con importante actividad antimicrobiana, como aquellas del género *Bacillus* o *Pseudomonas* (Potter et al. 2013; Grizard et al. 2014; Lee et al. 2014b), dos grupos de bacterias queratinolíticas (Ramnani et al. 2005; Stiborova et al. 2016) en los que se ha descrito la producción de metabolitos secundarios con propiedades antimicrobianas (Abriouel et al. 2011; Naz et al. 2015).

Conocidos los efectos que la incubación tiene sobre las comunidades bacterianas en los nidos, nos planteamos la posibilidad de que la incubación pueda tener efectos sobre la actividad antimicrobiana de las plumas y de las plantas en los nidos de las aves. Para ello estudiamos de forma experimental las diferencias en los efectos de las plantas y de las plumas en términos de

carga bacteriana de huevos y de actividad antimicrobiana de bacterias aisladas de nidos con actividad reproductora y sin ella.

Objetivos

En este escenario, en el que el ambiente bacteriano puede influir en la salud y viabilidad de los pollos y los huevos, nuestro interés se centra en el estudio de los posibles mecanismos que las aves adultas pueden desarrollar para reducir la carga bacteriana en los nidos. Más concretamente nos centramos en el estudio del posible papel de las plumas (pigmentadas y no pigmentadas) y de las plantas aromáticas como materiales del nido con propiedades antimicrobianas. Los objetivos específicos que nos planteamos son:

1- Determinar experimentalmente el efecto de las plumas y las plantas aromáticas en los nidos, y de su interacción, sobre la carga bacteriana de los huevos, dependiendo de la actividad reproductora, del año y la zona de estudio y del aumento experimental de la densidad bacteriana (**capítulo 1**).

2- Explorar experimentalmente el efecto de las plumas y las plantas aromáticas en los nidos, y de su interacción, sobre la carga bacteriana de la piel de los pollos, así como los efectos sobre variables relacionadas con el estado de salud de los pollos (peso corporal, sistema inmune y éxito de vuelo) (**capítulo 2**).

3- Determinar el efecto de las plumas y las plantas usadas como materiales del nido sobre la longitud y dinámica de los telómeros de los pollos, dependiendo de la carga parasitaria presente en los nidos, estimada a través de la carga bacteriana y la abundancia del ectoparásito *Carnus hemapterus* (**capítulo 3**).

4- Estudiar la capacidad antimicrobiana de bacterias aisladas de las plumas y de las cáscaras de huevos dependiendo de la manipulación experimental del material del nido (composición de plumas pigmentadas y no pigmentadas en el nido y actividad reproductora), además de la pigmentación de las plumas de las que proceden las colonias bacterianas (**capítulo 4**).

5- Determinar la preferencia del estornino negro por el color de plumas (pigmentadas y no pigmentadas), y por el tipo de plantas aromáticas para construir sus nidos, dependiendo de la etapa reproductora (antes y después del inicio de la puesta) (**capítulo 5**).

Para llevar a cabo estos objetivos específicos, hemos realizado una serie de experimentos en una población de estornino negro, una especie que como ya se ha destacado, lleva a sus nidos tanto plumas como plantas (Polo & Veiga 2006). Los objetivos los hemos desarrollado en cinco capítulos y más abajo destacamos brevemente la importancia de los experimentos en el marco teórico de esta Tesis.

Capítulo 1: La función que normalmente se ha atribuido a las plumas y a las plantas en los nidos del estornino negro ha sido la función sexual, pero debido a las evidencias existentes en otras especies sobre la capacidad antimicrobiana de plumas y plantas como materiales de nido, nos planteamos explorar esa función en el estornino negro con el interés adicional de plantear experimentos de manipulación de plumas y de plantas en los mismos nidos en un diseño factorial completo que nos permitiera explorar posibles efectos combinados (aditivos y/o antagónicos) de ambos materiales sobre la carga bacteriana de los huevos. En este capítulo, además, estimamos los efectos experimentales de plumas y plantas en nidos con actividad reproductora y en

nidos artificiales, lo que nos permite explorar experimentalmente los efectos de la incubación sobre la comunidad bacteriana de los huevos, y su posible influencia sobre el papel de las plumas y de las plantas.

Capítulo 2: De la misma forma que en el capítulo anterior, debido a las propiedades antibacterianas de las plumas y las plantas, se esperaría que estos materiales afectaran a la carga bacteriana de la piel de los pollos, predicción que exploramos experimentalmente. En este capítulo, también exploramos los efectos de los materiales del nido sobre el peso corporal, la respuesta inmune y el éxito de vuelo de los pollos. Debido a los efectos antibacterianos de estos materiales, predecimos que los pollos que han crecido en nidos con plantas y también con plumas estarán en mejor condición física que los pollos crecidos en nidos sin plumas ni plantas añadidas.

Capítulo 3: La longitud y la dinámica de los telómeros pueden utilizarse como una medida del estrés de los pollos en el nido y de su calidad fenotípica, ya que en muchas especies se ha encontrado una relación con la esperanza de vida y/o la probabilidad de reclutamiento a la población reproductora. Por tanto, el ambiente en el que crezcan los pollos, incluyendo por ejemplo la presencia o no de parásitos en el nido, o los diferentes materiales del nido, pueden afectar a la dinámica de los telómeros. Durante la fase de pollos añadimos plumas y plantas a los nidos de estornino de dos poblaciones que diferían en la carga de ectoparásitos y de bacterias. Esperamos que en nidos con plumas y plantas, los pollos presenten una mayor longitud y un menor acortamiento de los telómeros y que este efecto sea más acusado en las áreas con mayor parasitismo. Además, cuantificamos la abundancia de ectoparásitos en el nido y la carga bacteriana en la piel de los pollos, y predecimos una relación negativa entre la abundancia de estos patógenos y la longitud de los telómeros, y una relación positiva con la tasa de acortamiento de los telómeros en pollos al final de su estancia en el nido.

Capítulo 4: Existían argumentos que sugerían diferentes capacidades antimicrobianas para bacterias procedentes de plumas pigmentadas y plumas no pigmentadas y en este capítulo nos planteamos comprobar distintas predicciones asociadas. Además, debido a que la actividad antimicrobiana modificaría el ambiente del nido, esta actividad también podría influir en la actividad antimicrobiana de la comunidad bacteriana del nido. Para comprobar estas hipótesis, añadimos plumas pigmentadas, no pigmentadas, una mezcla de ambos tipos de plumas, o no añadimos plumas, a nidos naturales de estornino y a nidos artificiales con huevos de codorniz. Tanto de los nidos artificiales como de los naturales aislamos bacterias de la cáscara de los huevos y de las plumas y estimamos su actividad antimicrobiana. Esperamos una mayor capacidad antimicrobiana para las colonias aisladas de plumas blancas de nidos naturales.

Capítulo 5: La construcción del nido es un proceso costoso, con lo que la selección de materiales para el nido debe ser la adecuada para obtener los mayores beneficios. Uno de estos beneficios puede estar relacionado con las propiedades antiparasitarias que las plumas y las plantas puedan ofrecer a las aves. Esta selección de materiales para la construcción del nido la estudiamos experimentalmente ofreciendo a los estorninos plumas pigmentadas y no pigmentadas, así como plantas aromáticas y no aromáticas *ad libitum,* y evaluamos el uso de dichos materiales en sus nidos. Esperábamos que seleccionasen plumas no pigmentadas y plantas aromáticas, ya que teóricamente, estos son los materiales con mejores capacidades antimicrobianas.

Material y Métodos Generales

Especie de estudio: Estornino negro (*Sturnus unicolor*)

El estornino negro es un ave de la familia *Sturnidae* del orden de los *Passeriformes*. Es una especie endémica de la región mediterránea occidental, asociada en muchas ocasiones a construcciones humanas. Es un ave de mediano tamaño (longitud pico-cola = 21-23 cm, envergadura alar = 38-42 cm). El macho es de mayor peso (media = 93.4 g) que la hembra (media = 88.5 g) (Cramp 1998; Veiga & Polo 2016). Además del peso, existe un leve dimorfismo sexual en la coloración del plumaje; el macho tiene un color negro brillante mientras que el plumaje de las hembras es de un negro más apagado (Fig. 1). Durante la época de cortejo, el macho tiene la base del pico azulado, mientras que el de la hembra es amarillento rosáceo, siendo la punta del pico amarilla en ambos sexos (Veiga & Polo 2016). La intensidad de tonalidades amarillas en el pico se relaciona con la concentración de carotenoides y vitamina A en el plasma sanguíneo y, por tanto, informaría de la capacidad antioxidante de los individuos en contextos sexuales (Navarro et al. 2010). Existe también diferenciación sexual en las plumas de la garganta; las de los machos son más estrechas y alargadas que las de las hembras y los juveniles (Hiraldo & Herrera 1974; Lezana et al. 2000). La longitud de las plumas de la garganta de los machos se considera un carácter sexual ya que está relacionada con la probabilidad de selección por parte de la hembra y con el número de pollos (Aparicio et al. 2001). Además, estas plumas alargadas de la garganta de los machos son aparentemente señales honestas de calidad fenotípica, ya que se degradan mucho más fácilmente por bacterias que el resto de las plumas, y sólo machos en buen estado de salud serán capaces de mantenerlas en buenas condiciones (Ruiz-Rodríguez et al. 2015).

Figura 1: (A) Hembra (Foto: Gustavo Fernando Durán) y (B) macho (Foto: Sbas Ayllón) de estornino negro (*Sturnus unicolor*)

El estornino negro es un ave colonial que nidifica en cavidades; aprovecha para ello huecos en las construcciones humanas y en árboles adaptándose bien al uso de cajas-nido (Veiga & Polo 2016). Tanto el macho como la hembra participan en la construcción del nido, siendo el macho el que comienza a llevar los materiales, pero es la hembra la que construye la mayor parte del nido, completándolo en un período de entre 2 y 7 días (Veiga & Polo 2016). Para la construcción del nido utilizan diferentes tipos de materiales. Para la estructura, suelen utilizar fragmentos de plantas secas, tallos y ramas secas, siendo los de gramíneas los más frecuentes. También utilizan plumas y plantas frescas, sobre todo como revestimiento del nido (Polo & Veiga 2006; Peralta-Sánchez et al. 2012). En la especie filogenéticamente más cercana, el estornino pinto (*Sturnus vulgaris*), se ha descrito una función de automedicación para las plantas verdes que revisten el nido, encontrando efectos como una disminución de la carga de ectoparásitos y de bacterias, y una mejora del estado físico de los pollos (ver introducción, Clark & Mason 1985; Gwinner et al. 2000). Estas aves seleccionan las plantas que introducen en los nidos de forma no aleatoria (Clark & Mason 1985), siendo capaces de discriminarlas por el olor (Clark & Mason 1987).

Las evidencias experimentales que existen en estorninos negros sobre funciones de los materiales del nido (i.e., plumas y plantas) apuntan a que

actúan en el ámbito de la selección sexual. Desde casi el comienzo de la elaboración del nido el macho lleva plantas frescas a la cavidad. Este comportamiento se interpreta como una señal de estatus hacia la hembra (Veiga et al. 2006) en la que provoca un aumento en el nivel de testosterona (Polo et al. 2010), en el tamaño de puesta, y en la concentración de pigmentos y andrógenos depositados en los huevos (López-Rull & Gil 2009). Además, también tiene efectos positivos en el esfuerzo constructor de las hembras acarreando plumas al nido; comportamiento que, a su vez, se interpreta como indicador de la calidad fenotípica de la hembra, ya que se relaciona positivamente con un incremento en el tamaño de la puesta y una reducción de mortalidad de los pollos (Veiga & Polo 2011). Por tanto, la función de este comportamiento de las hembras también se interpreta en contextos de selección sexual (Polo & Veiga 2006). En nuestra zona de estudio el número de plumas que aparecen en los nidos de estornino varía normalmente entre 0 y 35 plumas, aunque algunos nidos pueden llegar a tener más de 50 plumas (observación personal). Las plumas, también podrían tener una función facilitando la detección de los huevos ya que, aparentemente, la disposición de las plumas en el nido no es aleatoria sino que se disponen maximizando su reflectancia en el ultravioleta (Veiga & Polo 2005; Avilés et al. 2010).

En nuestra zona de estudio, los estorninos comienzan a construir sus nidos en marzo y la puesta de los primeros huevos suele ser a mediados de Abril. La hembra de estornino pone un huevo al día y el tamaño medio de la puesta es de 4-5 huevos (Soler et al. 2008b; Veiga & Polo 2016). La coloración de los huevos es azul verdosa y sirve como señal indicadora de la condición física de la hembra hacia el macho (López-Rull et al. 2007a; Soler et al. 2008b) y de la cantidad de antioxidantes y vitamina E depositados en el huevo (Navarro et al. 2011). El macho ajusta su esfuerzo reproductor a la intensidad de coloración de los huevos, por lo que se considera una señal sexual de las hembras de estornino (Moreno & Osorno 2003; Soler et al.

2008b). La incubación la lleva a cabo casi completamente la hembra y comienza antes de finalizar la puesta, normalmente tras la puesta del penúltimo huevo, lo que conlleva la eclosión asincrónica de los huevos hacia primeros de mayo (Soler et al. 2008b). La incubación dura entre 10 y 15 días (Veiga & Polo 2016), mientras que la estancia de los pollos en el nido se prolonga por unos 18-25 días (Veiga & Polo 2016). Los pollos son principalmente alimentados con insectos tanto por la hembra como por el macho (Motis et al. 1997; Cramp 1998), aunque la participación del macho es mayor durante la primera mitad de la estancia de los pollos en el nido (Soler et al. 2008b).

Área de estudio

El estudio lo llevamos a cabo durante la época de reproducción de los estorninos de los años 2012 a 2014 en la Hoya de Guadix (Granada), en el sureste de la Península Ibérica. Se trata de una altiplanicie entre la Sierra de Baza y Sierra Nevada, a 1000 m sobre el nivel del mar, y con un clima típicamente semiárido. La vegetación típica de la zona consiste en plantaciones de secano con cereales, principalmente de cebada (*Hordeum vulgare*), almendros (*Prunus dulcis*) y pinos (*Pinus halepensis* y *P. pinaster*, principalmente) y otras de regadío como la lechuga (*Lactuca sativa*). La vegetación natural se compone principalmente de encinares (*Quercus ilex*), retamales (*Retama sphaerocarpa*) donde las bolinas (*Genista speciosa* y *Genista spartioides*) son las especies dominantes, y un espartal donde domina casi de forma exclusiva el esparto (*Stipa tenaccisima*). Además, en la zona es abundante una gran diversidad de plantas aromáticas típicas de la vegetación mediterránea. La precipitación media diaria durante los años de estudio fue de 0.93 mm y la temperatura media diaria fue de 13.2 °C, pudiendo alcanzar los -10.4 °C de mínima y los 38.6 °C de máxima. La humedad media anual fue del 56.6% (datos obtenidos de la estación más cercana, Jerez del Marquesado:

http://www.juntadeandalucia.es/agriculturaypesca/ifapa/ria/servlet/FrontController?action=Static&url=coordenadas.jsp&c_provincia=18&c_estacion=6).

Estudiamos dos poblaciones de estornino negro, una situada en la estación de tren de La Calahorra (37º 15' N, 3º 01' W) con 78 cajas-nido disponibles (Fig. 2A) y la otra en la estación de tren de Huéneja (37º13' N, 2º56' W) con 62 cajas-nido (sólo en **capítulo 3**) (Fig. 2B). Ambas zonas están separadas entre sí por unos 20 km y no se utilizan como apeadero desde hace décadas. El tamaño de las cajas-nido es de 350 mm de altura * 180 mm de ancho * 210 mm de profundidad y la altura desde el suelo de la caja-nido hasta el agujero de entrada es de 240 mm. Están hechas de corcho y colocadas en troncos de árboles y paredes de edificios a 3-4 m del suelo (Fig. 2C). Excepto intentos esporádicos de reproducción por parte del gorrión común (*Passer domesticus*) estas cajas-nido son ocupadas exclusivamente por estorninos negros.

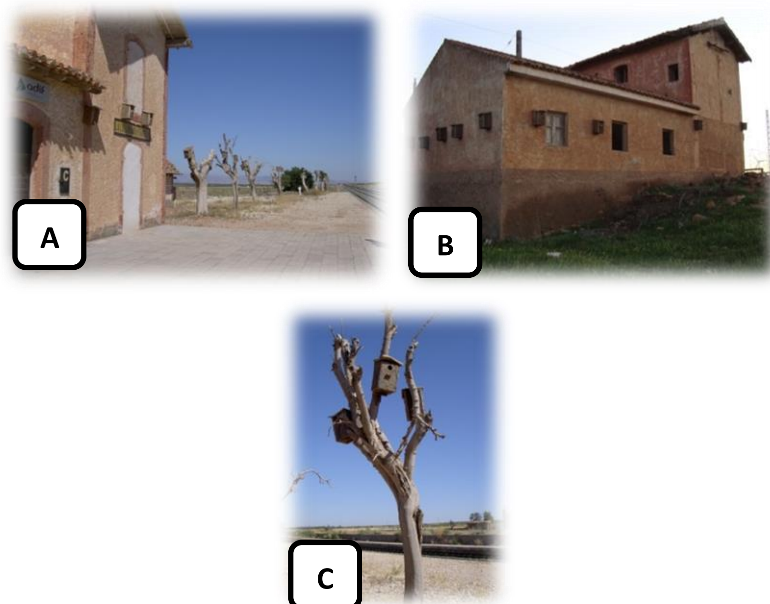


Figura 2: Área de estudio, Hoya de Guadix; (A) Estación de La Calahorra (B) Estación de Huéneja (Foto: D. Martín-Gálvez) y (C) cajas-nido en árboles de la estación de La Calahorra.

Tareas generales de campo

Al comienzo de la estación reproductora, la última semana de marzo, visitábamos las cajas-nido para así establecer el estado de construcción de los nidos. A partir de esta visita, revisábamos las cajas-nido una vez por semana hasta encontrar el nido completo. Las cajas con nidos completos, las revisábamos cada 3 días para detectar la puesta de los primeros huevos, antes de que comenzara la incubación. Una vez aparecían los huevos, los marcábamos con un rotulador permanente para su identificación individual. La siguiente revisión la hacíamos a los 5 días del inicio de la puesta, momento en que medíamos la longitud y anchura de los huevos con un calibre digital (precisión de 0.01 mm). Los nidos se volvían a visitar a los 12 días del inicio de la puesta y en días sucesivos para determinar la fecha de eclosión, la cual se establecía como el día en el que la mitad o más de los huevos habían eclosionado (Tomás 2015). Gracias a la información recogida durante estas visitas obteníamos el éxito de eclosión como el porcentaje de huevos eclosionados.

Durante la etapa de pollos las visitas a los nidos las realizamos a los 3, 5, 8 y 14 días de edad de los pollos. Excepto a los 5 días de edad, en el resto de las visitas obtuvimos el peso corporal de todos los pollos del nido con una balanza electrónica (precisión de 0.01 g). Los pollos los marcábamos individualmente en la primera visita cortándoles en una combinación única el plumón de la cabeza, espalda y alas. Dichas marcas eran patentes en pollos de 8 días, cuando se marcaban con anillas metálicas numeradas proporcionadas por el Ministerio de Medio Ambiente. Los nidos se volvían a visitar a los 14 días donde además de obtener nuevamente el peso de los pollos, también mediamos la longitud del tarso (± 0.01 mm) y del ala (± 1 mm), y estimamos el éxito de vuelo como el número de pollos vivos respecto al número de pollos nacidos.

En cada una de las visitas a los nidos, tanto en etapa de huevos como de pollos, recogimos información sobre el número de plumas no pigmentadas (blancas) y pigmentadas (negras) que había en los nidos. También pesamos con una balanza (precisión 0.1 g) la cantidad de plantas verdes que había revistiendo el nido.

Diseños experimentales

Los objetivos planteados en esta Tesis los exploramos en nidos naturales de estornino negro, en los que manipulamos la composición de las plumas y las plantas. Además, en los objetivos relacionados con la influencia de los materiales de los nidos sobre la carga bacteriana de huevos (**capítulo 1**) y sobre la capacidad antagónica de las bacterias aisladas de huevos y de plumas (**capítulo 4**), utilizamos nidos artificiales con un diseño muy similar al de nidos naturales (ver más abajo) para evitar los efectos relacionados con el comportamiento de los adultos. Entre los efectos que pretendíamos eliminar o poner de manifiesto, destacan los originados por la actividad de incubación, ya que se ha demostrado que ésta disminuye o modifica la carga bacteriana de los huevos, y por tanto la probabilidad de contaminación del embrión (Cook et al. 2005a; Shawkey et al. 2009; Grizard et al. 2014). En este apartado, primero explicaré el modo de preparación de las plumas y de las plantas que utilizábamos en los nidos experimentales y posteriormente los diseños experimentales.

Material empleado

1. Preparación de plumas

Las plumas que utilizamos para revestir los nidos naturales y artificiales fueron de gallina (*Gallus gallus domesticus*), y se recogieron de granjas cercanas a nuestra zona de estudio. Utilizamos plumas de gallina porque son las más comunes en los nidos de nuestra población de estornino. Distinguimos

entre plumas no pigmentadas y plumas pigmentadas, y usamos aquellas de un tamaño similar a las utilizadas por los estorninos. En el laboratorio, marcamos el raquis de cada una de las plumas con un rotulador permanente y las esterilizamos en una cabina de flujo laminar (Burdinola, BV-100) con luz ultravioleta durante 10 minutos por cada cara de la pluma (Fig. 3). A continuación, rociamos las plumas con una solución de *Bacillus licheniformis* D13 (excepto para las utilizadas en el **capítulo 4**). Dicha solución se obtenía al inocular una colonia de *B. licheniformis* D13 de una placa de TSA (Tripticase Soy Agar) en 6 ml de BHI (Brain Heart Infusion), dejándola crecer durante 12 h a 37 °C. Para asegurarnos una densidad homogénea de colonias en las plumas utilizadas en todos los nidos, rociamos con 84 mL de la solución de *B. licheniformis* una superficie de un metro cuadrado completamente cubierta por plumas. Posteriormente guardamos las plumas en bolsas herméticas y a 4 °C hasta su uso en el campo.

Figura 3: Esterilización de las plumas experimentales

2. *Preparación de plantas*

Las plantas introducidas en los nidos fueron una mezcla de las cuatro especies más utilizadas por los estorninos de nuestra población: marrubio (*Marrubium vulgare*), boja (*Artemisia barrelieri*), lamio (*Lamium amplexicaule*) y margarita (*Anacyclus clavatus*) (Fig. 4). Todas estas plantas presentan

compuestos volátiles o aceites esenciales con capacidad antimicrobiana (Laggoune et al. 2008; Bakkali et al. 2008; Meyre-Silva & Cechinel-Filho 2010; Selles et al. 2013; Bouterfas et al. 2014). Para los experimentos realizados en nidos artificiales (**capítulo 1**, ver más adelante "Nidos artificiales"), además utilizamos una planta no aromática, la cebada (*Hordeum vulgare*). Cada día, antes de visitar los nidos, recogíamos las plantas en nuestra zona de estudio, las cortábamos de un tamaño similar al empleado por los estorninos, y preparábamos una mezcla homogénea de las cuatro especies aromáticas (o de cebada). A los nidos se añadían 1.7 g de plantas durante la incubación de los huevos (**capítulo 1**) y 1.6 g durante la fase de pollos (**capítulos 2 y 3**), ya que eran la cantidades máximas de plantas que encontramos en nuestra población durante la incubación y durante la fase de pollos, respectivamente.

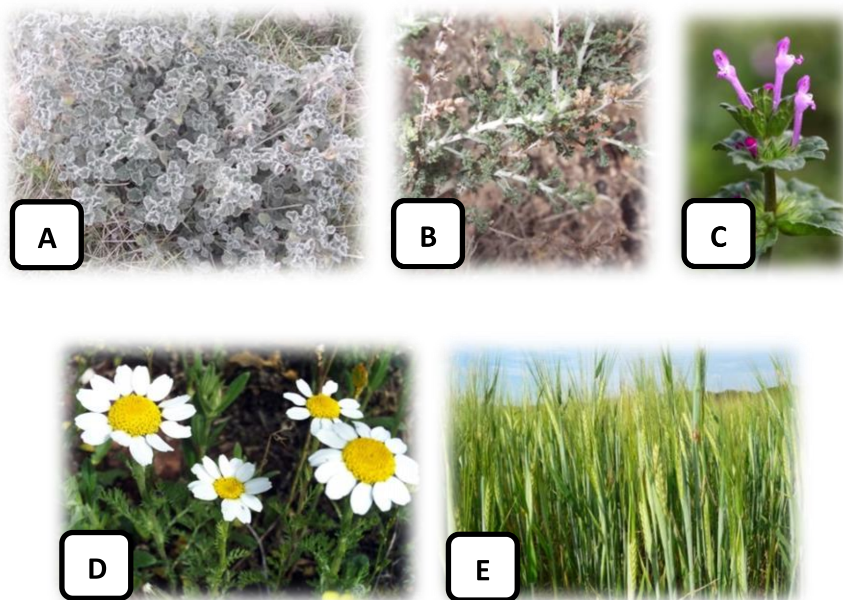


Figura 4: (A) *Marrubium vulgare* (Foto: G. Tomás), (B) *Artemisia barrelieri* (Foto: G. Tomás) (C) *Lamium amplexicaule* (Foto: Masaki Ikeda), (D) Anacyclus clavatus (Foto: Javier Martin), (E) *Hordeum vulgare* (Foto: Zauber)

Diseños experimentales

1. Plantas y plumas en nidos naturales

Para determinar el efecto de las plumas y las plantas usadas como materiales del nido sobre la carga bacteriana de los huevos y pollos (**capítulos 1, 2 y 3**), y sobre las variables relacionadas con la calidad fenotípica de los pollos poco antes de abandonar el nido (**capítulos 2 y 3**), manipulamos la composición de plumas y plantas en los nidos en un diseño factorial completo. Los tratamientos de plumas consistieron en poner en el nido (i) 15 plumas pigmentadas, (ii) 15 plumas no pigmentadas, o (iii) dejar el nido sin plumas. Los tratamientos de plantas consistieron en meter en el nido (i) 1.7 g (**capítulo 1**) o 1.6 g (**capítulos 2 y 3**) de una mezcla de plantas aromáticas comunes en nuestra zona de estudio, o (ii) dejar el nido sin plantas.

En el día 3 tras la puesta del primer huevo (**capítulo 1**), o en el día 3 de edad de los pollos (**capítulos 2 y 3**), asignábamos aleatoriamente a los nidos los tratamientos de plumas y plantas. Después de contar las plumas y pesar las plantas presentes en los nidos, las retirábamos y aplicábamos los tratamientos experimentales correspondientes (Fig. 5A y B). Durante las siguientes visitas a los nidos, de nuevo contábamos las plumas y pesábamos las plantas que hubieran metido los adultos, las retirábamos y renovábamos los tratamientos. Tanto en la fase de huevos (**capítulo 1**) como en la de pollos (**capítulos 2 y 3**) realizamos dos muestreos de la carga bacteriana. Uno de ellos en la primera visita al nido para determinar la carga bacteriana antes del tratamiento y el segundo muestreo antes de que los huevos eclosionaran (**capítulo 1**), o con pollos de 8 días de edad (**capítulo 2 y 3**) para evaluar el efecto de los materiales de plumas y plantas experimentales sobre las comunidades bacterianas. En la fase de pollos, además, obtuvimos dos muestras de sangre para estimar el efecto de los materiales sobre dos variables relacionadas con la supervivencia de los pollos, la longitud de los telómeros y

la respuesta inmune. La primera muestra la tomamos de pollos con 3 días de edad, para estimar la longitud de los telómeros antes del tratamiento y la segunda cuando tenían 14 días de edad para determinar la longitud y la tasa de acortamiento de los telómeros de los pollos que habían crecido en los diferentes tratamientos experimentales (**capítulo 3**). La muestra de sangre de los pollos de 14 días, también nos sirvió para estimar el efecto de los tratamientos de plumas y plantas sobre la respuesta inmune de los pollos (**capítulo 2**).

Durante la estación reproductora del año 2014 realizamos de nuevo una manipulación de las plumas que revisten el nido, para estimar la influencia de este material en la capacidad antimicrobiana de las colonias que crecían en las plumas y en la cáscara de los huevos (**capítulo 4**). El diseño experimental consistió en poner en los nidos (i) 20 plumas pigmentadas, (ii) 20 plumas no pigmentadas, (iii) 10 plumas pigmentadas y 10 no pigmentadas en el mismo nido (mixto), o (iv) dejar el nido sin plumas. Las plumas las dispusimos una semana antes de la fecha estimada de la puesta del primer huevo y, al igual que hemos descrito anteriormente, eliminábamos las plumas y plantas que hubiera en el nido. Los tratamientos se renovaban con la puesta del primer huevo y 5 días después de la puesta del primer huevo. Al final de la incubación muestreamos la carga bacteriana de uno de los huevos de la puesta elegido al azar y recogimos una pluma pigmentada y una no pigmentada de cada nido para, posteriormente en el laboratorio, poder estimar la densidad bacteriana y analizar la actividad antimicrobiana de las colonias aisladas de la cáscara de los huevos y de la superficie de las plumas (ver más abajo).

2. Plantas y plumas en nidos artificiales

Para la preparación de los nidos artificiales (**capítulos 1 y 4**) utilizamos 73 cajas-nido en el año 2012, 156 en el 2013 y 40 en el 2014. Las cajas fueron rellenadas con fibra de poliéster previamente esterilizada en la campana de

flujo laminar con luz ultravioleta. Este material artificial fue colocado de forma que simulara el cuenco del nido a un cuarto de la altura de la caja, donde dispusimos huevos experimentales de codorniz previamente limpiados con toallitas desinfectantes.

A cada una de las cajas se le aplicó el tratamiento experimental correspondiente (Fig. 5C). Los tratamientos experimentales fueron los mismos que en nidos naturales, aunque en los años 2012 y 2013 (**capítulo 1**) añadimos un tratamiento de plantas no aromáticas, que consistió en colocar 1.7 g de cebada (*Hordeum vulgare*). Visitamos los nidos cada dos días para mover los huevos y de esta forma asegurarnos de que toda la superficie estuviera en contacto con el material de plumas y plantas experimentales. Además, también se reponía el tratamiento de plantas con material fresco.

Cada huevo experimental se muestreaba solo una vez, por lo que el número de huevos dispuestos en nidos experimentales dependía del número de muestreos que se planteaba realizar en cada uno de los experimentos. En el año 2012 se colocaron tres huevos de codorniz y en el 2013 se colocaron seis huevos (**capítulo 1**). Las muestras de la carga bacteriana de la cáscara de los huevos, las recogimos a los 5 y 9 días tras el inicio del tratamiento. En el año 2013, se estimó la carga bacteriana de los huevos una tercera vez, a los 17 días para intentar observar un mayor efecto de las plumas y las plantas sobre la carga bacteriana de los huevos. En el año 2014 (**capítulo 4**), para estimar el efecto de las plumas y las plantas sobre la capacidad antimicrobiana de las colonias aisladas de las plumas y de la cáscara de los huevos, en los nidos artificiales pusimos un solo huevo de codorniz que, al igual que en nidos naturales, muestreamos a los 12 días de ponerlo en el nido. Además, en este día recogimos una pluma pigmentada y/o una no pigmentada (según el nido tuviera un tratamiento de plumas pigmentadas, no pigmentadas, o mixto).

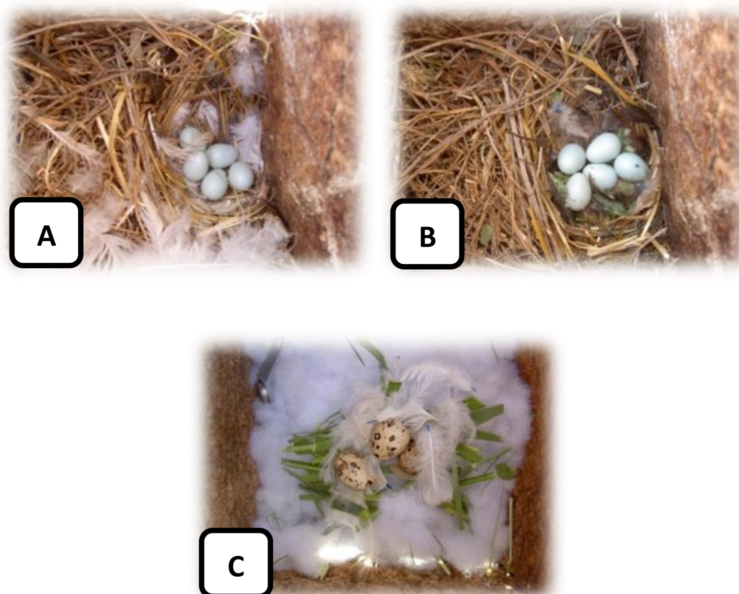


Figura 5: (A) Nido natural de estornino antes del tratamiento experimental, (B) mismo nido de estornino tras aplicarle el tratamiento experimental (plumas pigmentadas y plantas aromáticas) y (C) nido artificial con huevos de codorniz y con tratamiento de plumas no pigmentadas y cebada.

3. Manipulación de la carga bacteriana

Durante la temporada de cría del año 2013 (**capítulo 1**) realizamos un tercer experimento, para determinar si con un aumento de la carga bacteriana en la cáscara de los huevos los efectos de los materiales del nido eran mayores o más fáciles de detectar. Para ello, aumentamos la carga bacteriana de la superficie del huevo con una solución del interior de un huevo de gallina que previamente había sido contaminado por bacterias. Por tanto, en nuestro experimento utilizábamos cepas de bacterias capaces de atravesar la cáscara de los huevos. Para favorecer la contaminación de los huevos de gallina con bacterias de la zona de estudio, los pusimos durante dos o tres semanas en cajas-nido utilizadas por los estorninos en años anteriores. El interior de estos huevos los sembramos en medio TSA durante 72 h a 37 °C para comprobar la

presencia de bacterias en el interior del huevo de gallina. Con los huevos de gallina en los que se detectó contaminación bacteriana, preparamos una solución en la que mezclamos 300 µL del interior del huevo con 300 µL de tampón fosfato. Este cultivo lo guardábamos a 4 °C hasta el día anterior a su utilización en los nidos experimentales, cuando lo incubábamos a 37 °C durante toda la noche. Esta solución la extendíamos con un hisopo sobre la cáscara de dos de los huevos de las puestas de estorninos y en tres de los seis huevos de codorniz que dispusimos en los nidos artificiales. De este modo, aumentamos la carga bacteriana en dos tercios de los nidos naturales y artificiales, dejando el resto de nidos como controles. Los efectos del experimento se determinaron muestreando la carga bacteriana de la cáscara de los huevos de nidos naturales de estornino y de los huevos de codorniz de nidos artificiales.

En nidos naturales la manipulación de la carga bacteriana la hicimos a los 3 días del inicio de la puesta (nidos con 3 huevos) y renovamos el tratamiento el día 8 (5 días después). Realizamos dos muestreos, el primero en el día 3 después de iniciar la puesta donde muestreamos la carga bacteriana de un huevo al azar, para determinar la carga bacteriana antes de los tratamientos. Posteriormente extendimos la solución de huevo de gallina con bacterias experimentales sobre la superficie de otro huevo, y aplicamos los tratamientos de plumas y plantas en los nidos. El segundo muestreo lo hicimos a los 12 días, antes de que los huevos eclosionaran para determinar el efecto de las plumas y plantas sobre la carga bacteriana de huevos untados o no con la solución de bacterias experimentales.

En los nidos artificiales la manipulación de la carga bacteriana la hicimos el día en que pusimos los huevos en las cajas-nido y renovamos el tratamiento a los 5 y 9 días. Durante estos días, además muestreamos la carga bacteriana de un huevo que no se hubiese muestreado anteriormente para determinar el efecto de los materiales de plumas y plantas (sin el efecto de los

adultos; i.e., incubación) sobre la carga bacteriana de los huevos. Por último, hicimos un tercer muestreo a los 17 días para determinar si los efectos esperados de las plumas y plantas experimentales en la carga bacteriana eran más fáciles de detectar después de un mayor tiempo de exposición de los huevos a las condiciones ambientales.

4. Selección de plumas y plantas

Para evaluar la preferencia de los estorninos sobre distintos tipos de material para construir sus nidos (**capítulo 5**), en el año 2013 les ofrecimos plumas pigmentadas y no pigmentadas, y plantas aromáticas y no aromáticas *ad libitum*. Intentando poner de manifiesto posibles diferencias en la selección de material antes y después de comenzar la puesta de los huevos, tanto las plumas como las plantas se las ofrecimos desde una semana antes de la fecha estimada del inicio de la puesta en nuestra zona de estudio.

Las plumas se las ofrecimos en un total de 10 mallas de cada tipo de pluma de un tamaño de 24*24 cm y distribuidas por toda nuestra zona de estudio (Fig. 6A). Cada una de las mallas contenía 50 plumas de pavo (*Meleagris gallopavo*) de unos 10 cm de longitud y marcadas en el raquis con rotulador permanente para poder distinguirlas de las llevadas por los adultos. Las plumas las reponíamos cuando en las mallas quedaban 15 plumas o menos. De esta forma, siempre había plumas disponibles para los estorninos, y el número de veces que reponíamos cada malla fue utilizado como otro indicador de la preferencia.

Las plantas se las ofrecimos en contenedores de plástico de un tamaño de 6.5 cm de largo, 7 cm de ancho y 6 cm de alto, rellenos con espuma aislante (Sika Boom, Sika, S.A.U.) que, por su naturaleza porosa, permitía el anclaje de los tallos de las plantas y el relleno con agua para mantener las plantas frescas el mayor tiempo posible (Fig. 6B). Cada uno de los contenedores tenía cuatro tallos de unos 10 cm de largo, marcados

previamente con tinta de sellos, de cada una de las especies ofrecidas. Las plantas ofrecidas fueron cuatro aromáticas (*Marrubium vulgare*, *Artemisia barrelieri*, *Lamium amplexicaule, Anacyclus clavatus*) y una no aromática (*Hordeum vulgare*). Los contenedores fueron colocados sobre 2/3 de las cajas-nido (Fig. 6C) y reponíamos las plantas cada tres días, momento en el que contábamos los tallos que los estorninos se habían llevado.

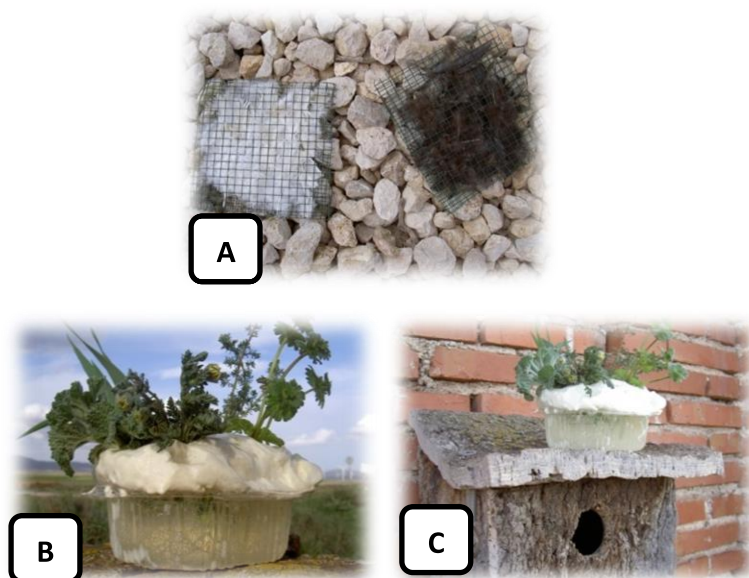


Figura 6: (A) Mallas con plumas de pavo pigmentadas y no pigmentadas disponibles para los estorninos. (B) Contenedor de plástico donde ofrecíamos los cinco tipos de plantas. (C) Contenedor sobre la tapa de la caja-nido con las cinco especies de plantas disponibles.

Las cajas-nido fueron visitadas cada tres días. En cada visita contábamos el número de plumas pigmentadas y no pigmentadas en los nidos y cuántas de ellas eran experimentales. También contábamos el número de tallos experimentales de plantas que nos encontrábamos en los nidos, anotando su especie y el peso de todas las plantas que hubiera en el nido.

Recogida y análisis de las muestras en el laboratorio

Carga bacteriana y actividad antimicrobiana

Para llevar a cabo los objetivos de esta Tesis estimamos la carga bacteriana de la cáscara de los huevos (**capítulos 1 y 4**), de la piel de los pollos (**capítulos 2 y 3**) y de la superficie de las plumas (**capítulo 4**) por medio de técnicas de cultivo. Aunque en los medios de cultivo solo una pequeña proporción de las bacterias totales pueden ser cultivadas, algunos estudios han observado que la densidad de bacterias estimada por medio de técnicas dependientes de cultivo y por medio de técnicas independientes de éstos, como los basados en PCR, estaban positivamente relacionadas (Lee et al. 2013). En esta Tesis, nos planteamos explorar los efectos de los materiales de nido, como son las plumas y las plantas sobre la carga bacteriana de los huevos y los pollos, con lo que la estima a través de técnicas dependientes de cultivo puede ser una buena aproximación para nuestros propósitos (Gwinner & Berger 2005; Cook et al. 2005a; Mennerat et al. 2009a; Peralta-Sánchez et al. 2010; González-Braojos et al. 2012b).

Todas las muestras recogidas en el campo, además de los diferentes manejos que hicimos de los materiales del nido y de los huevos y los pollos, los realizamos con guantes de látex nuevos y lavados con etanol al 96% para evitar la contaminación entre nidos.

1. *Recogida de muestras y estima de la carga bacteriana en huevos y pollos*

Los muestreos de la carga bacteriana de huevos y pollos los hicimos limpiando la superficie de un huevo (**capítulos 1 y 4**) o de la barriga de un pollo (**capítulo 2 y 3**) con ayuda de un hisopo estéril humedecido en tampón fosfato sódico (0.2 M, pH 7.2). Cada huevo o pollo lo muestreamos sólo una vez. A continuación, guardábamos el hisopo en un tubo Eppendorf con 1.2

mL de tampón fosfato a 4 °C hasta su posterior análisis en el laboratorio durante las 24 horas siguientes.

Una vez en el laboratorio, en la cabina de flujo laminar, después de agitar cada muestra en el vortex (Boeco V1Plus), realizamos diluciones seriadas (hasta 10^{-6}) y sembramos 100 µL de cada dilución en cuatro medios diferentes (Scharlau Chemie S.A. Barcelona): uno general para bacterias mesófilas (TSA), y tres medios restrictivos, Hektoen (HK) para enterobacterias, Kenner Fecal (KF) para enterococos y Vogel-Johnson (VJ) para estafilococos (las muestras de la superficie de huevos del **capítulo 4** se sembraron en otros medios, ver en el siguiente apartado). Estas placas las incubamos en la estufa a 37 °C durante 72h.

Transcurrido este tiempo de incubación, para la estimación de la carga bacteriana utilizamos el número de unidades formadoras de colonias (CFU) y estandarizamos por cm^2 de superficie muestreada. En el caso de los huevos, la superficie la estimamos con la siguiente fórmula: $S = (3.155 - 0.0136 * L + 0.0115 * W) * L * W$, siendo S la superficie del huevo, L la longitud y W la anchura (Narushin 2005). La superficie de la barriga de los pollos fue estimada con la misma fórmula, considerando la barriga como la mitad de un ovoide.

2. *Procesamiento y siembra de muestras bacterianas de plumas y estimación de la actividad antimicrobiana*

Uno de los principales objetivos del **capítulo 4** precisaba de la cuantificación de la actividad antimicrobiana de las comunidades bacterianas presentes en las plumas y en la cáscara de los huevos. Para ello, recogimos plumas pigmentadas y no pigmentadas de los nidos naturales de estornino y de los nidos artificiales de las que aislamos colonias bacterianas a las que posteriormente se les realizaron pruebas de antagonismo (ver más abajo). Las plumas las guardamos separadamente en bolsas herméticas a 4 °C hasta su

análisis en el laboratorio en los siguientes 30 días (las muestras de huevos fueron tomadas siguiendo el método del apartado anterior).

En el laboratorio, en condiciones de esterilidad, cortamos un trozo de aproximadamente 1 cm^2 de cada una de las plumas y lo introducimos en un tubo Eppendorf previamente esterilizado y tarado en el que determinamos el peso de la pluma cortada en una báscula de precisión (Metler Toledo, AB135-S/FACT Classic Plus; precisión 0.00001 g). A continuación le pusimos 1 mL de tampón fosfato sódico para la siembra. Tanto con las muestras de plumas como con las tomadas de los huevos, una vez agitadas en el vortex, hicimos diluciones seriadas (hasta 10^{-4}) y las sembramos en tres tipos de medio: un medio mesófilo (TSA) que nos permitía una estima de la densidad general de bacterias, y dos medios restrictivos para bacterias queratinolíticas fabricados con harina de pluma pigmentada (P-FMA) y con harina de pluma no pigmentada (UN-FMA). La estimación de la carga bacteriana de las muestras de huevos la obtuvimos por superficie muestreada, mientras que la estimación de la carga bacteriana de las plumas la obtuvimos en relación al peso de pluma utilizado.

Una vez teníamos las colonias crecidas en los diferentes medios aislamos un máximo de 5 colonias de morfología diferente de cada uno de los medios de cultivo. El método de aislamiento consistió en picar cada una de las colonias a aislar y transferirlas a un tubo con 6 mL de BHI que se incubaba durante 24 h a 37 °C. Una vez crecidas, las sembramos en placas de TSA en estría y las dejamos crecer durante 24 h a 37 °C. Cuando crecieron las guardamos a 4 °C hasta el ensayo de la capacidad antimicrobiana en las siguientes 72 h. La capacidad antimicrobiana de las colonias aisladas se estimó frente a 18 cepas bacterianas (ver más abajo) siguiendo el método de la doble capa (Gratia & Fredericq 1946). Para cada una de las colonias a testar hicimos 18 réplicas sembrándolas de nuevo en medio TSA y las incubamos a 37 °C durante 24 h. Una vez crecidas, depositamos sobre ellas una segunda

capa de BHA (Brain Heart Agar) que previamente habíamos inoculado con 100 µL de un cultivo de la cepa indicadora en BHI durante 12 h. Y de nuevo estas placas fueron incubadas a 37 °C durante 24 h. La actividad antimicrobiana se infería por la presencia de un halo de inhibición alrededor de la colonia testada (Fig. 7).

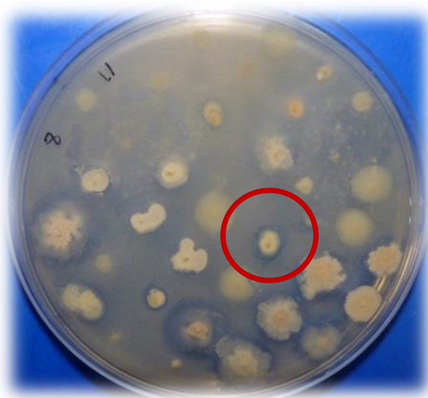


Figura 7: Colonias bacterianas testadas frente a una cepa indicadora, el círculo marca el halo de inhibición

Estimamos la capacidad antimicrobiana de estas colonias frente a 18 cepas indicadoras que dividimos en dos grupos: bacterias potencialmente patógenas (*Enterococcus faecalis MRR-103, Escherichia coli CECT774, Listeria monocytogenes CECT4032, Salmonella choleraesuis CECT443, Staphylococcus aureus CECT240*), y no patógenas (*Bacillus licheniformis D13, Bacillus megatherium, Bacillus thuringiensis, Enterococcus faecium UJA34, Klebsiella sp., Lactobacillus lactis lactis LM2301, Lactobacillus paracasei 11-2, Lactobacillus plantarum CECT784, Listeria inocua CECT340, Micrococcus luteus 241, Mycobacterium sp., Proteus sp., Pseudomonas putida*) (Pinowski et al. 1994; Hubálek 2004; Benskin et al. 2009).

Muestra sanguínea para estimar la capacidad de respuesta inmune y la longitud y dinámica de los telómeros

Para abordar los objetivos relacionados con los efectos de los materiales del nido sobre la respuesta inmune de los pollos y la longitud y dinámica de sus telómeros (**capítulos 2 y 3**), recogimos muestras de sangre poco después de la eclosión (a los 3 días de edad) y poco antes de abandonar al nido (a los 14 días de edad). Debido a la dificultad implícita en la obtención de muestras de sangre de pollos de 3 días de edad, el muestreo lo realizamos recogiendo una pequeña gota de sangre en un papel de filtro, mediante punción de la vena braquial, que guardamos a 4 °C hasta la extracción de ADN.

A los 14 días de edad obtuvimos una segunda muestra de sangre de todos los pollos que hubieran sobrevivido en el nido. En esta ocasión, con ayuda de capilares heparinizados, recogíamos 75 µL de sangre en tubos Eppendorf que guardábamos a 4 °C hasta su procesamiento en el laboratorio, dentro de las 12 h siguientes. Ya en el laboratorio, centrifugábamos la sangre durante 5 minutos a 17.000 g para separar el plasma de las células sanguíneas. El plasma lo guardábamos a -80 °C para su utilización en la estima de la respuesta inmune (**capítulo 2**), y las células en etanol a temperatura ambiente hasta su posterior utilización en la estima de la longitud y dinámica de telómeros (**capítulo 3**).

1. Estimación de la respuesta inmune

Como estimación de la capacidad de respuesta inmune seguimos el protocolo de Matson et al. (2005) que permite determinar componentes de la inmunidad humoral innata. Esta es la primera línea de defensa que tienen los pollos en el nido frente a bacterias y otros microorganismos (Playfair & Bancroft 2004). Esta primera línea defensiva está constituida por los anticuerpos naturales (NAbs) y el sistema del complemento cuya primera función es reconocer el antígeno e iniciar la reacción inmunitaria (Carroll & Prodeus 1998).

Brevemente, la técnica consiste en realizar diluciones seriadas de 50 µL de plasma en tampón fosfato rellenando dos placas de 96 pocillos y posteriormente añadir 25 µL de sangre de conejo (Hemostat laboratories, Dixon, CA 95620, USA, con anticoagulante de Alsevers y guardada a 4 °C hasta su uso en el ensayo). La estimación de respuesta humoral innata se relaciona con la capacidad de lisis y de aglutinación que se obtiene a partir del último número de pocillo en el que las reacciones de lisis y aglutinación con la sangre de conejo son observadas (Fig. 8). La lisis estima la acción del complemento por la cantidad de hemoglobina que libera de los eritrocitos de la sangre de conejo, mientras que la aglutinación estima la interacción entre los anticuerpos naturales y el antígeno, que en este caso es la sangre de conejo (Matson et al. 2005).

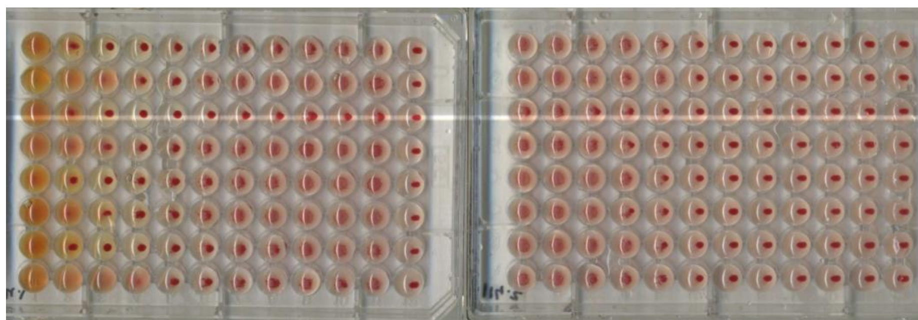


Figura 8: Placa en la que se muestra la reacción de lisis y aglutinación. Por ejemplo en la segunda fila, del pocillo 1 a 3 mostraría el proceso de lisis, los pocillos 4-5 mostrarían la forma más compacta de aglutinación, del 6 al 16 se mostraría una forma menos compacta de la aglutinación (sin contar el pocillo de la última columna de la primera placa, que junto con la última columna de la segunda placa son los controles, solo con tampón fosfato y sangre de conejo), el resto de pocillos del 17 al 22 muestran la falta de lisis y aglutinación. Por tanto, en este ejemplo las estimas de respuesta inmune tendrían valores de 3 para la lisis y 16 para la aglutinación.

2. *Estimación de la longitud de los telómeros*

Recientemente se está estudiando el acortamiento de los telómeros como una medida del estrés que pueden sufrir los pollos en el nido (Monaghan &

Haussmann 2006; Monaghan 2014). Los telómeros consisten en la repetición de una pequeña secuencia de ADN no codificante de las bases TTAGGG, y que se encuentra al final de los cromosomas protegiendo la información genética durante la replicación del ADN en la división celular (Blackburn 1991). La longitud de los telómeros se ha relacionado negativamente con los niveles basales de corticosterona, estrés oxidativo, etc. (Badás et al. 2015; Kim & Velando 2015; Quirici et al. 2016). Además, la longitud de los telómeros en los primeros días tiene un fuerte componente maternal (Asghar et al. 2015a), pudiendo afectar también otros factores como la fecha de puesta, la competencia por la comida (Soler et al. 2015a) o la infección por parásitos (Asghar et al. 2015b). En esta Tesis analizamos la posibilidad de que los diferentes materiales del nido afecten también a la longitud y dinámica de los telómeros. Para estimar estos efectos recogimos muestras de sangre de los pollos con 3 y 14 días de edad.

El ADN de las células sanguíneas de las muestras recogidas a los pollos fue extraído siguiendo el protocolo del alcohol cloroformo-isoamyl (Ferraguti et al. 2013; Soler et al. 2015a). La concentración de ADN la ajustamos a 20 ng/µL usando agua destilada. Y mediante PCR cuantitativa (qPCR) estimamos la longitud relativa de los telómeros siguiendo el protocolo de Criscuolo et al. (2009). Esta estimación por medio de qPCR es una estimación relativa debido a que la longitud de los telómeros se expresa en función de un gen control que no varía entre individuos de la misma especie o en un mismo individuo a lo largo del tiempo. En este caso utilizamos una copia del gen gliceraldehído-3-fosfato deshidrogenasa (GAPDH) para normalizar la cantidad de secuencias de telómeros a la cantidad de ADN utilizada en la reacción de PCR. Durante la qPCR se estiman para cada una de las muestras los valores C_t (número mínimo de ciclos de PCR que necesita una muestra para acumular el suficiente producto para superar el umbral de señal de fluorescencia), los cuales transformamos a NRQs (cantidad

normalizada relativa) siguiendo el procedimiento de Hellemans et al. (2007). Este procedimiento permite controlar las estimas por la eficiencia de amplificación de cada una de las qPCR, las cuales en nuestro caso siempre estuvieron dentro de los márgenes establecidos de 1.85-2.2. Utilizamos los valores de NRQs como indicadores de longitud relativa de los telómeros. En cuanto a la dinámica de los telómeros, aunque las estimas de longitud de telómeros de los pollos recién eclosionados fueron menores que en los de 14 días (N = 131; NRQ = 0.0776, SE = 0.020; NRQ = 1.00, SE = 0.039, respectivamente), debido a las diferencias en la forma de guardar la sangre obtenida de los pollos de 3 y 14 días, estas estimas pudieron verse afectadas. Por lo que para calcular la diferencia entre la longitud de telómeros de ambas edades, transformamos la variable NRQ en rangos, asignando el valor 1 al valor más bajo.

Estimación de abundancia de ectoparásitos

En el **capítulo 3** relacionamos la carga parasitaria en los pollos de estornino con la longitud y dinámica de los telómeros. La estima de abundancia de parásitos la realizamos a los 8 días de edad de los pollos, cuantificando el número de *Carnus hemapterus* (en adelante *Carnus*) en los nidos (Fig. 9). *Carnus* es un pequeño díptero hematófago de unos 2 mm que se distribuye por todo el Paleártico y gran parte de Norte América (Grimaldi 1997; Brake 2011). Por su ciclo de vida (Calero-Torralbo 2011) prefiere parasitar aves trogloditas (Grimaldi 1997) de las que parasita un gran número de especies (Grimaldi 1997; Dawson & Bortolotti 1997; Brake 2011). *Carnus* puede realizar su ciclo completamente en el nido de los hospedadores, y aunque tiene una fase dispersiva, ésta no es obligatoria (Calero-Torralbo 2011). Las larvas son detritívoras y se alimentan de restos o productos derivados de la actividad reproductora de las aves (i.e., restos derivados del crecimiento de los pollos) y pupan en el mismo nido. Las pupas son ápodas y se mantienen en los nidos hasta la emergencia de los adultos, la fase parásita de la especie (Calero-

Torralbo 2011). El adulto puede dispersarse y, cuando encuentra un nido apropiado, pierde las alas (Roulin 1998). La principal fuente de alimento de las moscas adultas son los pollos de edades tempranas (Václav et al. 2016), aunque también pueden alimentarse de las aves adultas cuando están incubando (López-Rull et al. 2007b; Avilés et al. 2009) y de pollos ya emplumados (Dawson & Bortolotti 1997; Roulin 1998; Liker et al. 2001). Tienen efectos nocivos sobre el desarrollo de los pollos (Avilés et al. 2009; Hoi et al. 2010), aunque estos efectos no son siempre patentes (Dawson & Bortolotti 1997; Roulin 1998).

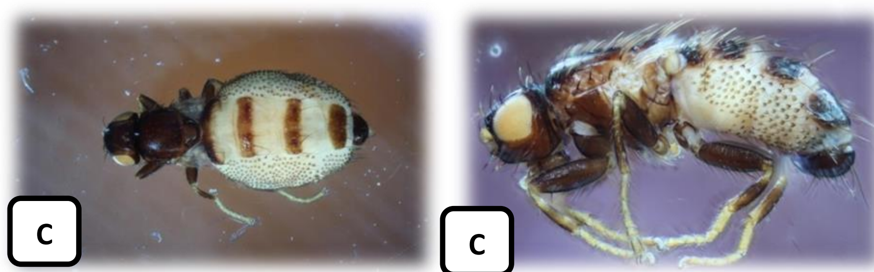


Figura 9: *Carnus hemapterus* (A) vista dorsal y (B) vista lateral (Fotos: Irina Brake)

Para estimar la abundancia de *Carnus*, extraíamos los pollos del nido con rapidez y cuidado. Los poníamos en una bolsa de captura limpia y de color blanco para, en el caso de que las *Carnus* abandonaran a los pollos, fueran fácilmente detectables dentro. A continuación cogíamos los pollos de uno en uno y contábamos las moscas que tenían en el cuerpo, las cuales principalmente se localizan en los pliegues de la piel y entre las alas y las patas. Por último, contábamos las moscas que pudieran haberse quedado en la bolsa (Avilés et al. 2009).

Estadística

Para analizar los efectos de los materiales experimentales de plumas y plantas sobre la carga bacteriana en pollos y huevos, y sobre las variables biométricas y estimas de éxito de vuelo y respuesta inmune (**capítulos 1 y 2**), utilizamos tres tipos de modelos estadísticos. En el caso de las variables de densidad de bacterias mesófilas, biométricas y de respuesta inmunitaria los datos se ajustaron a distribuciones normales después de una trasformación logarítmica y por ello, las incluimos como variables dependientes en análisis de modelos lineales generales (GLM). Para el caso de las bacterias crecidas en los medios restrictivos (enterobacterias, estafilococos y enterococos), la prevalencia fue relativamente baja, por lo que utilizamos la presencia o ausencia de estas bacterias como variable binomial en análisis de modelos lineales generalizados (GLZ). Por último, para el análisis del éxito de vuelo se asumió una distribución de Poisson en modelos GLZ. Las variables explicativas en todos estos modelos fueron los tratamientos de plumas y plantas y la presencia de plantas añadidas por los adultos como factores fijos. La fecha de puesta de los huevos o de nacimiento de los pollos y el número de plumas pigmentadas y no pigmentadas de cada muestreo se incluyó en los análisis como variables independientes continuas.

En el caso del efecto de los tratamientos de plumas sobre la carga bacteriana de los pollos (**capítulo 2**), hicimos dos aproximaciones complementarias: una primera para explorar el efecto de la presencia de plumas en los nidos (comparando nidos con plumas frente a nidos sin plumas), y una segunda aproximación para explorar el efecto de la pigmentación de las plumas (comparando nidos con plumas pigmentadas frente a nidos con plumas no pigmentadas). El razonamiento subyacente a estas aproximaciones se basa en los diferentes efectos encontrados previamente en relación a la pigmentación de las plumas en la fase de huevos

en nidos de golondrina (Peralta-Sánchez et al. 2010) y en nidos de estornino en el **capítulo 1**.

Para estimar el efecto de los materiales del nido, plumas y plantas, sobre la longitud y dinámica de los telómeros (**capítulo 3**), utilizamos las mismas variables explicativas que en el caso de la carga bacteriana de huevos y pollos, pero debido a que había más de un pollo por nido utilizamos modelos lineales generales mixtos (GLMM) con el nido como factor aleatorio y la abundancia del ectoparásito *Carnus* como covariable. Además, debido a que el experimento lo realizamos en dos poblaciones con distintos condicionantes ambientales (Huéneja y La Calahorra), incluimos el área de estudio como factor fijo.

Para explorar los efectos de los tratamientos de plumas experimentales, y el tipo de nido (artificial o natural), sobre la carga bacteriana de mesófilas y queratinolíticas en plumas y cáscaras de los huevos (**capítulo 4)** utilizamos GLMM con el método de Monte Carlo basado en las cadenas de Markov (MCMCglmm) en R (R Core Team 2015), con las librerías "MCMCglmm" (Hadfield 2010), "MASS" (Venables & Ripley 2002) y "mvtnorm" (Genz & Bretz 2009). Utilizamos el tipo de nido y los tratamientos como factores fijos, y la identidad del nido como factor aleatorio (i.e., más de una pluma era analizada por nido). La actividad antimicrobiana de las colonias aisladas frente a cada una de las cepas testadas fue utilizada como información binaria asumiendo una distribución multinomial en análisis MCMCglmm. En un primer modelo exploramos los efectos del origen de las colonias (pluma o cáscara de huevo), tipo de nido y tratamientos de plumas experimentales (factores fijos), y la identidad del nido como factor aleatorio. Y en un segundo modelo estudiamos el efecto de la pigmentación de la pluma de la cual habíamos aislado la colonia, incluyendo como factores fijos, el tipo de nido, el tratamiento experimental de plumas y la pigmentación de la pluma, con la identidad del nido como factor aleatorio.

Por último, para analizar la selección por parte de los estorninos de los materiales del nido (coloración de las plumas y plantas aromáticas; **capítulo 5**), utilizamos ANOVAs de medidas repetidas. El número de plumas de diferente pigmentación (experimentales y totales) y el peso de las plantas presentes en los nidos, se consideraron como variables dependientes. La disponibilidad de las plumas y las plantas, la etapa reproductiva (antes de la puesta o durante la puesta de los huevos) y la pigmentación de la pluma las incluimos como factores fijos. Y en un modelo separado estimamos el efecto de la fecha de puesta sobre las medidas repetidas. La utilización hecha por los estorninos de las plumas pigmentadas y no pigmentadas que les ofrecimos, estimada en base al número de veces que fue necesario reponer las plumas en las mallas, se analizó con un modelo GLM. Por su parte, la utilización de las plantas ofrecidas a los estorninos se analizó con un modelo GLZ con distribución binomial, considerando los tallos de las distintas especies de plantas presentes en los nidos.

Todos los análisis, excepto los mencionados con R, los llevamos a cabo con Statistica 8.0 (Statsoft Inc. 2011).

CAPÍTULOS

Capítulos

1. **Nest material shapes eggs bacterial environment**

   Cristina Ruiz-Castellano, Gustavo Tomás, Magdalena Ruiz-Rodríguez, David Martín-Gálvez & Juan J. Soler

   PLoS ONE 2016, 11(2): e0148894. doi:10.1371/journal.pone.0148894

2. **Nest materials influence the bacterial load and fitness of nestling birds**

   Cristina Ruiz-Castellano, Gustavo Tomás, Magdalena Ruiz-Rodríguez & Juan J. Soler

   Journal of Animal Ecology (submitted)

3. **Telomere length and dynamics of spotless starling nestlings depend on nest building materials used by parents**

   Juan J. Soler, Cristina Ruiz-Castellano, Jordi Figuerola, Manuel Martín-Vivaldi, Josué Martínez-de la Puente, Magdalena Ruiz-Rodríguez & Gustavo Tomás

   Animal Behaviour (submitted; 2nd revision)

4. **Parental activity and nest-lining feathers enhance antimicrobial capabilities of bacterial communities in avian nests**

   Cristina Ruiz-Castellano, Magdalena Ruiz-Rodríguez, Gustavo Tomás & Juan J. Soler

   Functional Ecology (submitted)

5. **Adaptive nest material selection by spotless starlings**

   Cristina Ruiz-Castellano, Gustavo Tomás, Magdalena Ruiz-Rodríguez & Juan J. Soler

   Behavioral Ecology (submitted)

CAPÍTULO 1

Nest material shapes eggs bacterial environment

Cristina Ruiz-Castellano, Gustavo Tomás, Magdalena Ruiz-Rodríguez,

David Martín-Gálvez & Juan José Soler

Abstract

Selective pressures imposed by pathogenic microorganisms to embryos have selected in hosts for a battery of antimicrobial lines of defenses that includes physical and chemical barriers. Due to the antimicrobial properties of volatile compounds of green plants and of chemicals of feather degrading bacteria, the use of aromatic plants and feathers for nest building has been suggested as one of these barriers. However, experimental evidence suggesting such effects is scarce in the literature. During two consecutive years, we explored experimentally the effects of these nest materials on loads of different groups of bacteria (mesophilic bacteria, *Enterobacteriaceae*, *Staphylococcus* and *Enterococcus*) of eggshells in nests of spotless starlings (*Sturnus unicolor*) at the beginning and at the end of the incubation period. This was also explored in artificial nests without incubation activity. We also experimentally increased bacterial density of eggs in natural and artificial nests and explored the effects of nest lining treatments on eggshell bacterial load. Support for the hypothetical antimicrobial function of nest materials was mainly detected for the year and location with larger average values of eggshell bacterial density. The beneficial effects of feathers and plants were more easily detected in artificial nests with no incubation activity, suggesting an active role of incubation against bacterial colonization of eggshells. Pigmented and unpigmented feathers reduced eggshell bacterial load in starling nests and artificial nest boxes. Results from artificial nests allowed us to discuss and discard alternative scenarios explaining the detected association, particularly those related to the possible sexual role of feathers and aromatic plants in starling nests. All these results considered together confirm the antimicrobial functionality mainly of feathers but also of plants used as nest materials, and highlight the importance of temporally and geographically environmental variation associated with risk of bacterial proliferation determining the strength of such effects. Because of costs associated to nest building, birds

should adjust nest building effort to expected bacterial environments during incubation, a prediction that should be further explored.

Keywords: aromatic plants, eggshell bacterial load, egg incubation, *Enterobacteriaceae*, *Enterococcus*, nest-lining feathers, mesophilic bacteria, spotless starling, *Staphylococcus*.

Introduction

Bird nests are infected by numerous parasites that affect dramatically their reproductive output (Clark & Mason 1985; Tomás et al. 2007; Soler et al. 2008). The best known are nest-dwelling ectoparasites, like mites and fleas (Lafuma et al. 2001; Cantarero et al. 2013). Microorganisms are also common in nests (González-Braojos et al. 2012), some of them being highly pathogenic for developing embryo (Pinowski et al. 1994). They can cross the eggshell (Godard et al. 2007), cause diseases in embryos (Bonisoli-Alquati et al. 2010) and, thus, reduce egg viability (Cook et al. 2005b). However, eggs have numerous defensive traits against pathogens like the eggshell and antimicrobial contents (Board et al. 1994; Shawkey et al. 2008; Wellman-Labadie et al. 2008; Soler et al. 2010; Horrocks et al. 2014). Although costly immunological barriers of eggs are quite effective fighting off potential pathogens (Saino et al. 2002; Playfair & Bancroft 2004), parents have also evolved additional defensive mechanisms to maintain their eggs free of parasites and pathogenic bacteria. For instance, birds can modulate their incubation behaviour in order to reduce humidity and thus conferring protection from precipitation or water that favour bacterial penetration (Cook et al. 2005a; Shawkey et al. 2009).

Some other birds like hoopoes (*Upupa epops*) preen their eggs with their own uropygial gland secretions to reduce density of pathogenic bacteria on the eggshell (Soler et al. 2008; Soler et al. 2012; Martín-Vivaldi et al. 2014). Others build a new –free of parasites– nest every year (González-Braojos et al. 2012), or remove the old nest materials from cavities before breeding (Pacejka & Thompson 1996; Mazgajski 2007). Some other bird species use substances produced by other animal or plant species for protection against pathogens (self-medication; Clayton & Wolfe 1993; De Roode et al. 2013).

A type of self-medication is the use of nest material with antimicrobial properties (Mainwaring et al. 2014). There are numerous materials used by birds with antimicrobial properties among which cigarette butts has been recently added to the list (Suárez-Rodríguez et al. 2013). The most studied nest materials with known antipathogenic effects are green plants (Clark & Mason 1985; Tomás et al. 2012; Dubiec et al. 2013). Most of the used green plants are aromatic plants that contain volatile compounds or essential oils (Clark & Mason 1985), which can play a repellent, fumigant or toxic role reducing abundance or minimizing the effect of pathogenic bacteria (Gwinner & Berger 2005; Mennerat et al. 2009a; Mennerat et al. 2009b) and parasites (Clark 1990; Tomás et al. 2012). Experimental evidence on antimicrobial properties of green plants reducing risk of bacterial infection on developing nestlings (Gwinner & Berger 2005; Mennerat et al. 2009a; Mennerat et al. 2009b) and embryos (Møller et al. 2013) is however scarce.

Nest lining feathers have traditionally been studied for their thermoregulatory properties (Hilton et al. 2004; Windsor et al. 2013) or their function as sexual display (Veiga & Polo 2005; Sanz & García-Navas 2011; García-López de Hierro et al. 2013; García-Navas et al. 2015). More recently, evidence of an antimicrobial function has been found in barn swallow nests (*Hirundo rustica*) (Peralta-Sánchez et al. 2010; 2011). This function may be due to bacterial strains, like *Bacillus licheniformis*, that live on feathers and digest the keratin (the main component of feathers), and are able to outcompete other bacteria by producing antibiotic agents (Gálvez et al. 1994; Soler et al. 2010). Those antimicrobials can help to fight off other bacteria with potentially stronger negative effects on developing embryos and nestlings. It is even known that the antimicrobial properties of bacteria degrading pigmented and unpigmented feathers differ depending on the nest lining feather composition (Peralta-Sánchez et al. 2014). Thus, the effects on the nest bacterial environment would depend on the abundance of pigmented

and unpigmented feathers lining the nest of birds (Soler et al. 2010). Evidences of the antimicrobial benefits of feathers are only known for barn swallow nests (Peralta-Sánchez et al. 2010; 2011; 2014), and exploring the expected effect on nests of other species is needed.

Some avian species such as blue tits (*Cyanistes caeruleus*) or spotless starlings (*Sturnus unicolor*) use both green plants and feathers as nest material (Polo & Veiga 2006; Mennerat et al. 2009b; Sanz & García-Navas 2011). Since antimicrobials of plants may affect not only pathogenic, but also antibiotic-producing bacteria of feathers, an interaction between both materials explaining bacterial environment of nests may be expected; a hypothesis that has not been hitherto investigated. In addition, since incubation activity can affect bacterial environment of nests (i.e., reducing eggshell bacterial load, e.g., (Cook et al. 2005a; Shawkey et al. 2009; D'Alba et al. 2010)), this behaviour may also modulate the effect of nest materials on bacterial density on eggshells. Thus, taking into account the effects of incubation is crucial to explore the isolate effect of nest materials on eggshell bacterial loads.

Here, we tried to fill these gaps with a study in spotless starlings, a species in which adults introduce green plants and feathers during the nest building and incubation stages. The use of feathers and green plants as nest material acts as sexual signals (Veiga & Polo 2005; Polo & Veiga 2006), and here we explore the possibility of additional antimicrobial functionality. Experimentally, we explore the combined effect of feathers and green plants explaining eggshell bacterial load in presence and absence of incubation. Each natural and artificial nest was randomly assigned to one of three feather treatments (only unpigmented feathers, only pigmented feathers or without feathers) and to one of two aromatic plants treatments (with or without aromatic plants). These experiments were performed in two different years and in different areas. Because of the presumed antibacterial effects of plants

and feathers, we expected a reduced eggshell bacterial load in nests where either plants or feathers were included. We also expected an interacting effect between experimental treatments because the antimicrobial compounds of plants may clean beneficial bacteria of feathers. Moreover, we expected that the effects of antimicrobial compounds should be more easily detected in high density bacterial environments (i.e., years or areas where the highest bacterial density were detected).

Materials and Methods

Ethics statement

The study was performed according to relevant Spanish national (Decreto 105/2011, 19 de Abril) and regional guidelines. The protocol was approved by ethics committee of Spanish National Research Council (CSIC) and all necessary permits for nest and egg manipulations were obtained from Consejería de Medio Ambiente de la Junta de Andalucía, Spain (Ref: SGYB/FOA/AFR/CFS and SGMN/GyB/JMIF). Our study area is not protected but privately owned, and the owners allowed us to work in their properties. This study did not involve endangered or protected species.

Time spent in each starling nest was the minimum necessary for bacterial sampling and for treatment application. This experiment did not show detectable effects in adult incubation behaviour or egg viability.

Study area and field work

The study was performed during the breeding seasons 2012 and 2013 in Hoya de Guadix, southeast Spain (37º18' N, 3º11' W), a high-altitude plateau 1000 m a.s.l, with a semi-arid climate. There were 80 cork-made nests boxes (internal height * width * depth: 350 * 180 * 210 mm, bottom-to-hole height: 240 mm) available for spotless starlings attached to tree trunks or walls at 3-4

m above ground. Our starling population usually commences to build their nests in March and they use green plants and feathers as nest material. Green plants and feathers are embedded in the nests, forming part of both their structural and lining layer. Our starling population laid eggs at mid-April, and since April 10th we visited nest boxes every three days until the first egg was laid. Laying dates were later in 2012 than in 2013 (2012: 27.45 ± 0.96; 2013: 23.00 ± 0.90 (April 1st = 1); ANOVA: $F = 11.24$, $df = 1,115$, $P = 0.001$). Incubation period in spotless starlings starts before the clutch is finished, usually with the third or fourth egg, and lasts for 7-12 days after laying the third egg. Environmental conditions in our study area differed between years. Mean daily temperatures, as well as minimum and maximum temperatures, were higher in 2012 (14.7 ± 0.9 ºC, 7.8 ± 0.7 ºC and 21.7 ± 1.1 ºC) than in 2013 (11.9 ± 0.7 ºC, 6.3 ± 0.6 ºC and 18.3 ± 0.9 ºC) (ANOVA: $F = 6.20$, $df = 1,76$, $P = 0.015$, $F = 2.56$, $df = 1,76$, $P = 0.114$, and $F = 6.27$, $df = 1,76$, $P = 0.015$, respectively). Total rainfall during the laying period was higher in 2013 (36.8 mm) than in 2012 (25.2 mm). Thus, mean humidity was higher in 2013 (70.89 ± 2.10%) than in 2012 (49.41 ± 2.51%) (ANOVA: $F = 43.43$, $df = 1,76$, $P < 0.001$) (data was obtained from the nearest climatological station, sited in Jerez del Marquesado: http://www.juntadeandalucia.es/agriculturaypesca/ifapa/ria/servlet/FrontController?action=Static&url=coordenadas.jsp&c_provincia=18&c_estacion=6).

Experimental procedures

Preparation of experimental nest lining feathers and aromatic plants

We collected white (i.e., unpigmented) and non-white (i.e., pigmented) body feathers of similar size as those used by starlings as lining material from chickens that grew in small farms close to the study area, which are common nest materials used by starlings in our population. Feathers were sterilized in the laboratory using a UV sterilizer chamber (Burdinola BV-100) during 10

minutes on each feather side. Subsequently, to homogenize the bacterial load and colonies on feathers, we sprayed, with an atomizer, approximately 84 ml of a high concentration solution of *Bacillus licheniformis* D13 per m^2 of surface completely covering experimental feathers. Solution was made from an overnight growth of a *Bacillus* colony in 6 ml of BHI (Brain Heart Infusion) media at 37 ºC on an orbital shaker. Finally, in separate hermetic bags we stored 15 pigmented or unpigmented feathers (i.e., the average number of feathers found in starling nests in previous years in the study area) at 4 ºC until its use in experimental nests. We used *Bacillus licheniformis* because it is a common feather-degrading and antimicrobial-producing bacterium (Soler et al. 2010).

Plants introduced in nests were a mixture of the four plant species most used by starlings in our population (personal observation); *Marrubium vulgare*, *Artemisia barrelieri*, *Lamium amplexicaule* and *Anacyclus clavatus*. All these plants have volatile compounds or essential oils with known antimicrobial activity (Meyre-Silva & Cechinel-Filho 2010; Fiamegos et al. 2011; Chipeva et al. 2013; Selles et al. 2013). Fragments of plants of similar size as those used by starlings were collected the same day of the experiment in the surroundings of the study area and therefore were placed fresh in nests in the nest cup, below the eggs. We weighed 1.7 g of plant mixture for each nest (approx. 0.425 g of each plant species) because this is the maximum amount of green plants that we found in starling nests in previous years in the study area (personal observation).

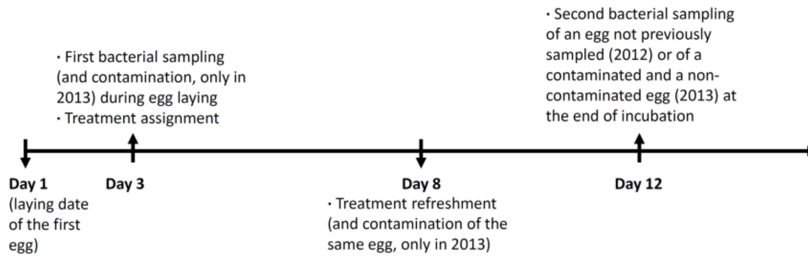
Experimental design in natural nests

Our experiment followed a factorial design with feathers and aromatic plants (Fig. 1A). Feathers treatments consisted of allocating (i) 15 pigmented, or (ii) 15 unpigmented feathers to the nest, or (iii) leave the nest without feathers. Plants treatments consisted of (i) introducing 1.7 g of a mixture of aromatic

plants or (ii) leave the nest without plants. Our experiment started on day 3 (i.e., nests had three eggs), by removing all plants and feathers that starlings had visible in their nests. Each nest was assigned to each of the experimental treatments for feathers and plants (Fig. 1A, see below). On day 3, we also numbered each egg with a permanent marker (Staedtler permanent Lumocolor), and, before experimental manipulation, we sampled the eggshell of an egg to characterize the nest bacterial environment at the beginning of the experimental treatment. On day 8 (i.e., at the beginning of incubation), we remarked each egg and measured length and breadth of all eggs in nests with a digital caliper to the nearest 0.01 mm. We also counted nest-lining feathers (distinguishing between pigmented and unpigmented), removed those added by adults, and refreshed the experimental treatment by adding pigmented and unpigmented feathers up to achieve the initial numbers. Finally, we removed and weighted green plants that were added by adults to nest materials and refreshed the experimental treatment. On day 12 (i.e., at the end of incubation), we sampled again the eggshells of one egg per nest that was not sampled during the first visit to characterize the bacterial environment of the nest after the experimental treatment. We also counted all lining feathers in the nest. A reliable estimation of green plant weight was not possible because small pieces were included as lining material on nest cup but also inserted within the nest material, being impossible to extract them without affecting nest structure. Thus we did not quantify green material.

A) Starling nests

Feathers / Plants	Unpigmented	Pigmented	Without feathers	Total nests
Aromatic plants	11/11	9/12	9/11	29/34
Without plants	10/9	6/11	8/10	24/30
Total nests	21/20	15/23	17/21	53/64



B) Quail eggs in non-incubated nests

Feathers / Plants	Unpigmented	Pigmented	Without feathers	Total nests
Aromatic plants	8/15	9/18	9/20	26/53
Non-aromatic plants	10/17	9/16	9/16	28/49
Without plants	5/15	5/18	9/21	19/54
Total nests	23/47	23/52	27/57	73/156

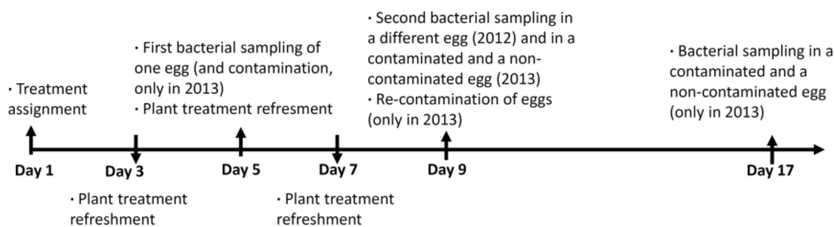


Figure 1.1: Experimental design of artificial and natural nests. Experimental protocols designed for exploring the effects of feathers and aromatic plants as nest materials on bacterial loads on spotless starling eggs (A) and on quail eggs in non-incubated nests (B). Numbers within the tables indicate sample sizes of different experimental treatment for 2012/2013 study years.

We collected information on eggshell bacterial loads for 117 starling nests, 53 in 2012 and 64 in 2013 (see S1 Appendix). The experiments were effective in causing differences between nests under different treatments in the number of pigmented and unpigmented feathers at the time of hatching (Table 1).

Table 1. Influence of experimental treatments on nest lining feathers and aromatic plants. Influences of (A) feathers treatment (pigmented, unpigmented and without feathers) (B) and aromatic plants treatment (with or without) on nest lining feathers found in spotless starling nests at the end of incubation. Statistical tests were performed with log-transformed variables. Significant P-values are in bold.

(A) NEST LINING FEATHERS

		TREATMENTS			Statistical tests			
		No feathers	Pigmented	UnPigmented				
Year		Mean, SE (N)	Mean, SE (N)	Mean, SE (N)	F	df	P	Tolerance
2012	Number of feathers	19.0, 3.3 (17)	21.7, 10.9 (15)	23.2, 2.6 (21)	1.18	2.50	0.315	0.955
	Pigmented feathers	9.9, 1.6 (17)	14.3, 1.8 (15)	10.4, 2.5 (21)	2.50	2,50	0.092	0.909
	Unpigmented feathers	**9.1, 2.2 (17)**	**7.5, 1.5 (15)**	**12.8, 1.4 (21)**	**4.61**	**2,50**	**0.015**	**0.844**
2013	Number of feathers	23.6, 3.0 (21)	27.5, 3.2 (23)	26.2, 3.3 (20)	0.35	2,61	0.703	0.989
	Pigmented feathers	**3.1, 0.7 (21)**	**10.0, 0.9 (23)**	**2.3, 0.8 (20)**	**25.25**	**2,61**	**0.000**	**0.547**
	Unpigmented feathers	20.5, 2.9 (21)	17.4, 2.7 (23)	24.0, 2.9 (20)	2.46	2,61	0.094	0.727

(B) AROMATIC PLANTS

		TREATMENTS		Statistical tests			
		No aromatic plants	Aromatic plants				
Year		Mean, SE (N)	Mean, SE (N)	F	df	P	Tolerance
2012	Number of feathers	19.1, 2.3 (24)	23.4, 2.3 (29)	1.51	1,51	0.224	0.971
	Pigmented feathers	10.4, 2.1 (24)	12.1, 7.5 (29)	1.26	1,51	0.268	0.976
	Unpigmented feathers	8.7, 1.1 (24)	11.3, 1.6 (29)	0.65	1,51	0.423	0.970
2013	Number of feathers	29.7, 2.9 (30)	22.3, 2.1 (34)	3.36	1,62	0.072	0.949
	Pigmented feathers	6.0, 1.1 (30)	4.7, 0.8 (34)	0.16	1,62	0.699	0.997
	Unpigmented feathers	23.7, 2.7 (30)	17.7, 1.9 (34)	0.63	1,62	0.428	0.990

Experimental design in artificial nests

The experimental design for artificial nests was similar to that of natural nests. We included an additional treatment to the aromatic plant experiment consisting of adding 1.7 g of green barley (*Hordeum vulgare*) leaves (i.e., a non-aromatic plant, see Tomás et al. (2013)) as a control of aromatic plants.

This experiment was performed at two different localities in each of the two study years (Pinos (i.e., Area 1) and Pocico (i.e., Area 2) in 2012 and Calahorra (i.e., Area 3) and Area 1 in 2013). These areas are relatively close to each other and belong to Hoya de Guadix area. 73 new nest-boxes in 2012 and 156 in 2013 were placed in the study area (see S1 Appendix) for this experiment. The entrance of these nest-boxes was closed with a plastic mesh to prevent birds´ and/or predators´ access. They were filled (one fourth of the volume) with previously ultraviolet sterilized polyester fiberfill, on top of which experimental nest material (aromatic/non aromatic plants and/or feathers) were placed in a hollow simulating a nest cup. Two and three quail eggs previously cleaned with disinfectant wipes (Aseptonet, LaboratoiresSarbec, Cod.998077-51EN) were laid on top of the experimental nest material in 2012 and 2013 respectively.

Experiments in nest-boxes with no incubation activity were all performed the same day (the 2nd of April in 2012 and the 11th of April in 2013, hereafter day 1) (Fig. 1B). Nest-boxes were visited every second day to refresh aromatic and non-aromatic plants. Also, every second day the eggs were gently moved ensuring contact of the whole eggshell surface with nest lining material. Bacteria from shells of each egg were sampled only once. Thus, different experimental eggs were sampled on day 5 and on day 9. In 2013, we collected a third sample on day 17.

Contamination experiment procedure

Only in 2013, we performed an additional experiment consisting on infecting starling and quail eggshells with bacterial strains known to be able to cross avian eggshells (Fig. 1). These bacteria were collected from the interior of hen eggs that were kept in nest-boxes in the study area for two-three weeks (i.e., were exposed to the environmental conditions of the study area). Briefly, with a sterile rayon swab (EUROTUBO® DeltaLab) wet with a solution of 300 µl of sodium phosphate buffer (0.2 M; pH = 7.2) and 300 µl of egg contents with bacteria (we confirmed a high bacterial load in this solution by overnight cultivation at 37 ºC), we besmeared two starling and three quail eggs in two-thirds of the nests under different plants' and feathers' experimental treatments leaving the other nests as controls.

Bacterial sampling

For each nest visiting and sampling we wore new gloves sterilized with 96% ethanol to prevent contamination of eggshell bacterial samples among nests. For eggshell bacterial sampling we cleaned the complete egg surface with a sterile rayon swab (EUROTUBO® DeltaLab) slightly wet with sterile sodium phosphate buffer (0.2 M; pH = 7.2). After cleaning, we introduced the swab in an Eppendorf tube with the buffer solution and preserved it at 4-6 ºC in a portable refrigerator until being processed in the laboratory within 24 h after collection.

Laboratory work

After vigorously shaken in a vortex (Boeco V1 Plus), eggshell bacterial samples of starlings and quails were cultivated in four different solid media (Scharlau Chemie S.A., Barcelona). For that, we spread homogeneously 100 µl of serially diluted samples until 10^{-6}.

We used Tryptic Soy Agar, a broadly used general medium to grow aerobic mesophilic bacteria, and three specific media: Hektoen Enteric Agar for *Enterobacteriaceae*, Vogel-Johnson Agar for *Staphylococcus*, and Kenner Fecal Agar for *Enterococcus*. Plates were incubated at 37 ºC and after 72 h the number of colonies on each plate was counted. For more details see (Peralta-Sánchez et al. 2010).

Eggshell bacterial density was estimated by standardization of the number of colonies per cm^2 of sampled eggshell (CFU, Colony Forming Units). Eggshell surface was estimated following Narushin formula (1997) from length and width of each egg ($S = 3 * L^{0.771} * W^{1.229}$, where S is the egg surface in cm^2, W is the egg width and L is the egg length). Characterization of bacterial environments by traditional culture techniques have been demonstrated as appropriate for exploring associations between eggshell bacterial density and risk of embryo infection (Cook et al. 2005a; Cook et al. 2005b; Martín-Vivaldi et al. 2014) and, thus, for our purposes.

Sample sizes and statistical analysis

Mesophilic bacteria and number of feathers did approximately follow normal distributions after log10 transformation. The effects of feather and plant treatments on mesophilic bacterial loads and growth during the incubation period (i.e., changes in bacterial load between sampling events) were separately analysed for different study years by means of General Linear Models (GLM). Experimental treatments were included as fixed discrete factors, and the following variables as continuous predictors: (i) date of sampling, log-transformed (ii) number of pigmented and (iii) unpigmented feathers at the time of experimental manipulation, and (iv) number of pigmented and (v) unpigmented feathers found in starling nests soon before hatching. Including the number of feathers at the end of incubation together with experimental treatments does not imply collinearity problems because of

relatively low correlation coefficient among these two factors (Table 1) (Quinn & Keough 2002).

Contrary to one of our predictions, the interaction between treatments was far from statistical significance for all models tested ($P > 0.2$) and, thus, it was not considered for the final analyses. Non-significant terms in the models with the highest p-value were removed one by one up to p-values lower than 0.1. Results are shown for both complete (in Appendices) and reduced statistical models.

Prevalence of bacteria growing in specific media was relatively low in starlings eggshells (*Enterobacteriaceae*: 9.7% and 3.7%; *Staphylococcus*: 7.5% and 10.4%; and *Enterococcus*: 14.9% and 10.4% for first and second sampling, respectively). Consequently, we analysed presence/absence rather than density in relation to experimental treatments in Generalized Linear Models (GLZ) with binomial error and logic link function. Factors in these models were those included in GLM models explaining mesophilic bacterial loads without log-transformation and the analyses controlled for overdispersion. In the model of first sampling, effects of nest material are shown for the complete model, because we did not detected any effect of the experiment. In the other models non-significant terms with the highest p-value were removed in the same way than for GLM. Chi-square Maximum Likelihood values were estimated in a type III analysis.

Prevalence of specific bacteria on non-incubated nest boxes was very low (< 2% in all cases) and, thus, the effects of experimental treatments on eggshell bacterial loads and on probability of trans-shell colonization were analysed only for mesophilic bacteria. Since all experimental boxes were explored the same day and the whole nest lining material was experimental (i.e., no covariable that varied among sampling date was necessary), we explored these effects in Repeated Measures ANOVAs.

Results

Eggshell bacterial loads in natural starling nests

Nest material and mesophilic bacterial loads comparisons between first and second sampling

In first sampling, at time of egg laying pigmented feathers were more abundant in 2012 than in 2013, whereas unpigmented and total number of feathers did not differ between study years (Table 2). Density of mesophilic bacteria on the eggshell was higher in 2013 than in 2012 breeding season (Table 2).

In second sampling, at the end of incubation, density of mesophilic bacteria and number of unpigmented feathers were higher in samples from 2013 than in those from 2012 (Table 2). Number of pigmented feathers were however lower in 2013 than in 2012 (Table 2).

Nest material and experimental treatments effect on mesophilic bacterial load

At the time of laying (day 3), in 2012 we found a negative relationship between number of unpigmented feathers and mesophilic bacterial load on eggshells (Beta (SE) = -0.167 (0.089), $F = 3.73$, df = 1,50, $P = 0.068$; Fig. 2A). However, in 2013 unpigmented feathers did not affect mesophilic bacterial load ($F = 0.70$, df = 1,60, $P = 0.407$). In this year, mesophilic bacterial load increased as the season progressed (Beta (SE) = 0.025 (0.009), $F = 7.48$, df = 1,60, $P = 0.008$; Fig. 2B). No other variables affected mesophilic bacterial load at the time of laying in 2012 ($F < 1.91$, df = 1,49, $P > 0.173$) and 2013 ($F < 0.69$, df = 1,60, $P > 0.407$).

Table 2. Among years variation in nest feathers and bacteria. Inter-annual differences in nest lining materials (total, pigmented and unpigmented feathers) and density of mesophilic bacteria on spotless starling eggshells during the laying stage, at the end of the incubation period and changes experienced during the incubation period. Significant p-values are in bold.

	2012		2013		Comparisons	
Laying (day 3)	Mean (SE)	N	Mean (SE)	N	F	P
log number of feathers	2.243 (0.121)	53	2.074 (0.094)	64	1.25	0.266
log pigmented feathers	**1.715 (0.136)**	**53**	**1.195 (0.121)**	**64**	**8.21**	**0.005**
log unpigmented feathers	1.371 (0.124)	53	1.517 (0.091)	64	0.93	0.336
log mesophilic bacterial density	**0.994 (0.079)**	**53**	**1.302 (0.062)**	**64**	**9.69**	**0.002**
End of incubation (day 12)						
log number of feathers	2.973 (0.074)	53	3.162 (0.063)	64	3.80	0.054
log pigmented feathers	**2.264 (0.102)**	**53**	**1.420 (0.126)**	**64**	**25.71**	**<0.0001**
log unpigmented feathers	**2.173 (0.103)**	**53**	**2.832 (0.098)**	**64**	**21.25**	**<0.0001**
log mesophilic bacterial density	**1.016 (0.056)**	**53**	**1.277 (0.051)**	**64**	**12.00**	**0.001**
Along incubation changes (Δ day 3-day 12)						
log number of feathers	**0.730 (0.141)**	**53**	**1.088 (0.112)**	**64**	**4.05**	**0.047**
log pigmented feathers	0.549 (0.164)	53	0.224 (0.181)	64	1.71	0.194
log unpigmented feathers	**0.801 (0.146)**	**53**	**1.315 (0.121)**	**64**	**7.48**	**0.007**
log mesophilic bacterial density	0.022 (0.082)	53	-0.025(0.064)	64	0.21	0.649

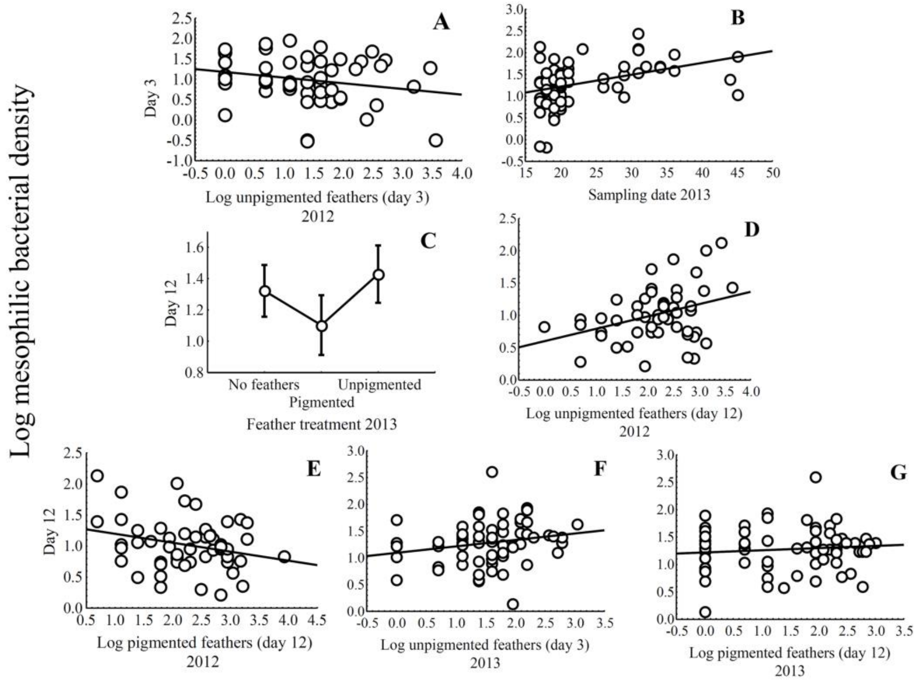


Figure 1.2: Nest material and experimental treatments effect on mesophilic bacterial load. Statistically significant relationships between loads of mesophilic bacteria of starling eggshells at day 3 in relation to number of unpigmented feathers in 2012 (A) and date of sampling in 2013 (B). The relationships between eggshell mesophilic bacterial load at day 12 (± 95% CI) in relation to feather treatment in 2013 (C), unpigmented feathers at day 12 in 2012 (D), pigmented feathers at day 12 in 2012 (E), unpigmented feathers at day 3 in 2013 (F) and pigmented feathers at day 12 in 2013 (G) are also shown.

At the end of incubation (day 12), the reduced model showed that eggshells of experimental nests with pigmented feathers treatment harboured lower mesophilic bacterial load (Table 3; Beta (SE) = -0.182 (0.088), t = -2.07, P = 0.042; Fig. 2C). However, experimental feather manipulation in 2012, and manipulation of aromatic plant material in 2012 and 2013, did not significantly affect mesophilic bacterial load on the eggshell at the end of incubation (see S2 Appendix).

Table 3. Results from GLM explaining mesophilic bacterial density on incubated spotless starling eggshells at the end of incubation (day 12). Nest lining materials (pigmented and unpigmented feathers) before incubation started (1st) and at the end of incubation (2nd) were included as continuous independent factors. Experimental treatments of aromatic plants (with or without) and of feathers (pigmented, unpigmented and without feathers) were included as fixed factors. In 2013, we used a third experimental treatment that consisted on eggshell contamination at the time of egg laying. Interactions between treatments did not reach statistical significance (2012: $P = 0.23$; 2013: $P > 0.15$) and are not shown. We only show final models with retained factors with p-values < 0.1. However, associated statistical significance of different factors did not change in full models (see S2 Appendix). Significant associations are in bold.

	Beta (SE)	df	F	P
2012				
log pigmented feathers (2nd)	**-0.151 (0.070)**	**1,50**	**4.74**	**0.034**
log unpigmented feathers (2nd)	**0.196 (0.069)**	**1,50**	**8.07**	**0.006**
2013				
date of first sampling (1=1 April)	**0.015 (0.008)**	**1,57**	**3.59**	**0.063**
log unpigmented feathers (1st)	**0.150 (0.072)**	**1,57**	**4.41**	**0.040**
log pigmented feathers (2nd)	**0.153 (0.066)**	**1,57**	**5.40**	**0.023**
log unpigmented feathers (2nd)	-0.127 (0.070)	1,57	3.26	0.076
Feather treatment		2,57	2.38	0.101

Feather nest material did also affect mesophilic bacterial load at the end of incubation in both years. In 2012, mesophilic bacterial load was positively related to number of unpigmented feathers at the end of incubation (Table 3; Fig. 2D) and negatively related to number of pigmented feathers at the end of incubation (Table 3; Fig. 2E). In 2013, mesophilic bacterial load was higher in nests with more unpigmented feathers at time of laying (Fig. 2F), and tended to be negatively and positively related to number of unpigmented and pigmented feathers at day 12, respectively (Table 3; Fig. 2G). Finally, in 2013 coating eggshells with a solution of bacteria on egg contents did not affect mesophilic bacterial loads (S2 Appendix).

When we explored the variation in eggshell bacterial loads along incubation (variation between day 3 and day 12) we found that in 2012, the performed experiments with nest lining materials (aromatic plants or feathers) did not affect changes in mesophilic bacterial loads of eggshells along the incubation period (S3 Appendix). In 2013, experimental manipulation of nest lining feathers, but not that of aromatic plants, did explain changes in mesophilic bacterial loads along incubation period (Table 4): only experimental nests with pigmented feathers, but not nests without feathers or those with unpigmented feathers, did reduce eggshell bacterial loads from laying to the end of incubation (Fig. 3A).

Table 4. Results from GLM explaining changes in mesophilic bacterial density on spotless starling eggshells along the incubation period (changes between day 3 and day 12). Nest lining material (pigmented and unpigmented feathers) before incubation started (1st) and few days before hatching (2nd) were included as continuous independent covariates. Experimental modification of green plants (i.e. with or without aromatic plants) and of feathers (i.e. pigmented, unpigmented or without feathers treatment) were included as factors with fixed effects. In 2013, we used a third experimental treatment that consisted on eggshell contamination at the time of egg laying. We only show final models with retained factors with p-values < 0.1. However, associated statistical significances of different factors did not change in full models (see S3 Appendix). Significant associations are in bold.

	Beta (SE)	df	F	P
2012				
log pigmented feathers (1st)	**-0.202 (0.077)**	**1,50**	**6.85**	**0.012**
log unpigmented feathers (1st)	**0.305 (0.085)**	**1,50**	**11.17**	**0.002**
2013				
log pigmented feathers (2nd)	**0.216 (0.082)**	**1,60**	**6.91**	**0.011**
Feather treatment		**2,60**	**5.36**	**0.007**

Variation in eggshell bacterial loads along incubation in 2012 were however explained by nest materials at day 3 (i.e., negatively related with number of pigmented feathers (Fig. 3B), and positively related with number

of unpigmented feathers (Fig. 3C)) (Table 4). In 2013, the final model did retain the number of pigmented feathers at the time of second sampling, which was positively related with change in mesophilic bacterial load (Fig. 3D).

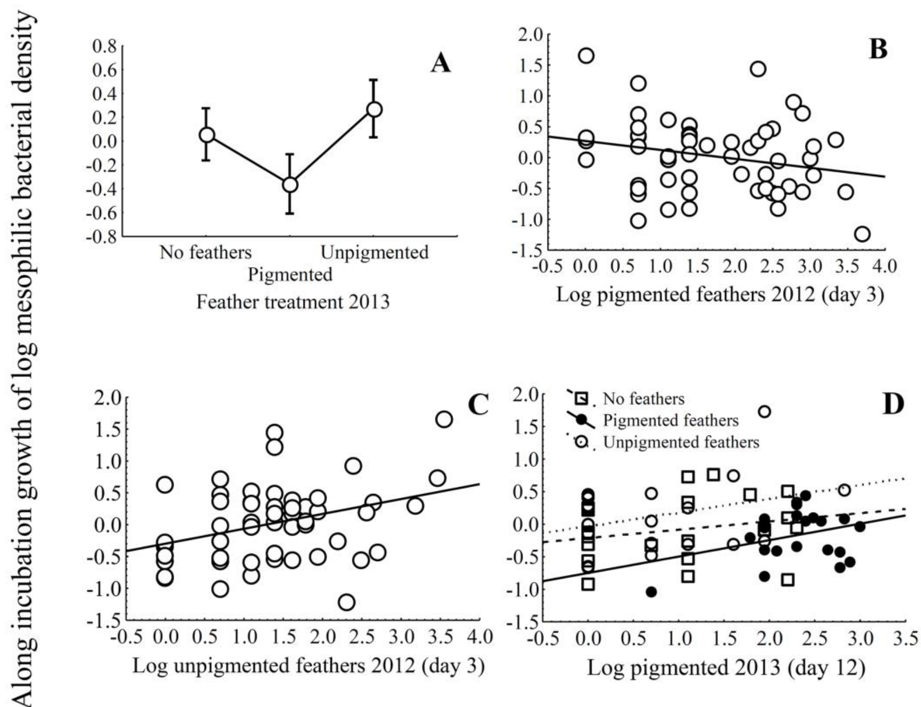


Figure 1.3: Results of mesophilic bacterial load along incubation (variation between day 3 and day 12). Average values of eggshell mesophilic bacterial growth (± 95% CI) on starling eggshells in relation to feathers' experimental treatments (unpigmented, pigmented or without feathers) in 2013 (A, D). The associations of eggshell mesophilic bacterial growth with number of pigmented (B) and unpigmented (C) feathers in starling nests in 2012 are also shown. Figure only shows the most relevant associations detected.

Nest material and experimental treatments effects on bacteria in specific media (Enterobacteriaceae, Enterococcus and Staphylococcus)

At time of laying (day 3), prevalence of bacteria in specific media was relatively low, and did not differ between years for *Enterobacteriaceae* (2012 = 9.43%, N = 53; 2013: 7.81%, N = 64, Fisher-exact test; P = 0.75) or *Enterococcus* (2012 = 18.87%, N = 53; 2013: 10.94%, N = 64, Fisher-exact test: P = 0.29). However, prevalence of *Staphylococcus* was higher in 2012 (15.09%, N = 53) than in 2013 (0%, N = 64) (Fisher-exact test: P = 0.0013). Because of the low prevalence of *Staphylococcus* in 2013, we did not explore its association with considered factors.

When we explored the effect of nest material at time of laying on bacteria prevalence, we found that number of unpigmented feathers was positively related with *Enterobacteriaceae* presence in 2013 ($\chi^2 = 7.58$, df = 1, P = 0.006; Fig. 4A) and tended to be positively related with *Enterococcus* presence in 2013 ($\chi^2 = 2.97$, df = 1, P = 0.085).

Nests with higher number of pigmented feathers tended to have lower prevalence of *Enterobacteriaceae* in 2012 ($\chi^2 = 3.67$, df = 1, P = 0.055), but it did not affect other kind of bacteria in 2012 or 2013 ($\chi^2 < 1.61$, df = 1, P > 0.205).

As the season progressed, prevalence of *Enterococcus* in 2012 increased ($\chi^2 = 6.37$, df = 1, P = 0.012; Fig. 4B). However, prevalence of *Enterobacteriaceae* and *Enterococcus* in 2013 were lower in late laying nests (*Enterobacteriaceae*: $\chi^2 = 18.43$, df = 1, P < 0.0001; Fig. 4C; *Enterococcus*: $\chi^2 = 5.13$, df = 1, P = 0.023; Fig. 4D). Sampling date was not significantly related to prevalence of *Enterobacteriaceae* or *Staphylococcus* in 2012 ($\chi^2 < 2.30$, df = 1, P > 0.129).

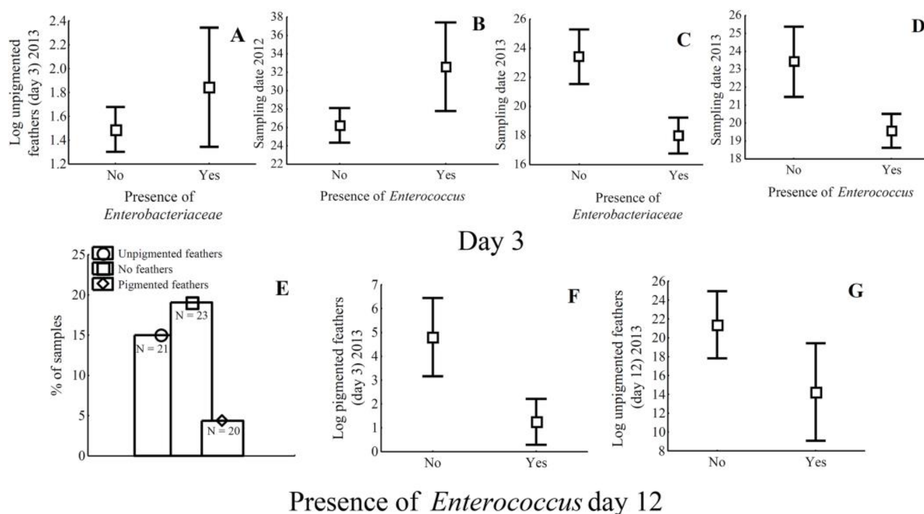


Figure 1.4: Nest material and experimental treatments effects on bacteria in specific media. Average number (± 95% CI) of unpigmented feathers at day 3 in 2013 in relation to prevalence of *Enterobacteriaceae* (A), and effects of sampling date in relation to prevalence of *Enterococcus* (in 2012, B), *Enterobacteriaceae* (in 2013, C), and *Enterococcus* (in 2013, D). Prevalence of *Enterococcus* at day 12 in relation to feather treatment (in 2013, E), number of pigmented feathers at day 3 (in 2013, F) and number of unpigmented feathers at day 12 (in 2013, G) is also shown. Figures show the most relevant associations detected.

At the end of incubation (day 12), prevalence of bacteria in specific media were very low and no year differences were found for *Enterobacteriaceae* (2012: 1.83%, N = 53; 2013: 3.13%, N = 64), *Enterococcus* (2012: 3.77%, N = 53; 2013: 2.50%, N = 64; P = 0.101) and *Staphylococcus* (2012: 0%, N = 53; 2013: 1.56%, N = 64) (Fisher-exact tests: P > 0.99). Because of the very low prevalence of specific bacteria groups in samples from incubated eggs in 2012 and *Enterobacteriaceae* and *Staphylococcus* in 2013, we did not explore its association with considered factors or variation along incubation (variation between day 3 and 12).

At the end of incubation, the experimental manipulation of feather nest material had an effect on *Enterococcus* prevalence in 2013. The reduced

model showed that eggshells of experimental nests with pigmented feathers treatment had lower *Enterococcus* prevalence than those in nests without feathers or with unpigmented feathers (χ^2 = 7.25, df = 2, P = 0.027; Fig. 4E). However, experimental manipulation of aromatic plant material did not significantly affect *Enterococcus* prevalence in 2013 (complete model: χ^2 = 0.41, df = 1, P = 0.52).

Nest feather material did also affect *Enterococcus* bacteria in 2013. The reduced model showed that nests with higher number of pigmented feathers at time of laying (χ^2 = 80.4, df = 1, P = 0.005; Fig. 4F) and with higher number of unpigmented feathers at the end of incubation (χ^2 = 6.30, df = 1, P = 0.012; Fig. 4G) were those with the lowest *Enterococcus* prevalence.

Eggshell bacterial loads in artificial nests with no incubation activity

In 2012, mesophilic bacterial loads on quail eggshells increased from first to second sampling, mainly for the study area number 2 (Table 5). The effects of experimental feathers on density and growth of mesophilic bacterial loads were apparent for samples from area number 2, but not for those from area number 1 (see interactions between study area and feather treatment, and between sampling events, study area and feather treatment in Table 5). Eggs in nest boxes with pigmented or unpigmented feathers treatments harbored lower density of bacteria than eggs in nests without feathers (Fig. 5). Post-hoc analyses did not reveal differences in the effects of pigmented and non-pigmented feathers on eggshell bacterial loads (Fisher LSD; area number 1: P = 0.942, area number 2: P = 0.635) or bacterial growth (area number 1: P = 0.926, area number 2: P = 0.616).

Experiments with green plants did not affect eggshell bacterial loads or growth during the study period in any of the study areas (S4 Appendix). Finally, we did not find evidence of the interaction between green plants' and feathers' experiments determining eggshell bacterial loads (S4 Appendix).

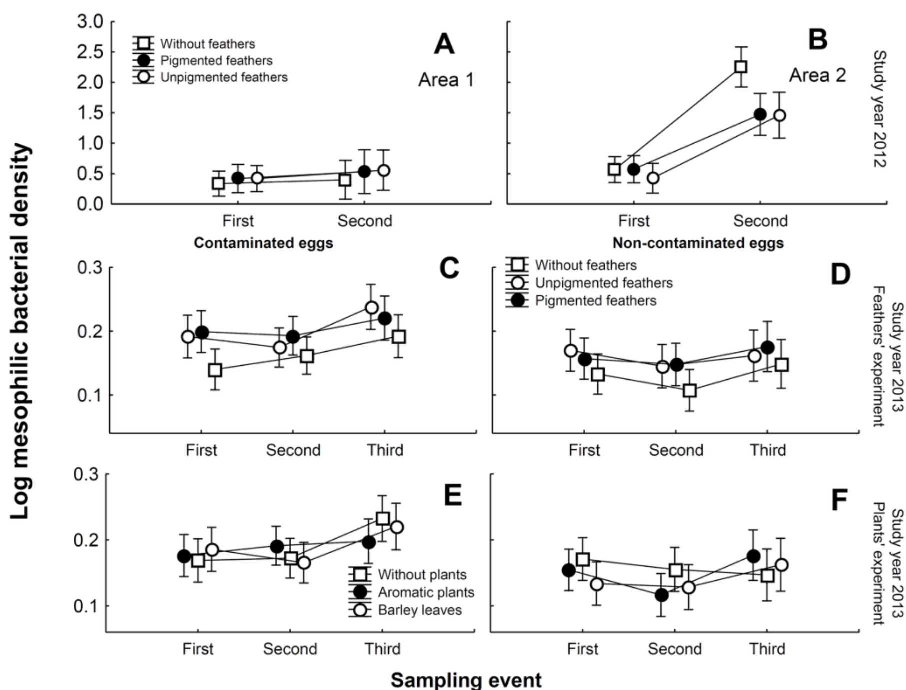


Figure 1.5: Results of mesophilic bacterial density of quail eggs. Eggshell mesophilic bacterial density (± 95% CI) estimated for experimental quail eggs during the two sampling events in relation to feathers experimental treatment and area for samples collected in 2012 (A and B). The effects of feathers (C and D) and plants (E and F) experimental treatments for contaminated and non-contaminated quail eggs during the three sampling events in 2013 are shown.

In 2013, we also found significant statistical differences between study areas and among experimental treatments (Table 5). However, the detected effects in this year were contrary to those detected in 2012. Bacterial density of quail eggs was higher in nests with feathers than in nests without feathers (Fig. 5). The effect of feather experiment in this case did not depend on the study area (S4 Appendix). Moreover, we found the expected effects of experimental contamination with pathogenic bacteria, which was independent of experimental treatment and study area (Table 5). We also detected an increase in eggshell bacterial loads from first to third samples (Table 5). The interaction between sampling time and contamination experiment only differed for nests under different green plants treatments (Table 5). Eggshell bacterial loads of nests without plants increased from first to third sampling date in experimentally contaminated eggs but decreased in non-contaminated eggs (Fig. 5).

Table 5 (next page). Results from Repeated Measures ANOVAs explaining mesophilic bacterial loads on quail eggshells. Quail eggs in experimental nest-boxes without incubation activity were subjected to two different treatments (plants (aromatic plants, non-aromatic plants or no plants) and feathers (pigmented, unpigmented or no feathers) as nest lining materials) in a full factorial design. The experiments were performed in two different areas and two different years. Samples were collected 5, 9 and 17 (only in 2013) days after the onset of the experiment. Thus, the models included study area and experimental treatments as between factors and sampling events and its interaction with study area and experimental treatments as within factors. In 2013, we included an additional within nest experimental treatment consisting on contaminating eggshells in the nests and, thus, contamination and the interaction with sampling event were included as additional within nest effects (repeated measures). We show final models that only include between-factors that alone or in interaction with other variables did result associated with eggshell bacterial loads, at least partially ($P < 0.1$). Statistically significant factors did not differ from those shown in the full models. Significant associations are in bold.

	F	df	P
Study year: 2012			
Between effects			
Area (1)	**53.94**	**1, 67**	**< 0.0001**
Feathers' treatment (3)	1.37	2, 67	0.26
(1) x (3)	**4.07**	**2, 67**	**0.021**
Within effects (Repeat measures – sampling)			
(Sampling)	**78.56**	**1, 67**	**< 0.0001**
Sampling x (1)	**55.59**	**1, 67**	**< 0.0001**
Sampling x (3)	2.42	2, 67	0.097
Sampling x (1) x (3)	**3.33**	**2, 67**	**0.042**
Study year: 2013			
Between effects			
Area (1)	**97.37**	**1, 54**	**< 0.0001**
Aromatic plants' treatments (2)	0.27	2, 54	0.764
Feathers' treatment (3)	**6.00**	**2, 54**	**0.004**
First within effects (Repeat measures - Contamination)			
(Contamination)	**22.56**	**1, 54**	**< 0.0001**
Contamination x (1)	0.50	1, 54	0.483
Contamination x (2)	0.27	2, 54	0.764
Contamination x (3)	0.11	2, 54	0.900
Second within effects (Repeat measures – Sampling)			
(Sampling)	**6.42**	**2, 108**	**0.002**
Sampling x (1)	1.83	2, 108	0.165
Sampling x (2)	0.15	4, 108	0.961
Sampling x (3)	0.23	4, 108	0.920
First x Second within effects (Repeat measures)			
(Sampling x Contamination)	1.88	2, 108	0.157
Sampling x Contamination x (1)	0.24	2, 108	0.786
Sampling x Contamination x (2)	**3.20**	**4, 108**	**0.016**
Sampling x Contamination x (3)	0.96	4, 108	0.432

Discussion

Our experimental modification of nest material in starling nests and in artificial nest boxes did affect nest bacterial environments as estimated as eggshell bacterial loads. The expected associations between eggshell bacterial loads and nest materials were most obvious for nest lining feathers' than for green plants' experiments. Although some of our bacterial quantifications do not distinguish between potentially pathogenic and non-pathogenic bacteria (i.e., mesophilic bacteria), we also detected evidence of expected associations for *Enterobacteriaceae* and *Enterococcus*, two groups of bacteria that include embryo pathogens. Below we discuss these results that varied depending on the study year and location under the hypothesis that nest lining feathers and green plants have antimicrobial functions.

Considering that transporting feathers and green plants to the nest are costly activities in terms of time and energy (Mainwaring & Hartley 2013), birds should adjust these efforts to the expected beneficial consequences. The expected beneficial effects of these nest materials on eggshell bacterial loads were only detected in particular study years and locations, especially those for which high bacterial densities were detected (2013 in starling nests and in artificial nest boxes sampled in the area 2 in 2012). Bacterial loads in starling nests were higher in 2013 than in 2012 and, in accordance with a possible nest building effort adjustment to bacterial environment, starlings carried to the nest more pigmented feathers in 2013 that in 2012 (see Results). Several clues may be used by adults to infer future risk of bacterial proliferation in their nests and accordingly adjust the effort dedicated to collect and transport nest materials with antimicrobial activity. We know for instance that humidity (D'Alba et al. 2010; Soler et al. 2015), temperature (Bruce & Drysdale 1994; Berrang et al. 1999), and characteristics related to laying date (Soler et al. 2015; Møller et al. 2015) are good predictors of bacterial growth. Thus, birds may adjust amount and composition of nest materials to environmental

conditions, which we found to differ between study years in terms of temperature and humidity. Our results fit at least partially this possibility since amount of antimicrobial nest material detected in starling nests before incubation started predicted the risk of infection during the incubation period, as well as eggshell bacterial loads soon before egg hatching, independently of experimental treatment.

Nest bacterial environments and thus risk of embryo infection also depend on other factors that may directly or indirectly be related to nest building material. Nest building activity has a sexually selected component in birds (Soler et al. 1998; Moreno 2012). Particularly for spotless starlings, there is experimental evidence that the use of feathers and green plants as nest material is sexually selected (Veiga & Polo 2005; Polo & Veiga 2006; Veiga et al. 2006; Veiga & Polo 2011). Thus, it is possible that the detected associations between nest materials and eggshell bacterial loads were a by-product of adult phenotypic characteristics implicated in sexual selection (Soler et al. 2011).

Incubation activity has also been suggested to have an important antimicrobial function (Cook et al. 2005a; D'Alba et al. 2010; Soler et al. 2015), which may be positively related to the expression of sexually selected characters (Møller & Cuervo 2000; Lislevand et al. 2004). The bacterial clearance effect of incubation was clearly pointed out with the contamination experiment performed in artificial and natural nests. The detected experimental effect of eggshell contamination on bacterial density in non-incubated artificial nests was counteracted in incubated starling eggs (Table 5). A very similar experiment (eggshell contamination) was recently performed in natural and artificial nests of black billed magpies (*Pica pica*) with exactly the same results (Soler et al. 2015), therefore, confirming the antimicrobial effects of avian incubation. Thus, the possible interaction between amount of nest lining materials and incubation efficiency of adults

reducing eggshell bacterial colonization and growth may explain our findings of bacterial environment modification. We take advantage of experimental results including nest boxes with and without incubation activity to conclude that even assuming a sexually selected component of the studied nest lining materials, these materials have independent effects on bacterial colonization and/or proliferation on starling eggshells.

The antimicrobial effects of particular aromatic plants have been experimentally demonstrated in nests of European starlings (*Sturnus vulgaris*) (Gwinner & Berger 2005) and on skin of nestling blue tits (*Cyanistes caeruleus*) (Mennerat et al. 2009a) but never in egg microbiota (D'Alba & Shawkey 2015). We here did not detect such expected effects in nests of spotless starlings. Thus, we cannot discard that the relatively soft manipulations performed made difficult, or was not adequate, to detect antimicrobial effects of aromatic plants in nests of spotless starlings. Another possible explanation is related to the large number of antimicrobial defense lines of natural avian nests against bacterial proliferation on the eggshells and trans-shell infection of embryos (D'Alba & Shawkey 2015). Absence of one of these lines of defense (i.e., green plants) will provoke slight negative effects, possibly requiring large sample sizes to be detected. In accordance with this possibility, we detected the expected effects of experimental green plants in artificial nests with no incubation activity, bacterial growth of previously contaminated experimental eggs that were in contact with aromatic plants was lower than that of eggs in nest boxes without aromatic plants (Table 5) (see also Møller et al. (2013)). Thus, we found experimental support for the antimicrobial effects of aromatic plants that would be more difficult to detect in natural nests.

Antimicrobial function of nest lining feathers has recently been suggested, but support for the hypothesis has only been detected in barn swallow (*Hirundo rustica*) nests (Peralta-Sánchez et al. 2010; 2011; 2014).

Here, we found experimental and correlative support to the hypothesis in nests of spotless starlings. Previous studies dealing with lining feathers of swallow nests, as well as theoretical work (Soler et al. 2010), suggested a relatively larger antimicrobial beneficial effect for unpigmented feathers. We here find out experimental support in natural nests for pigmented feathers, but not for unpigmented feathers. Moreover, the amount of pigmented feathers in spotless starling nests at different stages did result negatively related with eggshell bacterial loads and growth more frequently than that of unpigmented feathers, which also suggests larger effects for pigmented feathers. As we discussed for green plants, the detected associations may be a by-product of antimicrobial capability of birds and sexually selected traits (see above). However, this is also unlikely for feathers because the expected antimicrobial effect of nest lining feathers was more clearly detected in nest boxes with no incubation activity. In this case pigmented and unpigmented feathers produced similar effects.

The strength of the experimental effects and even the sign of the detected associations between nest lining materials and eggshell bacterial loads did greatly varied for different statistical models tested. It may simply be the consequence of partial effects in statistical models where independent factors share covariance with the dependent factor (eggshell bacterial loads). Another possibility is that detectable effect of particular nest material (i.e., unpigmented feathers) depends on whether or not other materials (pigmented feathers, or green plants) were present in the nest. We know for instance that bacteria isolated from unpigmented nest lining feathers have higher antimicrobial capabilities if collected from nests that at the beginning of incubation did only contain unpigmented feathers (Peralta-Sánchez et al. 2014). Thus, it is possible that particular compositions of nest lining feathers select for beneficial bacteria with different antimicrobial capacities. Even more, some of our bacterial quantifications do not distinguish between

potentially pathogenic and non-pathogenic bacteria, and some of the detected bacteria on the eggshells may be from nest lining material with the highest bacterial growth (i.e. unpigmented feathers, see Introduction). We predicted a possible effect of green plants on the antimicrobial properties of nest lining feathers and found no support, even in artificial nests without incubation. Thus, although more research is necessary before reaching firm conclusions, we concluded that this interaction is unlikely.

Summarizing, all these results considered together confirm an association between nest materials and bacterial environments of nests that depended on environmental conditions of different study years and localities. Particularly interesting is the association between variations in bacterial environments and in expected effects of nest lining materials, which suggests that birds should adjust nest building effort to bacterial environments. Finally, we hope that the detected experimental effects of feathers as antimicrobial material in avian nests encourage further research looking for mechanisms mediating such effects, including selection of bacterial strains with particular antimicrobial properties depending on nest lining material composition.

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Appendix

S1 Appendix. Samples sizes for artificial and natural nests in 2012 and 2013. Sample sizes for artificial non-incubated nests with quail eggs and for experimental starling nests under different experimental treatments during 2012 and 2013. Non-incubated nests were subjected to three different experiments: (1) eggshell contamination (contaminated (C) or not (NC) with egg contents with bacteria), (ii) feather experiment (with pigmented, unpigmented, or without (control) feathers) and (iii) plant experiment (with aromatic and non-aromatic plant and with no plants). Starling nests were subjected to the same feather's experimental treatments, but the plants treatment included only two groups (with and without aromatic plants).

			2012					2013				
Non-incubated nest-boxes												Total
Study area		Pin	Poc	Total	Pin	CH			Total			
Contamination treatments		NC	NC		C	NC	C	NC	Total-C	Total-NC		
Feather treatments	Plant treatments											
Control	Control	5	4	9	4	8	3	6	7	14	21	30
Control	Non-aromatic	5	4	9	3	5	3	5	6	10	16	25
Control	Aromatic	4	5	9	4	6	4	6	8	12	20	29
Pigmented	Control	2	3	5	4	6	3	5	7	11	18	23
Pigmented	Non-aromatic	4	5	9	5	5	2	4	7	9	16	25
Pigmented	Aromatic	5	4	9	4	6	3	5	7	11	18	27
Unpigmented	Control	3	2	5	4	6	2	3	6	9	15	20
Unpigmented	Non-aromatic	5	5	10	3	4	4	6	7	10	17	27
Unpigmented	Aromatic	5	3	8	3	4	3	5	6	9	15	23
Total	Total	38	35	73	34	50	27	45	61	95	156	229

Starling nests		2012			2013							Total
Study area		Pin	Poc	Total	Pin	CH			Total			
Contamination treatments		NC	NC		C	NC	C	NC	Total-C	Total-NC		
Feather treatments	Plant treatments											
Control	Plants			9							11	20
Control	Control			8							10	18
Pigmented	Plants			9							12	21
Pigmented	Control			6							11	17
Unpigmented	Plants			11							11	22
Unpigmented	Control			10							9	19
Total	Total			53							64	117

S2 Appendix. Results from GLM explaining mesophilic bacterial density on incubated eggshells of spotless starlings few days before hatching (day 12). Nest lining material (pigmented and unpigmented feathers) before incubation started (1st) and few days before hatching (2nd) were included as continuous independent factors. Experimental modification of green plants (i.e. with or without aromatic plants) and of feathers (i.e. pigmented, unpigmented and without feathers) were included as fixed effects. In 2013, we used a third experimental treatment that consisted on eggshell contamination at the time of egg laying. The interactions between experiments were far from statistical significance (2012: $P = 0.23$; 2013: $P > 0.15$) and are not shown. Reduced models show retained factors in the models with p-values < 0.1. Significant relationships are in bold.

2012	Beta (SE)	df	F	P
Date of first sampling (1=1 April)	-0.008 (0.008)	1,44	0.86	0.358
log number of pigmented feathers (1st)	-0.051 (0.056)	1,44	0.83	0.366
log number of unpigmented feathers (1st)	0.083 (0.061)	1,44	1.87	0.179
log number of pigmented feathers (2nd)	**-0.172 (0.080)**	**1,44**	**4.64**	**0.037**
log number of unpigmented feathers (2nd)	**0.189 (0.080)**	**1,44**	**5.61**	**0.022**
Feather treatment		2,44	0.70	0.501
Aromatic plant treatment		1,44	0.06	0.808
2013				
Date of first sampling (1=1 April)	0.014 (0.009)	1,54	2.56	0.115
log number of pigmented feathers (1st)	0.047 (0.054)	1,54	0.79	0.379
log number of unpigmented feathers (1st)	0.147 (0.077)	1,54	3.62	0.062
log number of pigmented feathers (2nd)	**0.160 (0.066)**	**1,54**	**5.91**	**0.018**
log number of unpigmented feathers (2nd)	-0.117 (0.073)	1,54	2.61	0.112
Eggshell contamination treatment		1,54	1.54	0.219
Feather treatment		2,54	2.10	0.133
Aromatic plant treatment		1,54	1.12	0.294

S3 Appendix. Results from GLM explaining changes in mesophilic bacterial density on eggshells of spotless starlings along the incubation period (variation between day 3 and day 12). Nest lining material (pigmented and unpigmented feathers) before incubation started (1st) and few days before hatching (2nd) were included as continuous independent factors. Experimental modification of green plants (i.e. with or without aromatic plants) and of feathers (i.e. pigmented, unpigmented and without feathers treatment) were included as fixed effects. In 2013, we used a third experimental treatment that consisted on eggshell contamination at the time of egg laying. The interactions between experiments were far from statistical significance (2012: $P > 0.88$; 2013: $P > 0.10$) and are not shown. Significant relationships are in bold.

2012	Beta (SE)	df	F	P
Complete model				
Date of first sampling (1=1 April)	-0.006 (0.012)	1,44	0.24	0.626
log number of pigmented feathers (1st)	**-0.214 (0.081)**	**1,44**	**6.97**	**0.011**
log number of unpigmented feathers (1st)	**0.255 (0.089)**	**1,44**	**8.26**	**0.006**
log number of pigmented feathers (2nd)	-0.130 (0.116)	1,44	1.26	0.267
log number of unpigmented feathers (2nd)	0.062 (0.116)	1,44	0.29	0.594
Feather treatment		2,44	1.09	0.344
Aromatic plant treatment		1,44	1.81	0.186
2013				
Date of first sampling (1=1 April)	-0.014 (0.012)	1,54	1.34	0.252
log number of pigmented feathers (1st)	0.041 (0.071)	1,54	0.34	0.565
log number of unpigmented feathers (1st)	0.111 (0.103)	1,54	1.16	0.285
log number of pigmented feathers (2nd)	**0.221 (0.088)**	**1,54**	**6.33**	**0.015**
log number of unpigmented feathers (2nd)	-0.144 (0.097)	1,54	2.21	0.143
Eggshell contamination treatment		1,54	2.68	0.108
Feather treatment		**2,54**	**5.64**	**0.006**
Aromatic plant treatment		1,54	1.23	0.273

S4 Appendix. Results from Repeated Measures ANOVAs explaining mesophilic bacterial loads of quail eggs in experimental nest-boxes without incubation activity. The artificial nests were subjected to two different experiments: plants (aromatic plants, non-aromatics plants or no plants) and feathers (pigmented, unpigmented or no feathers) as nest lining materials in a full factorial design. The experiments were performed in two different areas and two different years. Samples were collected 5, 9 and 17 (only in 2013) days after the onset of the experiment. Thus, the models included study area and experimental treatments as between factors and sampling events and its interaction with study area and experimental treatments as within factors. In 2013, we included an additional within nest experimental treatment consisting on contaminating some eggshells in the nests and, thus, contamination and the interaction with sampling event were included as additional within nest effects (repeated measures). We show full models. Significant relationships are in bold.

	Whole model			Reduced model		
2012	F	df	P	F	df	P
Between effects						
Area (1)	**54.40**	**1, 55**	**< 0.0001**	**53.94**	**1,67**	**< 0.0001**
Plants' treatment (2)	0.47	2, 55	0.629			
Feathers' treatment (3)	1.31	2, 55	0.279	1.37	2,67	0.26
(1) x (2)	0.04	2, 55	0.965			
(1) x (3)	**3.86**	**2, 55**	**0.027**	**4.07**	**2,67**	**0.021**
(2) x (3)	1.95	4, 55	0.115			
(1) x (2) x (3)	1.25	4, 55	0.301			
Within effects (Repeat measures – sampling)						
(Sampling)	**71.90**	**1, 55**	**< 0.0001**	**78.56**	**1,67**	**< 0.0001**
Sampling x (1)	**50.71**	**1, 55**	**< 0.0001**	**55.59**	**1,67**	**< 0.0001**
Sampling x (2)	0.24	2, 55	0.789			
Sampling x (3)	1.81	2, 55	0.174	2.42	2,67	0.097
Sampling x (1) x (2)	0.12	2, 55	0.890			
Sampling x (1) x (3)	**2.73**	**2, 55**	**0.074**	**3.33**	**2,67**	**0.042**
Sampling x (2) x (3)	1.20	4, 55	0.320			
Sampling x (1) x (2) x (3)	0.82	4, 55	0.521			

	Whole model			Reduced model		
2013	F	df	P	F	df	P
Between effects						
Area (1)	**90.87**	**1, 42**	**< 0.0001**	**97.37**	**1,54**	**< 0.0001**
Aromatic plants' treatment (2)	0.24	2, 42	0.787	0.27	2,54	0.764
Feathers' treatment (3)	**4.69**	**2, 42**	**0.015**	**6.00**	**2,54**	**0.004**
(1) x (2)	0.24	4, 42	0.914			
(1) x (3)	0.52	2, 42	0.601			
(2) x (3)	2.34	2, 42	0.109			
(1) x (2) x (3)	0.73	4, 42	0.577			
First within effects						
Repeat measures (Contamination)	**19.39**	**1, 42**	**< 0.0001**	**22.56**	**1,54**	**< 0.0001**
Contamination x (1)	0.58	1, 42	0.450	0.50	1,54	0.483
Contamination x (2)	0.18	2, 42	0.840	0.27	2,54	0.764
Contamination x (3)	0.07	2, 42	0.934	0.11	2,54	0.900
Contamination x (1) x (2)	0.66	4, 42	0.624			
Contamination x (1) x (3)	1.30	2, 42	0.284			
Contamination x (2) x (3)	0.32	2, 42	0.726			
Contamination x (1) x (2) x (3)	0.11	4, 42	0.978			
Second within effects						
Repeat measures (Sampling)	**5.56**	**2, 84**	**0.005**	**6.42**	**2,108**	**0.002**
Sampling x (1)	1.44	2, 84	0.243	1.83	2,108	0.165
Sampling x (2)	0.15	4, 84	0.961	0.15	4,108	0.961
Sampling x (3)	0.30	4, 84	0.880	0.23	4,108	0.920
Sampling x (1) x (2)	1.04	8, 84	0.416			
Sampling x (1) x (3)	0.72	4, 84	0.583			
Sampling x (2) x (3)	1.69	4, 84	0.159			
Sampling x (1) x (2) x (3)	0.70	8, 84	0.692			

	Whole model			Reduced model		
	F	df	P	F	df	P
First x Second within effects						
Repeat measures (Sampling x Contamination)	1.27	2, 84	0.287	1.88	2,108	0.157
Sampling x Contamination x (1)	0.14	2, 84	0.868	0.24	2,108	0.786
Sampling x Contamination x (2)	**2.71**	**4, 84**	**0.036**	**3.20**	**4,108**	**0.016**
Sampling x Contamination x (3)	0.70	4, 84	0.591	0.96	4,108	0.432
Sampling x Contamination x (1) x (2)	0.64	8, 84	0.738			
Sampling x Contamination x (1) x (3)	0.80	4, 84	0.531			
Sampling x Contamination x (2) x (3)	0.38	4, 84	0.824			
Sampling x Contamination x (1) x (2) x (3)	0.75	8, 84	0.651			

CAPÍTULO 2

Nest materials influence the bacterial load and fitness of nestling birds

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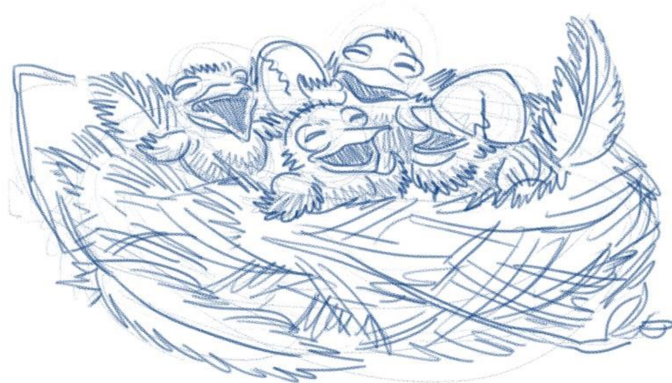

Abstract

Bird´s nests are infected by numerous parasites including bacteria. To counteract its deleterious effects, birds have developed several strategies aimed to avoid or reduce bacterial load, like the use of nesting materials with antimicrobial activity. These materials include aromatic plants rich in volatile compounds, and feathers that harbor antibiotic-producing bacterial strains. Experimental evidence on the antimicrobial effects of aromatic plants and feathers are however scarce in the literature. We explored experimentally the effects of these nest materials on density of cultivable skin bacteria and fitness related variables (body mass, immune response and fledging success) in spotless starling (*Sturnus unicolor*) nestlings. According to the predicted beneficial effects of these materials, the experimental addition of feathers decreased the bacterial load on nestling skin and improved their health and condition. The experimental addition of aromatic plants also reduced the bacterial load on nestling skin, especially in nests with no feathers added. We also detected negative associations between skin bacterial loads and nestling body mass, immune response and fledging success. Taken together, these results confirm the hypothetical antimicrobial function of feathers and plants used as nest materials by birds, and also its impact on fitness through a bacteria-mediated link in nestling health and condition.

Keywords: Aromatic plants, body mass, fledging success, immune response, nest building, nest feathers, skin bacterial load.

Introduction

Bird´s nests harbor numerous parasites (Loye & Zuk 1991, Clayton & Moore 1997, López-Rull & Macías-Garcia 2015) that include bacteria (Benskin et al. 2009, Soler et al. 2010). Some of these bacteria can be pathogenic for birds, especially during the breeding season. They can cross the eggshells and affect egg viability (Cook et al. 2003, Godard et al. 2007, Hansen et al. 2015), and also infect nestlings causing them diseases or reducing their survival prospects (González-Braojos et al. 2012b, Jacob et al. 2015). Nestlings, however, are not defenceless, and a number of adaptations have evolved to counteract the deleterious effects of bacteria, including physiological and behavioural defences. For example, lysozymes deposited in the eggs by laying females confer protection to the embryos (D'Alba & Shawkey 2015), and even to nestlings (Saino et al. 2002). The nestling immune system is also an effective barrier against pathogens (Sheldon & Verhulst 1996, Norris & Evans 2000, Merino 2010). Parent birds can also contribute to protect nestlings against pathogens with adaptive behaviours, such as building a new nest every year (González-Braojos et al. 2012a) or removing the old nest material from cavities before breeding (Pacejka & Thompson 1996, Mazgajski 2007).

The incorporation to the nest of certain materials with antimicrobial properties is a form of self-medication (Clayton & Wolfe 1993, De Roode et al. 2013). The most studied nest materials with antiparasitic properties are aromatic plants (Clark & Mason 1985, Dubiec et al. 2013). Aromatic plants have been hypothesized to remove or reduce the abundance of parasites and bacteria in nestlings due to their volatile compounds or essence oils (Clark 1990, Lafuma et al. 2001, Gwinner & Berger 2005, Scott-Baumann & Morgan 2015). Aromatic plants can also act as post-mating sexually selected traits in birds affecting parental effort and consequently nestling condition (Brouwer & Komdeur 2004, Polo & Veiga 2006, Tomás et al. 2013). In addition, aromatic plants can also improve nestling health (e.g., increasing

body mass, boosting immune system, etc.) through different metabolic routes, other than those related to parasite avoidance (Gwinner et al. 2000, Mennerat et al. 2009b). It is for instance known that *Rosmarinus officinalis* polyphenols increase brain protection against corticosterone-induced toxicity (Sasaki et al. 2013). However, experimental evidence of antimicrobial properties of aromatic plants reducing risk of bacterial infection on developing nestlings is scarce (Gwinner & Berger 2005, Mennerat et al. 2009a).

Feathers are another common nest material used by birds that may provide protection for nestlings against bacterial infections. Nest lining feathers have traditionally been studied for their thermoregulatory properties (Hilton et al. 2004, Windsor et al. 2013) or their function as sexual display (Veiga & Polo 2005, Sanz & García-Navas 2011, García-López de Hierro et al. 2013, García-Navas et al. 2015). More recently, a role of feathers in reducing risk of bacterial infection in the nest environment has been detected (Peralta-Sánchez et al. 2010, 2011, Ruiz-Castellano et al. 2016). A plausible explanation for this effect is that the keratinolytic bacterial community of feathers includes antibiotic producing strains that reduce proliferation of other microorganisms, some of which could be pathogenic (Soler et al. 2010). Different groups of keratinolytic bacteria are known to produce bacteriocins active against other bacteria, such as *Pseudomonas* (Ghequire & De Mot 2014), *Streptomyces* (Lee et al. 2014), and *Bacillus* (Barbosa et al. 2015). One of the most common feather degrading bacteria is *Bacillus licheniformis* (Burtt & Ichida 1999), which also produces bacteriocins (Callow & Work 1952, Gálvez et al. 1994, Lebbadi et al. 1994). The proliferation of keratinolytic bacteria is likely different in pigmented and unpigmented feathers because some pigments, like melanin, make more difficult the degradation of feathers (Goldstein et al. 2004, Gunderson et al. 2008). Thus, both types of feathers may have differential antibacterial properties mediated by their microbiome. The beneficial antimicrobial effects of nest-lining

feathers have already been shown in the barn swallow (*Hirundo rustica*) (Peralta-Sánchez et al. 2010, 2011, 2014), where unpigmented feathers decreased eggshell bacterial load and increased hatching success. In spotless starling (*Sturnus unicolor*) nests, however, pigmented feathers showed a greater antibacterial effect on eggs than unpigmented feathers (Ruiz-Castellano et al. 2016). Thus, although both studies confirm the expected beneficial effects of feathers reducing risk of bacterial infection of eggs, the direction of differential effects of pigmented and unpigmented feathers seems to depend on the study species and/or nest environment. Furthermore, despite until now the antimicrobial beneficial effects of nest lining feathers has only been demonstrated on the egg stage, it is also plausible that their effects extend to the nestling stage, which has not yet been explored. Indeed, adult starlings add both aromatic plants and feathers to the nest not only during nest building and incubation, but also during the nestling stage (Ruiz-Castellano et al. 2016).

In this study, we experimentally tested the combined fitness effects of aromatic plants and feathers as nest lining materials in spotless starling nests during the nestling phase. We expected a reduced bacterial load in nestlings from nests where plants or feathers were experimentally added. We also expected an interacting effect between aromatic plants and feathers because antimicrobial compounds of plants may remove beneficial bacteria living on feathers. Furthermore, we expected additional positive effects of feathers and plants, mediated by bacteria, on fitness-related traits such as nestling body mass, immune response or fledging success.

Materials and Methods

Study area and species

The study was performed in the Hoya de Guadix, southeast Spain (37º18' N, 3º11' W), a high-altitude plateau 1000 m a.s.l. with a semi-arid climate, during the 2012 breeding season. The spotless starling (hereafter starlings) population under study had 80 cork-made nest boxes available (internal height * width * depth: 350 * 180 * 210 mm, bottom-to-hole height: 240 mm) attached to tree trunks or walls at 3-4 m above ground. The starling is a hole-nesting passerine that mostly breeds in colonies. Starlings use a variety of nesting materials, including feathers and green plants that are used both for the structural and the lining layer of the nest (Veiga & Polo 2016). In the studied population, starlings start to lay their typical 4-5 eggs clutches in mid-April. Full incubation starts with the penultimate egg resulting in asynchronous hatching, which usually takes place from early May onwards (e.g. Soler et al. 2008). From the beginning of April, nest boxes were checked every second-third day until eggs were detected. Hatching date was estimated by daily visiting nest boxes when close to the expected dates (i.e., considering that the incubation period lasts for 7-12 days after clutch completion). The nestling period is quite variable in this species, ranging from 18 to 25 days (Veiga & Polo 2016).

Experimental procedures

Our experiment followed a full-factorial design with two treatments: the modification of number of feathers and amount of aromatic plants used as nest materials. On day 3 of nestling age (day 0 = hatching day, defined as the day when half or more of the brood had hatched, Tomás 2015), we counted the number of feathers and weighed (± 0.1 g) aromatic plants in nests, and removed both materials. Then, each nest was assigned to one of the feathers

and aromatic plants treatments. Feather treatments consisted of allocating (i) 15 pigmented, (ii) 15 unpigmented feathers to the nest, or (iii) leaving the nest without feathers. Aromatic plant treatments consisted of (i) introducing 1.6 g of a mixture of aromatic plants (see below) or (ii) leaving the nest without plants. On that day, all nestlings in the nest were individually marked by cutting some of their tuft feathers from the head, back or wings, and weighted to the nearest 0.1 g. The belly of one randomly selected nestling was sampled to characterize the bacterial environment of the nest before the experimental treatment (see below). Two days after the first visit (day 5) all plants and any feather added by birds were removed (experimental feathers were marked on the quill, see below) and plants for the aromatic-plant treatment were refreshed. When nestlings were 8 days old, all nestlings were ringed and weighed, and the belly of one nestling per nest (not sampled during the first visit) was sampled to characterize the bacterial environment of the nest after the experimental treatment. At this time, all lining feathers were again counted and all lining plants present in nests were weighted.

On day 14 of the nestling period, nestlings were weighed and blood samples were collected for immunity assays (see below) by puncturing the brachial vein and collecting 300-375 μL per nestling with sodium heparinized capillary tubes. The blood was stored in an Eppendorf tube at 4-6 ºC in a portable refrigerator until it was centrifuged (17000 g, 5 min) at the end of the day, and the plasma fraction was stored at -80 ºC until analysis.

Several variables were estimated as proxies of fitness, namely body mass of nestlings at ages of 8 and 14 days, innate immune response (i.e., lysis and agglutination titers derived from activity of natural antibodies and complement) at day 14, and percentage of hatchlings that fledge (i.e., fledging success).

Preparation of experimental nest lining feathers and aromatic plants

Experimental unpigmented and pigmented body feathers were of similar size and characteristics as those used by starlings as lining material in our population. These feathers were collected from chickens that grew in small farms close to the study area. In the laboratory, we sterilized the feathers in a UV sterilizer chamber (Burdinola BV-100), 10 minutes on each feather side. Subsequently, to homogenize density of bacterial colonies on feathers, with the help of an atomizer we sprayed approximately 84 mL of a high concentration solution of *Bacillus licheniformis* D13 per m^2 of surface completely covering experimental feathers. The solution was made from an overnight growth of a *B. licheniformis* colony in 6 ml of BHI (Brain Heart Infusion) media at 37 ºC on an orbital shaker. Finally, in separate hermetic bags we stored 15 pigmented or unpigmented feathers (i.e., the average number of feathers found in starling nests in previous years in the study area) at 4 ºC until its use in experimental nests. Experimental feathers were marked on the quill with a permanent marker allowing distinguishing them from those carried by adults to the nests.

Plants introduced in nests were a mixture of the four plant species most used by starlings in our population (unpublished data): *Marrubium vulgare*, *Artemisia barrelieri*, *Lamium amplexicaule* and *Anacyclus clavatus*. All these aromatic plants have volatile compounds or essential oils with known antimicrobial activity (Bakkali et al. 2008, Bouterfas et al. 2014, Laggoune et al. 2008, Meyre-Silva & Cechinel-Filho 2010, Selles et al. 2013). Fragments of plants of similar size as those used by starlings were collected the same day of the experiment in the surroundings of the study area and therefore were placed fresh in nests. We weighed 1.6 g of an homogeneous plant mixture for each nest because this is the maximum amount of fresh plants that we found in starling nests during the nestling stage in previous years in the study area (unpublished data). A new pair of gloves washed with

ethanol was employed to collect and manipulate feathers and plants used in each nest.

Bacterial sampling and laboratory work

For each nest visit and sampling we wore new gloves sterilized with 96% ethanol to prevent contamination of bacterial samples among nests. For sampling bacteria on the nestling belly we cleaned the complete belly surface with a sterile rayon swab (EUROTUBO® DeltaLab) slightly wet with sterile sodium phosphate buffer (0.2 M; pH = 7.2). After sampling, we introduced the swab in an Eppendorf tube with the buffer solution and preserved it at 4-6 ºC in a portable refrigerator until being processed in the laboratory within 24 h after collection.

Once in the laboratory, and after vigorously shaken in a vortex (Boeco V1 Plus), bacteria were cultivated by spreading 100 µL of serially diluted samples until 10^{-6} in four different solid media (Scharlau Chemie S.A. Barcelona). We used Tryptic Soy Agar, a broadly used general medium to grow aerobic mesophilic bacteria, and three specific media: Hektoen Enteric Agar for *Enterobacteriaceae*, Vogel-Johnson Agar for *Staphylococcus*, and Kenner Fecal Agar for *Enterococcus*. Plates were incubated at 37 ºC, and after 72 h the number of colonies on each plate was counted. For more details see Peralta-Sánchez et al. (2010).

Belly bacterial density was estimated by standardization of the number of colonies per cm^2 of sampled surface (CFU, Colony Forming Units). Belly surface was estimated from measurements of length and breadth of nestling belly, obtained with digital callipers to the nearest 0.01 mm, assuming that nestling belly is half of an ovoid (Narushin 2005). We measured surface area of 12 and 7 nestlings respectively sampled at the age of 3 and 8 days. Bacterial counts of these samples did depend on nestling age, but the relationships with belly area sampled at day 3 (mesophilic bacteria:

$F_{1,10}$ = 1.38, P = 0.266, *Enterococcus*: $F_{1,10}$ = 1.56, P = 0.240) or at day 8 (mesophilic bacteria: $F_{1,3}$ = 2.74, P = 0.454, *Enterococcus*: $F_{1,3}$ = 2.74, P = 0.666) were not significant. Thus, we quantified bacterial density in nestlings using the average belly area for each different nestling age (3 day old nestlings: 7.24 cm^2; 8 day old nestlings: 9.36 cm^2).

Characterization of bacterial environments by traditional culture techniques produces a relatively narrow picture of bacterial communities (Lee et al. 2013), but it has been demonstrated as an appropriate method for exploring associations between skin bacterial density and nestling infection and are thus useful for our purposes (Gwinner & Berger 2005, Mennerat et al. 2009a, González-Braojos et al. 2012a,2012b).

Estimation of natural antibodies and complement

We followed the procedure of Matson et al. (2005) for estimating the capacity of nestling plasma for lysis and agglutination of foreign antigens. In brief, 50 µL of nestling plasma was serially diluted in PBS buffer in two consecutive polystyrene 96-well assay plates (in contrast to one plate used by Matson et al. (2005)) and 25 µL of 1% rabbit blood cell suspension (Hemostat laboratories, Dixon, CA 95620, USA) in PBS buffer was added. Rabbit blood, with Alsever´s anticoagulant, was stored at 4 °C until the analyses. Quantification of lysis and agglutination titers was assessed as the number of titer with the last plasma dilution at which the lysis or agglutination reaction of rabbit blood was observed (Matson et al. 2005). In some cases, agglutination reaction did not stop in the last titer of the second plate and in these cases we assigned a value of one more than the last titer of the second plate. In some samples agglutination response could not be reliably estimated (N = 15) and these samples were not considered for estimating the average of agglutination value of nestlings in each nest.

Sample size and statistical analysis

We quantified the bacterial communities on the belly of nestlings in 56 starling nests (Table 1). Experimental effects of feathers on nestling bacterial load were analysed in two different general lineal models (GLM), one for presence of feathers and another for feather pigmentation. First, we explored the effects of feathers as a whole (i.e., factor with two-levels: with vs without feathers) and thus considered together nests under pigmented and unpigmented feather treatments (hereafter "feather models"). Secondly, we explored the effects of feather pigmentation (two-level factor: pigmented vs unpigmented feathers) and thus considered only nests with feathers experimentally added (hereafter "feather pigmentation models"). The rationale for this second approach is based on previous results suggesting differential effects of pigmented and unpigmented feathers in barn swallow (Peralta-Sánchez et al. 2010) and spotless starling nests (Ruiz-Castellano et al. 2016). Fitness-related variables were also analysed and we considered the average body mass and immune response of all nestlings at sampling as a dependent variable.

Table 1: Distribution of spotless starling nests among feather and plant treatments.

Feather treatment	Plant treatment	N
Control	Plants	10
Control	Control	9
Unpigmented	Plants	10
Unpigmented	Control	10
Pigmented	Plants	8
Pigmented	Control	9
Total		56

Density of mesophilic bacteria, density of *Enterococcus,* body mass (both at day 8 and day 14 of nestling age) and lysis response did approximately follow normal distributions after log10 transformation. Agglutination response did not follow a normal distribution, but the residuals of the statistical model did. For a considerable number of samples, *Enterobacteriaceae* and *Staphylococcus* were not detected (first sampling: 36.4% and 10.9%, respectively; second sampling: 63.8% and 38.3%, respectively). Thus, to avoid problems related with these zero-inflated distributions we used information of presence/absence for these variables.

For normally distributed dependent variables, we used General Linear Models (GLM) to explore expected associations in models that included feather and aromatic plant experimental treatments as fixed factors. In addition, to control for nest composition before experimental manipulations, and for nest building activity during the experiment, we included in the models information on the presence/absence of plants as a third discrete factor, and the number of feathers found in starling nests in the first and/or second sampling (experimental and no experimental) as a covariate. Finally, we also included date of first sampling (1 = 1st April) and brood size as continuous predictors. In models directed to explore possible differential effects of pigmented and unpigmented feathers (feather pigmentation models), number of pigmented and of unpigmented feathers found in starling nests in the first and/or second sampling were included as covariates instead of number of feathers.

GLM directed to estimate the effect of skin bacteria on fitness variables (body mass and immune response) included mesophilic bacteria and *Enterococcus* density as continuous predictors, while prevalence of *Enterobacteriaceae* and *Staphylococcus* were considered as categorical binomial factors (presence/absence). Fledging success was analysed in

Generalized Linear Models (GLZ) with a Poisson distribution and log-link function while controlling for overdispersion.

Presence/absence of *Enterobacteriaceae* and *Staphylococcus* were used as a dependent variable in GLZ models with binomial error and logic link function while controlling for overdispersion. Chi-square Maximun Likelihood values were estimated in a type III analysis. Feather and aromatic plant experimental treatments, as well as the presence/absence of plants, were included as fixed factors, and number of feathers in the first and/or second sampling (experimental and no experimental), date of first sampling (1 = 1st April) and brood size as continuous predictors. Due to the low prevalence of plants at day 8 in the nests (see Results), this variable was not included in the feather pigmentation model where we considered pigmented and unpigmented feathers separately.

We applied backward stepwise solution to identify important independent variables in our models, i.e., non-significant terms with the highest p-value were removed one by one up to p-values = 0.1, without removing the main effects of the interactions. We mainly report and discuss the reduced models, although the complete models are showed in appendices. All analyses were performed with the software Statistica 8.0 (Statsoft Inc. 2011). Values reported are means ± SE.

Results

Feathers and plants in nests during nestling growth

In the first sampling event, when nestlings were 3 days old, there was on average 28.8 ± 2.4 feathers in starling nests. Number of unpigmented feathers (16.2 ± 1.9) was not consistently larger than that of pigmented feathers (12.6 ± 1.5) within the same nests (t-test for dependent samples: $t_{55} = -0.99$, P =

0.324), and were not significantly related to each other (Pearson correlation: r = 0.11, P = 0.412). In the second sampling event, when nestlings were 8 days old, there was on average 8.4 ± 0.1 feathers (experimental and non-experimental) in nests. Again, no bias was detected for number of pigmented and unpigmented feathers in starling nests (3.6 ± 0.5 vs. 4.8 ± 0.9; t-test for dependent samples: t_{48} = -0.59, P = 0.559), and numbers of both types of feathers within the same nest were not significantly related to each other (Pearson correlation: r = -0.07, P = 0.657).

Aromatic plants added by starling adults during the nestling stage were detected in 32.1 % of nests in the first sampling and in 12.5 % of nests in the second sampling, averaging 0.11 ± 0.01 g and 0.16 ± 0.03 g, respectively.

Feathers, plants and skin bacterial loads of nestlings

Before the experiment; effects of nest materials on 3 day old nestlings

None of the analysed nest materials added by adults explained a significant proportion of variance of mesophilic bacterial density on the skin of starling nestlings before the manipulation (see complete model in Appendix S1). Nonetheless, nestlings tended to have lower density of *Enterococcus* on their belly in nests with plants than in nests without plants ($F_{1,51}$ = 3.50, P = 0.067; see Appendix S1 for complete model). *Enterobacteriaceae* was positively influenced by abundance of unpigmented feathers (Estimate = 1.63, χ_1^2 = 4.13, P = 0.042), after controlling for the non-significant effects of presence of aromatic plants (Estimate = 0.63, χ_1^2 = 3.66, P = 0.056) and date of sampling (Estimate = 0.11, χ_1^2 = 3.62, P = 0.057) (Appendix S1). None of the considered variables explained the prevalence of *Staphylococcus* before the experiment (See Appendix S1 for complete models).

Effects of nest material added by starlings and experimental treatment on 8 day old nestlings

Mesophilic bacterial density on nestling bellies was lower in nests with experimentally added feathers ($F_{1,36}$ = 9.29, P = 0.004, Fig. 1A), after controlling for the negative (Fig. 1B) and positive (Fig. 1C) associations with number of feathers in the nests at days 3 (Beta (SE) = -0.94 (0.41), $F_{1,36}$ = 5.21, P = 0.029) and 8 (Beta (SE) = 1.39 (0.43), $F_{1,36}$ = 10.35, P = 0.003) respectively, and the negative effect of sampling date (Beta (SE) = -0.06 (0.02), $F_{1,36}$ = 7.59, P = 0.009, Appendix S2). When only considering nests with experimentally added feathers (i.e., feather pigmentation model), plant and feather treatments (pigmented vs unpigmented) or their interaction did not explain significant proportion of variation in mesophilic bacterial density ($F_{1,16}$ = 1.48, P = 0.242), which suggests similar effects for pigmented and unpigmented feathers. No other experimental factors or nest materials in the models did explain a significant proportion of variance (Appendix S2).

Experimental treatments or their interaction and nest material variables did not significantly explain *Enterococcus* density on the nestling skin (see complete model in Appendix S2).

Enterobacteriaceae prevalence at day 8 was higher in nests with more feathers in their nests at the date of sampling (Estimate = 2.36, χ_1^2 = 5.26, P = 0.022; see complete model in Appendix S2). Moreover, in the feather pigmentation model, we found that *Enterobacteriaceae* prevalence was higher in nests with more unpigmented feathers in the nest at day 8 (Estimate = 2.33, χ_1^2 = 4.76, P = 0.029; see the complete model in Appendix S2), which suggests that the detected feather effects on *Enterobacteriaceae* were mainly due to unpigmented feathers added by birds. No other experimental factors or nest materials in the models explained a significant proportion of variance (Appendix S2).

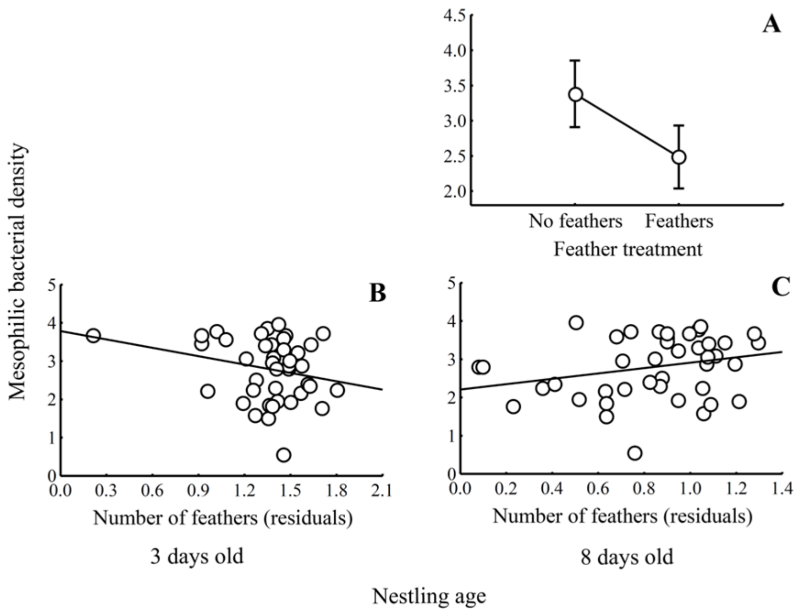

Figure 1: Feathers and bacteria. (A) Effect of the experimental addition of feathers to nests on mesophilic bacterial density on skin of spotless starling nestlings the day 8 after hatching (± CI 95%). (B-C) Relationship between the number of feathers (residuals of log number of feathers) present in the nest at two different ages and the bacterial load of nestlings at day 8.

Staphylococcus prevalence was affected by the interaction between treatments (χ_1^2 = 6.79, P = 0.009; Fig. 2A): it was higher in nests with only aromatic plants and no feathers, while those with both feathers and plants experienced the lowest prevalence. That was the case after controlling for the negative effects of sampling date (Estimate = -0.36, χ_1^2 = 11.36, P = 0.001), the number of feathers (Estimate = -4.25, χ_1^2 = 7.93, P = 0.005; Fig. 2B) and the presence of aromatic plants in the nest at day 3 (Estimate = -0.90, χ_1^2 = 4.35, P = 0.037; Fig. 2C). The presence of aromatic plants at day 8 resulted positively related to prevalence of *Staphylococcus* (Estimate = 3.69, χ_1^2 = 20.70, P < 0.001; Fig. 2C) (see Appendix S2 for complete model). For the feather pigmentation model, we only found an effect of feather treatment (χ_1^2 = 4.29, P = 0.038; Fig. 2D): *Staphylococcus* prevalence was lower in nests

with experimental pigmented feathers than in nests with experimental unpigmented feathers, after controlling for the negative non-significant effect of unpigmented feathers at day 8 (Estimate = -3.60, $\chi_1^2 = 3.65$, P = 0.056). No other variable in the models explained asignificant proportion of variance (see Appendix S2).

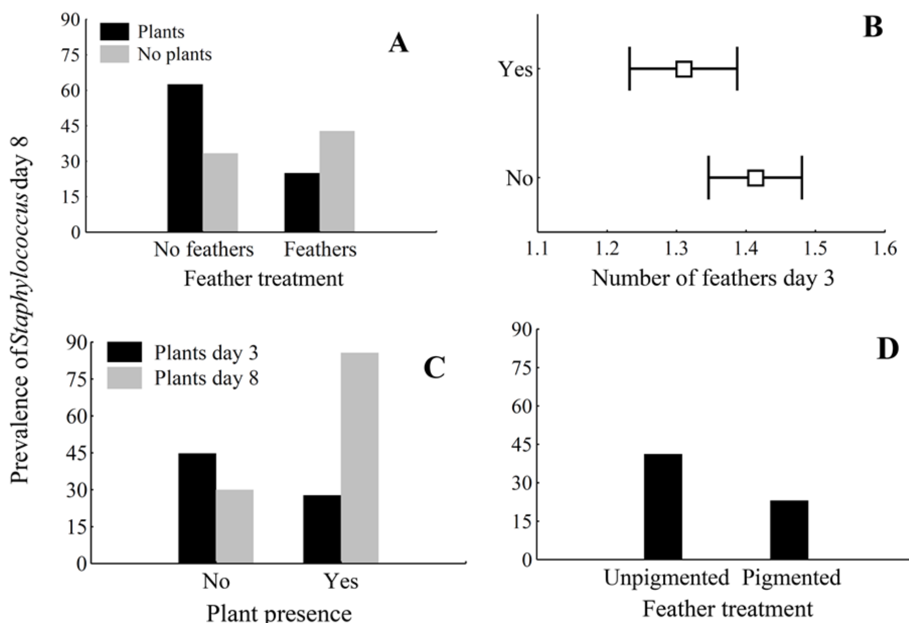

Figure 2: Feathers and plants, and *Staphylococcus*. Associations between presence of *Staphylococcus* (± CI 95%) in nests with feathers and plants treatments (i.e., feather model) (A), with log-transformed number of feathers in the nest at day 3 (B), presence of plants in the nests at day 3 and 8 (C) and with experimental pigmented and unpigmented feathers (D).

Effects of experimental treatments, nest materials and skin bacteria on fitness related variables

Nestling body mass

Body mass of 8 day old nestlings was not affected by experimental treatments but was negatively related to total number of feathers in the nest at day 8

(Beta (SE) = -8.40 (3.03), $F_{1,43}$ = 7.71, P = 0.008) and tended to be higher in nests with fewer nestlings (Beta (SE) = -2.20 (1.21), $F_{1,43}$ = 3.30, P = 0.076). For the feather pigmentation model, we found a significant effect of the interaction between treatments (Table 2). Nestling body mass was higher in nests with experimental unpigmented feathers, especially in nests that received experimental aromatic plants (Fig. 3A). That was the case after controlling for the negative effects of number of unpigmented feathers in the nest at day 8 (Fig. 3B) and brood size (Table 2). None of the bacterial estimates on days 3 or 8 explained body mass of 8 day old nestlings (see complete model in Appendix S3).

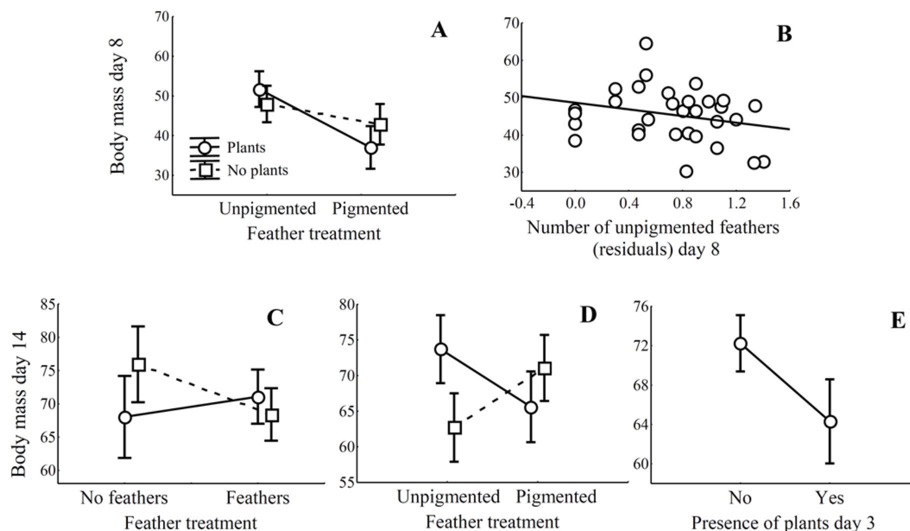

Figure 3: Feathers, plants and nestling body mass. Body mass (± CI 95%) of 8 days old spotless starling nestlings in experimental nests with pigmented or unpigmented feathers added, and with or without aromatic plants (A). The relation with residuals of log number of unpigmented feathers at day 8 in nests with feathers added is shown in B. The statistically significant relationships between body mass (± CI 95%) of 14 day old spotless starling nestlings and the interactions between feather and plant treatments (feather model), between feather pigmentation and plant treatments (feather pigmentation model) are shown in C and D respectively. Finally, body mass (± CI 95%) of 3 day old spotless starling nestlings in nests with feathers added, with and without aromatic plants detected is shown in E.

Table 2: Results of backward step models showing the effects of nest lining material, experimental treatments and their interaction on nestling body mass at days 8 and 14 of nestling age in spotless starlings.

	Body mass (day 8)			Body mass (day 14)		
	Beta (SE)	$F_{1,26}$	P	Beta (SE)	$F_{1,23}$	P
Feather treatment (1)		10.28	0.004		0.00	0.955
Plant treatment (2)		0.26	0.614		1.53	0.228
(1)*(2)		5.03	0.034		11.48	0.003
Plants presence (day 3)					9.08	0.006
Unpigmented feathers (day 8)	-13.11 (3.79)	11.99	0.002			
Brood size	-3.08 (1.24)	6.20	0.020	-6.85 (1.75)	15.38	0.001

Body mass of 14 day old nestlings was affected by the interaction between feather and aromatic plant treatments ($F_{1,43}$ = 4.49, P = 0.041; Fig. 3C) after controlling for the positive effects of number of feathers in the nest at day 3 (Beta (SE) = 8.40 (4.06), $F_{1,43}$ = 4.28, P = 0.041) and sampling date (Beta (SE) = 0.80 (0.22), $F_{1,43}$ = 12.64, P = 0.001). Nestling body mass was highest in nests without aromatic plants and without feathers and lowest in nests with only plants (see Appendix S4 for complete model). Moreover, in the feather pigmentation model, we found an effect of the interaction between feather and plant treatments (Table 2). Body mass of 14 days old nestlings was highest in nests with experimental unpigmented feathers and aromatic plants and lowest in nests with experimental pigmented feathers and aromatic plants (Fig. 3D) (Table 2). That was the case after controlling for the negative effects of plant presence in the nest at day 3 (Fig. 3E) and brood size (Table 2; see Appendix S4 for complete model). Moreover, in separate models analysing the association between bacterial loads and nestling body mass, we found that body mass of 14 days old nestlings was lower in nests with *Staphylococcus* detected at day 8 (reduced model, $F_{1,31}$= 4.68, P = 0.038: Fig.

4A) after controlling for the positive effect of sampling date (Beta (SE) = 0.48 (0.20), $F_{1,31}$ = 5.20, P = 0.030). No other bacterial group explained significant proportion of variance in nestling body mass (see Appendix S4 for complete model).

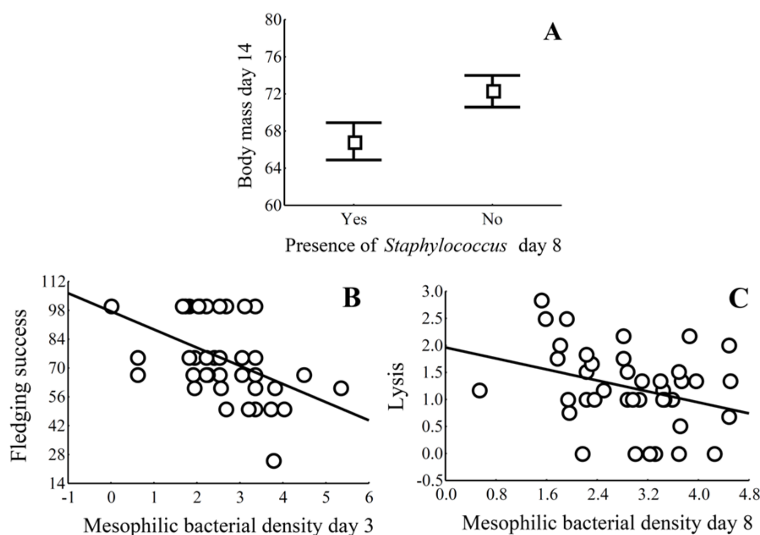

Figure 4: Bacteria and nestling traits. Significant relationships between body mass (± CI 95%) of 14 days old nestlings and presence of *Staphylococcus* at day 8 (A), between fledging success and mesophilic bacterial density at day 3 (B), and between lysis response and mesophilic bacterial density at day 8 (C).

Fledging success

Fledging success was not affected by experimental treatments or their interaction, but it was higher in nests that harbored less feathers at day 8 (Estimate = -0.23, χ_1^2 = 4.67, P = 0.031) after controlling for the negative effect of brood size (Estimate = -0.13, χ_1^2 = 11.05, P = 0.001) and the non-significant effect of the presence of aromatic plants at day 3 (Estimate = -0.06, χ_1^2 = 2.98, P = 0.084) (see Appendix S5 for full model). When only considering nests with experimentally added feathers (i.e., feather pigmentation model), fledging success was affected by the interaction between treatments: it was higher in nests with only pigmented feathers and

lower in nests with only unpigmented feathers (Estimate = 0.12, χ_1^2 = 6.25, P = 0.012; Fig. 5A), after controlling for the negative effects of the number of unpigmented feathers in the nest at day 3 (Estimate = -0.23, χ_1^2 = 4.29, P = 0.038), date of sampling (Estimate = -0.02, χ_1^2 = 4.16, P = 0.041), and the non-significant effects of number of pigmented feathers at day 3 (Estimate = -0.20, χ_1^2 = 2.86, P = 0.091) and brood size (Estimate = -0.11, χ_1^2 = 3.60, P = 0.058) (see complete model in Appendix S5).

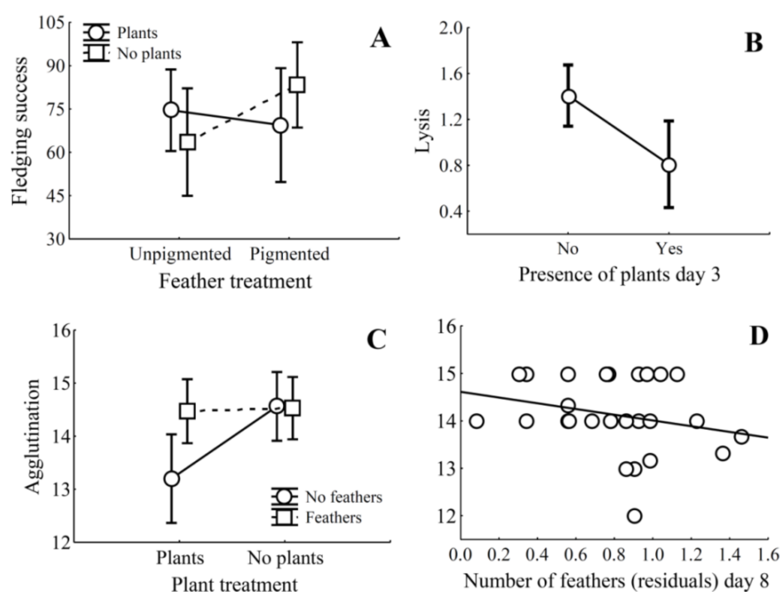

Figure 5: Feathers and plants, and nestling immune response. Fledging success (± CI 95%) in experimental nests with pigmented or unpigmented feathers added, and with or without aromatic plants (A). Statistically significant relationships between lysis activity as a measure of innate humoral immune response and presence of green plants in nests at day 3 (B), and the effect of plant and feather treatments (C) and the residuals of log number of feathers in the nest at day 8 (D) on agglutination response.

Finally, fledging success was negatively affected by density of mesophilic bacteria of 3 days old nestlings (Estimate = -0.13, χ_1^2 = 9.76, P = 0.002; Fig. 4B) and brood size (Estimate = -0.09, χ_1^2 = 3.89, P = 0.049). No other bacterial group explained significant proportion of variance (complete model in Appendix S5).

Innate humoral immunity

Regarding lysis response, experimental treatments or their interaction failed to explain a significant proportion of the variance, but aromatic plant presence at day 3 tended to be negatively associated with lysis (Beta (SE) = -0.23, $F_{1,39}$ = 3.98, P = 0.053). Similarly, in the feather pigmentation model, we did not find any experimental effect, but lysis response was again lower in nests with plant presence at day 3 (Beta (SE) = -0.33, $F_{1,27}$ = 5.07, P = 0.033; Fig. 5B; see Appendix S6 for complete model). In addition, in the bacterial model, lysis response was higher in nestlings with lower density of mesophilic bacteria at day 8 (Beta (SE) = -0.31 (0.14), $F_{1,31}$ = 4.74, P = 0.037; Fig. 4C). No other nest material or bacterial group explained a significant proportion of variance (see Appendix S6).

Agglutination response was explained by the interaction between treatments ($F_{1,18}$ = 4.94, P = 0.039). It was higher in nests without aromatic plants and with feathers, and was lower in nests with plants and without feathers. That was the case after controlling for the negative effects of the number of feathers in the nest at day 8 (Beta (SE) = -1.23, $F_{1,18}$ = 6.14, P = 0.023; Fig. 5C) and brood size (Beta (SE) = -0.51, $F_{1,18}$ = 4.86, P = 0.041; Fig. 5D) and the non-significant effect of plant presence at day 8 (Beta (SE) = 0.38, $F_{1,18}$ = 4.24, P = 0.054) (see Appendix S7 for complete model). These effects disappeared when only considering nests with experimentally added feathers (feather pigmentation model: see Appendix S7) suggesting that the detected interaction in the feather model were not due to differential effects of pigmented and unpigmented experimental feathers. None of the skin bacterial variables did associate with agglutination response (see Appendix S7 for complete model).

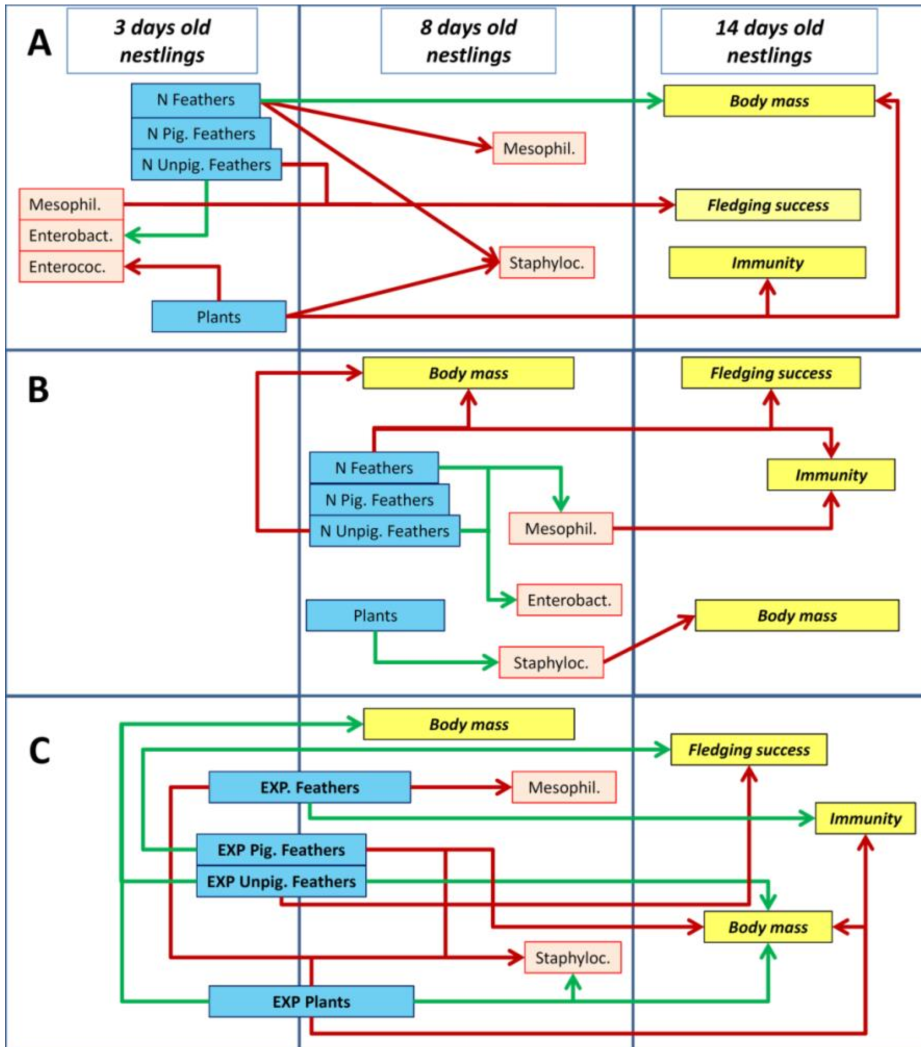

Figure 6: Effects of nest materials. Diagram summarizing the detected relationships between nest materials (blue boxes), the bacterial loads on the skin of 3 and 8 days old nestlings (red boxes), and variables used as proxies of nestling fitness (body mass of 8 and 14 days old nestlings, agglutination and lysis immune responses and fledging success, yellow boxes). The detected effects at days 3 (A) and at day 8 of nestling age (B), and experimental effects (C) are shown. Green arrows indicate positive relationships and red arrows indicate negative relationships. N = Number; Pig. = pigmented; Unpig. = unpigmented; Plants = presence of green plants; EXP = experimental manipulations; Mesophil. = Mesophilic bacteria; Enterobact. = *Enterobacteriaceae*; Enterococ. = *Enterococcus*; Staphyloc. = *Staphylococcus*.

Discussion

Our experimental manipulation of nest material (feathers and aromatic plants) in spotless starling nests did affect skin bacterial loads of starling nestlings and fitness-related variables (i.e., body mass at day 8 and 14, fledging success and immune response). The detected positive effects were larger for experimental feathers than for aromatic plants. Moreover, we found a significant interaction between experimental feathers and plants explaining prevalence of *Staphylococcus*, a group of bacteria that includes important avian pathogens (Hubálek 2004, Benskin et al. 2009). Nest material naturally added by adult birds was also associated with loads of some of the considered bacterial groups on nestlings, and with fitness related variables. Figure 6 summarizes the detected experimental effects and the complex relationships with nest material added by parents.

Antimicrobial effects of aromatic plants have been demonstrated in nestlings of other species. Gwinner and Berger (2005) experimentally replaced natural European starling (*Sturnus vulgaris*) nests with nests with or without green plants, and they found that nestlings in nests with green plants had lower bacterial loads than nests without plants. Similarly, Mennerat et al. (2009a) found that blue tit (*Cyanistes caeruleus*) nestlings in nests with experimentally supplied aromatic plants had lower bacterial density and richness than those in control nests. In our study, the effect of the aromatic plants treatment on skin bacteria was not that clear and depended on the feather treatment: in the case of *Staphylococcus,* the lowest prevalence was found in nests with experimental plants and feathers. It is possible that the relatively soft manipulations we performed here made difficult, or impeded, the detection of antimicrobial effects of aromatic plants in nests of spotless starlings in the absence of experimental feathers (Ruiz-Castellano et al. 2016). Behaviour of the adult birds, particularly their ability to add aromatic plants to the nest, may have interacted with our experimental treatment (Mennerat et al.

2009c, Tomás et al. 2012, see also Dawson 2004). In accordance with this possibility we found that the presence of aromatic plants in nests during the second sampling was positively related to presence of *Staphylococcus* on the skin of nestlings. Starling parents might use aromatic plants in a therapeutic form (Lozano 1998, De Roode et al. 2013), so that nests with high risk of bacterial infection should be those with more plants. Consequently, both experimental and correlational results are in accordance with previous results suggesting a role of aromatic plants limiting bacterial growth in avian nests.

We also explored the role of feathers as a nest material with antimicrobial functions, a characteristic recently suggested (Soler et al. 2010) but for which there is still little supporting evidence. This evidence was previously obtained only for bacteria in eggshells of barn swallows (Peralta-Sánchez et al. 2010, 2011, 2014) and spotless starlings (Ruiz-Castellano et al. 2016) though this role had never been explored in nestlings. Our results confirmed the expected antimicrobial effect of feathers since nestlings had lower bacterial loads in nests with experimentally added feathers than in experimental nests where feathers were removed. Moreover, nestlings in nests with experimental plants and feathers either pigmented or unpigmented, presented the lowest prevalence of *Staphylococcus,* suggesting similar effects for pigmented and unpigmented feathers (Fig. 6C). Thus, our experimental results confirm the antimicrobial effects of feathers in nest bacterial environment during the nestling phase. Correlational results were also in accordance with the hypothesis since the number of feathers in the nests at the time of bacterial sampling resulted positively related to bacterial loads. This suggests that, as stated above for aromatic plants, parents used feathers in a therapeutic form when risk of bacterial infection is high (Lozano 1998, De Roode et al. 2013). Interestingly, eight day old nestlings with the lowest bacterial loads were those with highest number of feathers in their nests soon after hatching, but with the lower number at the time of bacterial sampling

(Fig. 6), which is in agreement with a therapeutic use of feathers. The detected antimicrobial effects of feathers were predicted on the base of the antimicrobial capabilities of feather-degrading bacteria (Soler et al. 2010, Peralta-Sánchez et al. 2014), and these results should encourage further research on the microbiological particularities of such bacteria.

Our experimental manipulation of feathers and plants in spotless starling nests did also affect fitness-related variables in complex ways (Fig. 6). Remarkably, the experimental addition of feathers mostly resulted in beneficial effects in terms of fledging success, body mass and immunity of nestlings close to fledging the nests (Fig. 6C). Apparently, pigmented and unpigmented feathers have opposite effects in fledging success and body mass. Pigmented feathers provoked higher fledgling success of nestlings of lower body mass, while unpigmented feathers resulted in lower number of fledglings of higher body mass. It is generally assumed that body mass of nestlings close to fledging the nests is negatively influenced by brood size (Pettifor et al. 2001), while both variables are positively related to probability of nestling recruitment (Pettifor et al. 2001, Moreno et al. 2005). Thus, it is possible that pigmented and unpigmented feathers differentially affected parental decisions related to brood reduction. Further research on the effect of nest material on parental behaviour is necessary to clarify this possibility. Independently of pigmentation, the experimental addition of feathers resulted in nestlings having greater immune responses. Immune response is one of the main predictors of local recruitment of nestlings (Moreno et al. 2005, Cichon & Dubiec 2005, López-Rull et al. 2011) and, consequently, our experimental results as a whole strongly suggest a link between nest lining feathers and reproductive success of spotless starlings. Results were not so clear for experimental aromatic plants that resulted in positive and negative effects for body mass and immunity respectively. Nestling growth and immunity are in trade-off (Soler et al. 2003) and, thus, it is possible that the experimental

addition of aromatic plants influenced the resolution of such trade-off in favour of body mass. Previous experiments also detected positive experimental effects of aromatic plants on body mass (Gwinner & Berger 2005) and tarsus length (Polo et al. 2015; but this article also showed a negative effect on body mass) of fledglings, which further suggest that the negative effects we detected on immunity represent the outcome of the trade-off between immunity and growth. Correlational results showed mainly negative associations between nest materials and fitness related variables, which further suggest a therapeutic use of plants and feathers as nest building materials (Fig. 6A and Fig. 6B). However, since it was not always the case, and some positive associations appeared, further experimental research exploring parental responses to experimental risk of bacterial infection is necessary to reach more confident conclusions. Searching, selecting, carrying, and depositing these materials into the nest are costly activities for birds in terms of energy and time (Mainwaring and Hartley 2013). Therefore, birds should carefully adjust these efforts according to the balance between costs and benefits of the expected outcome, choosing the more appropriate combination of nest materials to reduce bacterial load (which will depend on risk of bacterial proliferation: see Ruiz-Castellano et al. 2016) and to improve nestling health and condition.

Remarkably, some of the fitness related variables (i.e. immune response and body mass of nestlings) were negatively associated with loads of mesophilic bacteria and of *Staphylococci*; exactly the two groups of bacteria that were negatively affected by the experimental nest material (Fig. 6). These results on the one hand confirm the expected negative association between bacterial load on nestling skin and nestling survival (González-Braojos et al. 2012, Jacob et al. 2015). On the other hand, it suggests that the detected experimental effects on fitness related traits of nestlings were mediated by the

effect of experimental nest material on the bacterial environment where nestlings developed.

A possibility to account for the observed effects of feathers and plants in the nests is the potential influence of these materials on adult behaviour. Research on different bird species has shown that aromatic plants and feathers in nests represent extended phenotypic signals (Dawkins 1982) with a role in sexual selection (Fauth et al. 1991, Brouwer & Komdeur 2004, Sanz & García-Navas 2011, Tomás et al. 2013). In spotless starlings, plant carrying by males and feather carrying by females can be viewed as sex-specific functionally related behaviours involved in mutual status signalling (Veiga et al. 2006). It is possible that starling adults increase their parental investment (e.g. increasing parental feeding rates or nest sanitation) to the nestlings in response to the feathers and plants found in the nests. However, previous studies in starlings have shown that the presence of plants or feathers did not affect provisioning rates or nest sanitation behaviours (Brouwer & Komdeur 2004, Veiga & Polo 2011), which makes this explanation unlikely.

To conclude, our study confirms beneficial effects of the presence of aromatic plants and feathers as nest materials on fitness related variables of nestlings that are likely mediated by the effects on the bacterial environment where nestlings develop. Our results emphasize that, due to the costs associated with nest building, adult birds should make a sensible selection of nest materials' combination that maintains their nest with a reduced pathogenic bacterial load and confer better survival prospects for their offspring. The detected experimental effects of feathers as antimicrobial material in avian nests should encourage further research to elucidate the mechanisms mediating such effects.

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Appendix

Appendix S1: GLM and GLZ complete models testing the effects of nest lining material added by spotless starlings on mesophilic bacteria and *Enterococcus* density and *Enterobacteriaceae* and *Staphylococcus* prevalence in skin of 3 day old nestlings. Brood size, sampling date and number of pigmented and unpigmented feathers were included as covariates and presence of green plants as a categorical factor. Associations with a P < 0.10 are in bold.

	Mesophilic bacteria			*Enterococcus*			*Enterobacteriaceae*		*Staphylococcus*	
	Beta (SE)	$F_{1,46}$	P	Beta (SE)	$F_{1,48}$	P	χ_1^2	P	χ_1^2	P
Pigmented (day 3)	0.02 (0.33)	0.00	0.952	**-0.03 (0.02)**	**3.12**	**0.084**	0.000	0.986	3.654	0.926
Unpigmented(day 3)	0.02 (0.30)	0.00	0.955	0.02 (0.02)	1.37	0.248	**4.359**	**0.037**	0.994	0.726
Plants (day 3)		0.56	0.457		**2.94**	**0.093**	**3.393**	**0.065**	2.245	0.984
Date	-0.01 (0.02)	0.16	0.687	0.00 (0.00)	0.02	0.900	2.694	0.101	0.264	0.607
Brood size	0.05 (0.13)	0.15	0.703	0.01 (0.01)	2.00	0.163	0.667	0.414	1.521	0.647

Appendix S2: GLM and GLZ complete models testing the effects of nest material and feather (feather and feather pigmentation models) and plants treatments on mesophilic bacteria and *Enterococcus* density and *Enterobacteriaceae* and *Staphylococcus* prevalence in skin of 8 day old spotless starling nestlings. Treatments were included as fixed factors, brood size, hatching date, total number of feathers and number of pigmented and unpigmented feathers were included as covariates and presence of green plants as a categorical factor. Associations with a P < 0.10 are in bold.

	Mesophilic bacteria			*Enterococcus*			*Enterobacteriaceae*		*Staphylococcus*	
	Beta (SE)	$F_{1,32}$	P	Beta (SE)	$F_{1,35}$	P	χ_1^2	P	χ_1^2	P
Feather model										
Feather treatment (1)		**9.07**	**0.005**		1.56	0.220	0.02	0.895	0.31	0.576
Plant treatment (2)		0.28	0.602		0.92	0.345	0.23	0.632	0.62	0.433
(1)*(2)					0.66	0.422	0.01	0.917	**6.57**	**0.010**
Feathers (day 3)	**-0.98 (0.43)**	**5.18**	**0.030**	-0.41 (0.36)	1.36	0.251	0.07	0.790	**8.08**	**0.004**
Plants (day 3)		1.74	0.196		1.74	0.196	0.22	0.639	**4.25**	**0.039**
Feathers (day 8)	**1.53 (0.46)**	**10.79**	**0.002**	**0.64 (0.37)**	**2.95**	**0.095**	2.40	0.122	0.44	0.507
Plants (day 8)		0.15	0.699		0.44	0.512	0.71	0.400	**18.00**	**0.000**
Date	**-0.06 (0.03)**	**4.78**	**0.036**	-0.02 (0.02)	1.24	0.272	0.54	0.464	**9.79**	**0.002**
Brood size	0.19 (0.15)	1.66	0.207	0.05 (0.12)	0.18	0.678	0.15	0.701	1.05	0.305

Feather pigmentation model	Mesophilic bacteria			*Enterococcus*			*Enterobacteriaceae*		*Staphylococcus*	
	Beta (SE)	$F_{1,16}$	P	Beta (SE)	$F_{1,18}$	P	χ_1^2	P	χ_1^2	P
Feather treatment (1)		0.02	0.887		0.44	0.515	0.20	0.651	**5.28**	**0.022**
Plant treatment (2)		0.30	0.592		0.02	0.898	0.23	0.635	**2.94**	**0.086**
(1)*(2)					0.88	0.362	2.56	0.110	0.00	0.969
Pigmented (day 3)	-0.12 (0.47)	0.07	0.800	0.15 (0.37)	0.17	0.683	0.29	0.591	0.99	0.321
Unpigmented (day 3)	-0.47 (0.49)	0.96	0.341	-0.09 (0.38)	0.05	0.825	0.23	0.632	0.04	0.833
Plants (day 3)		0.26	0.616		0.16	0.692	1.12	0.291	1.44	0.231
Pigmented (day 8)	1.07 (0.86)	1.57	0.228	0.71 (0.55)	1.53	0.231	1.42	0.234	1.14	0.285
Unpigmented (day 8)	1.10 (1.00)	1.20	0.290	-0.16 (0.68)	0.06	0.814	1.78	0.182	**5.07**	**0.024**
Date	-0.04 (0.05)	0.69	0.419	-0.02 (0.04)	0.22	0.646	0.04	0.839	0.92	0.337
Brood size	0.24 (0.28)	0.73	0.405	0.06 (0.22)	0.08	0.787	0.00	0.958	0.23	0.634

Appendix S3: GLM complete models testing the effects of feathers (feather and feather pigmentation models) and plants as nest material on body mass of 8 day old spotless starling nestlings. Treatments were included as fixed factors, brood size, hatching date, total number of feathers and number of pigmented and unpigmented feathers, and bacterial density were included as covariates and presence of green plants and bacterial prevalences as categorical factors. Associations with a P < 0.10 are in bold.

Feather model			
	Beta (SE)	$F_{1,36}$	P
Feather treatment (1)		0.01	0.934
Plant treatment (2)		1.68	0.203
(1)*(2)		2.81	0.102
Feathers (day 3)	1.05 (4.07)	0.07	0.798
Plants (day 3)		1.61	0.213
Feathers (day 8)	**-8.00 (4.01)**	**3.97**	**0.054**
Plants (day 8)		0.45	0.509
Date	0.11 (0.25)	0.20	0.657
Brood size	**-2.63 (1.30)**	**4.07**	**0.051**
Feather pigmentation model			
	Beta (SE)	$F_{1,21}$	P
Feather treatment (1)		3.15	0.091
Plant treatment (2)		0.15	0.698
(1)*(2)		2.68	0.116
Pigmented (day 3)	-0.98 (3.14)	0.10	0.759
Unpigmented (day 3)	-3.15 (3.07)	1.05	0.317
Plants (day 3)		1.74	0.202
Pigmented (day 8)	-1.29 (4.78)	0.07	0.790
Unpigmented (day 8)	**-11.13 (5.02)**	**4.91**	**0.037**
Date	-0.38 (0.27)	1.92	0.180
Brood size	**-3.89 (0.27)**	**7.26**	**0.014**

Bacteria			
	Beta (SE)	$F_{1,26}$	P
Mesophilic bacteria (day 3)	1.08 (2.25)	0.23	0.635
Enterococcus (day 3)	-45.80 (45.94)	0.99	0.328
Mesophilic bacteria (day 8)	-1.06 (2.22)	0.23	0.636
Enterococcus (day 8)	2.90 (3.02)	0.92	0.345
Enterobacteriaceae (day 3)		0.00	0.986
Staphylococcus (day 3)		0.01	0.907
Enterobacteriaceae (day 8)		0.02	0.904
Staphylococcus (day 8)		0.01	0.926
Date	0.00 (0.24)	0.00	0.995
Brood size	-2.11(1.84)	1.32	0.261

Appendix S4: GLM complete models testing the effects of feathers (feather and feather pigmentation models) and plants as nest materials on body mass of 14 day old spotless starling nestlings. Treatments were included as fixed factors, brood size, hatching date, total number of feathers and number of pigmented and unpigmented feathers, and bacterial density were included as covariates and presence of green plants and bacterial prevalences as categorical factors. Associations with a P < 0.10 are in bold.

Feathers model			
	Beta (SE)	$F_{1,30}$	P
Feather treatment (1)		0.83	0.370
Plant treatment (2)		0.11	0.746
(1)*(2)		**3.60**	**0.067**
Feathers (day 3)	**8.86 (4.56)**	**3.78**	**0.061**
Plants (day 3)		**3.98**	**0.055**
Feathers (day 8)	-3.46 (4.62)	0.56	0.460
Plants (day 8)		1.37	0.251
Date	**0.81 (0.28)**	**8.69**	**0.006**
Brood size	-3.05 (2.01)	2.29	0.141
Feather pigmentation model			
	Beta (SE)	$F_{1,18}$	P
Feather treatment (1)		0.77	0.392
Plant treatment (2)		1.50	0.236
(1)*(2)		**9.29**	**0.007**
Pigmented (day 3)	-0.43 (3.66)	0.01	0.907
Unpigmented (day 3)	0.00 (3.42)	0.00	1.000
Plants (day 3)		**7.40**	**0.014**
Pigmented (day 8)	4.03 (5.06)	0.63	0.436
Unpigmented (day 8)	-6.14 (5.81)	1.12	0.304
Date	0.11 (0.33)	0.10	0.755
Brood size	**-7.34 (2.49)**	**8.70**	**0.014**

Bacteria	Beta (SE)	$F_{1,26}$	P
Mesophilic bacteria (day 3)	0.09 (2.45)	0.00	0.971
Enterococcus (day 3)	-10.94 (47.16)	0.05	0.819
Mesophilic bacteria (day 8)	-3.09 (2.34)	1.78	0.200
Enterococcus(day 8)	4.29 (3.11)	1.90	0.181
Enterobacteriaceae (day 3)		0.00	0.946
Staphylococcus (day 3)		0.57	0.458
Enterobacteriaceae (day 8)		0.39	0.539
Staphylococcus (day 8)		2.81	0.107
Date	0.35 (0.24)	2.09	0.162
Brood size	-1.11 (2.21)	0.25	0.622

Appendix S5: GLZ complete models with Poisson distribution testing the effects of feathers (feather and feather pigmentation models) and plants as nest materials on fledging success in spotless starling nests. Treatments were included as fixed factors, brood size, hatching date, total number of feathers and number of pigmented and unpigmented feathers, and bacterial density were included as covariates and presence of green plants and bacterial prevalences as categorical factors. Associations with a $P < 0.10$ are in bold.

Feather model		
	χ_1^2	P
Feather treatment (1)	0.00	0.982
Plant treatment (2)	0.37	0.562
(1)*(2)	1.01	0.313
Feathers (day 3)	2.63	0.105
Plants (day 3)	2.00	0.157
Feathers (day 8)	1.06	0.304
Plants (day 8)	1.27	0.257
Date	**4.53**	**0.033**
Brood size	**8.57**	**0.003**
Feather pigmentation model		
Feather treatment (1)	0.92	0.336
Plant treatment (2)	0.01	0.925
(1)*(2)	**5.17**	**0.023**
Pigmented (day 3)	3.93	0.047
Unpigmented (day 3)	**2.79**	**0.095**
Plants (day 3)	**2.73**	**0.098**
Pigmented (day 8)	1.32	0.251
Unpigmented (day 8)	1.01	0.316
Date	**6.56**	**0.010**
Brood size	**6.52**	**0.011**

Bacteria	χ_1^2	P
Mesophilic bacteria (day 3)	2.49	0.114
Enterococcus (day 3)	1.39	0.238
Mesophilic bacteria (day 8)	0.07	0.938
Enterococcus (day 8)	0.06	0.813
Enterobacteriaceae (day 3)	0.01	0.915
Staphylococcus (day 3)	0.81	0.369
Enterobacteriaceae (day 8)	0.18	0.673
Staphylococcus (day 8)	0.35	0.555
Date	0.71	0.400
Brood size	2.49	0.115

Appendix S6: GLM complete models testing the effects of feathers (feather and feather pigmentation models) and plants as nest materials on lysis response in spotless starling nestlings. Treatments were included as fixed factors, brood size, hatching date, log total number of feathers and number of pigmented and unpigmented feathers, and bacterial density were included as covariates and presence of green plants and bacterial prevalence as categorical factors. Associations with a P < 0.10 are in bold.

Feather model			
	Beta (SE)	$F_{1,30}$	P
Feather treatment (1)		1.63	0.211
Plant treatment (2)		0.00	0.994
(1)*(2)		1.20	0.282
Feathers (day 3)	0.41 (0.42)	0.92	0.345
Plants (day 3)		**4.20**	**0.049**
Feathers (day 8)	-0.45 (0.43)	1.10	0.303
Plants (day 8)		0.02	0.877
Date	-0.01 (0.03)	0.01	0.919
Brood size	0.23 (0.19)	1.52	0.228
Feather pigmentation model			
	Beta (SE)	$F_{1,18}$	P
Feather treatment (1)		0.67	0.424
Plant treatment (2)		0.38	0.543
(1)*(2)		0.16	0.694
Pigmented (day 3)	-0.02 (0.49)	0.00	0.965
Unpigmented (day 3)	-0.24 (0.46)	0.27	0.610
Plants (day 3)		2.19	0.156
Pigmented (day 8)	-0.04 (0.68)	0.00	0.954
Unpigmented (day 8)	0.82 (0.78)	1.12	0.305
Date	-0.02 (0.04)	0.31	0.587
Brood size	0.16 (0.33)	0.24	0.631

Bacteria	Beta (SE)	$F_{1,23}$	P
Mesophilic bacteria (day 3)	0.18 (0.21)	0.72	0.407
Enterococcus (day 3)	-0.14 (4.02)	0.00	0.973
Mesophilic bacteria (day 8)	-0.38 (0.20)	3.53	0.073
Enterococcus (day 8)	-0.14 (0.27)	0.28	0.602
Enterobacteriaceae (day 3)		1.12	0.301
Staphylococcus (day 3)		0.89	0.355
Enterobacteriaceae (day 8)		0.92	0.349
Staphylococcus (day 8)		0.00	0.997
Date	-0.02 (0.02)	0.90	0.354
Brood size	0.27 (0.19)	2.00	0.171

Appendix S7: GLZ complete models testing the effects of feathers (feather and feather pigmentation models) and plants as nest materials on agglutination response in spotless starling nestlings. Treatments were included as fixed factors, brood size, hatching date, total number of feathers and number of pigmented and unpigmented feathers, and bacterial density were included as covariates and presence of green plants and bacterial prevalences as categorical factors. Associations with a P < 0.10 are in bold.

Feather model			
	Beta (SE)	$F_{1,15}$	P
Feather treatment (1)		**3.85**	**0.069**
Plant treatment (2)		2.85	0.112
(1)*(2)		**4.55**	**0.050**
Feathers (day 3)	0.57 (0.69)	0.67	0.426
Plants (day 3)		1.37	0.259
Feathers (day 8)	-1.71 (0.66)	**6.73**	**0.020**
Plants (day 8)		3.77	0.071
Date	0.00 (0.03)	0.00	0.994
Brood size	-0.72(0.28)	**6.36**	**0.023**

Feather pigmentation model			
	Beta (SE)	$F_{1,7}$	P
Feather treatment (1)		0.17	0.694
Plant treatment (2)		0.06	0.815
(1)*(2)		0.15	0.715
Pigmented (day 3)	0.07 (0.77)	0.01	0.925
Unpigmented (day 3)	-0.99 (0.73)	1.84	0.217
Plants (day 3)		0.65	0.447
Pigmented (day 8)	-0.11(1.00)	0.01	0.914
Unpigmented (day 8)	0.51 (1.04)	0.24	0.641
Date	0.01 (0.07)	0.03	0.864
Brood size	-0.27 (0.63)	0.19	0.676

Bacteria	Beta (SE)	$F_{1,9}$	P
Mesophilic bacteria (day 3)	-0.63 (0.48)	1.75	0.218
Enterococcus (day 3)	1.51 (7.99)	0.04	0.855
Mesophilic bacteria (day 8)	0.42 (0.40)	1.10	0.321
Enterococcus (day 8)	-0.17 (0.51)	0.11	0.751
Enterobacteriaceae (day 3)		0.06	0.811
Staphylococcus (day 3)		0.00	0.970
Enterobacteriaceae (day 8)		0.19	0.677
Staphylococcus (day 8)		0.20	0.664
Date	0.02 (0.04)	0.17	0.686
Brood size	-0.56 (0.43)	1.72	0.222

CAPÍTULO 3

Telomere length and dynamics of spotless starling nestlings depend on nest building materials used by parents

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Abstract

1. Nest materials used by animals can have profound effects on developing offspring. They can modify the bacterial and parasitic environment of the nest, and can influence parental investment through sexual signalling processes, therefore impacting on the quality of parental care received by offspring.

2. In spotless starlings (*Sturnus unicolor*), green plants and feathers are known nest materials with such functions. The aim of this work is to experimentally assess their influence on telomere length and attrition of nestlings, which are good predictors of their survival prospects.

3. In a full factorial experiment, we explored these effects in two different populations, together with the potential effects of hatching date, ectoparasitism, bacterial environment and nestling growth.

4. After correcting for a curvilinear association between hatching date and telomere length and dynamics, which may underlie the effects of territory and/or parental quality, the addition of feathers (pigmented and unpigmented) resulted in higher rates of telomere attrition in both populations, while the addition of unpigmented feathers resulted in fledglings with longer telomeres. Moreover, the experimental addition of green plants resulted in longer telomeres. All these effects greatly depended on the population. Moreover, prevalence of staphylococci on the skin of 8 days old nestlings was negatively related to telomere lengths of fledglings.

5. Taken together, these results suggest a direct link between nest material composition and nestling telomere length and dynamics. This relationship could be partially mediated by the antimicrobial and/or antiparasitic properties of nest materials or by sexual signalling processes. We discuss possible roles

of maternal effects, parasites, immunity and nestling growth explaining the detected experimental effects.

Keywords: Antimicrobial properties, *Carnus* flies, feathers, green plants, hatching date, nest building behaviour, nest-dwelling ectoparasites, sexual selection, telomere dynamics.

Introduction

Environmental conditions that offspring experience during growth have important consequences for their survival and reproductive prospects (Monaghan 2008). These environmental conditions include indirect genetic effects of parents on offspring phenotypes through parental behaviours that in birds include, among others, nest building, nest defence against predators, nest sanitation, incubation and brooding, or feeding effort (Mousseau & Fox 1998). From an evolutionary perspective, research on the effects of nest building behaviour on offspring survival prospects and recruitment is of particular interest because nests are extended phenotypes of builders (Dawkins 1982) on which natural and sexual selections are working (Collias & Collias 1984; Palomino et al. 1998; Soler et al. 1998; Hansell 2000; Moreno 2012). On the one hand, nest building behaviour may have indirect consequences on developing offspring because nests may signal phenotypic quality of builders (Collias 1964; Moreno 2012). This would affect reproductive decisions of their mates through differential investment (Burley 1986; Sheldon 2000) in a typical post-mating sexual selection process (Soler et al. 1998). On the other hand, nests may directly influence nestling phenotypes due to nest structures and materials having thermoregulatory, antipredatory, antimicrobial, or antiparasitic properties (Moreno 2012; Dubiec et al. 2013; Heenan 2013).

The use of nest materials with antimicrobial and/or antiparasitic 70 properties is considered a form of self-medication (Clayton & Wolfe 1993; De Roode et al. 2013). Special attention has received the use of green plants with known antipathogenic effects (Clark & Mason 1985; Tomás et al. 2012; Dubiec et al. 2013). Most green plants used for nest building are aromatic plants that contain volatile compounds or essential oils with repellent or toxic effects on blood-sucking arthropods and microorganisms and, therefore, could

play a role minimizing the effects of pathogenic bacteria and nest parasites on developing offspring (Clark & Mason 1985; Clark 1990; Tomás et al. 2012). Evidence of green plants reducing risk of bacterial and parasitic infection of nestlings is compiled in Dubiec et al. (2013). More recently, the use of feathers as nest material has also been proposed to have antimicrobial effects (Soler et al. 2010). Evidence of this function has accumulated during the last years. We know, for instance, that bacterial colonies from unpigmented feathers have higher antimicrobial activity than those from pigmented feathers in particular nest environments (Peralta-Sánchez et al. 2014). This property would explain the reduced bacterial loads and hatching failures of barn swallow (*Hirundo rustica*) eggs in nests with experimentally supplied unpigmented feathers (Peralta-Sánchez et al. 2010; 2011). More recently, experimental addition of feathers demonstrated a reduction in bacterial loads and trans-shell infection of eggs in nests of spotless starlings (*Sturnus unicolor*) and in artificial nests without parental influence (Ruiz-Castellano et al. 2016). Thus, effects related to the antimicrobial and antiparasitic properties of nest materials are key candidates to explain the expected associations between nest building behaviour of parents and variables related to survival prospects of nestlings (Gwinner & Berger 2005; Mennerat et al. 2009a; 2009b; Dubiec et al. 2013; Polo et al. 2015).

Nest building is however a costly activity (Mainwaring & Hartley 2013) and net benefits of carrying feathers and green plants to the nest would depend on the level of selection pressure that these materials are expected to counteract with. For instance, selection pressure due to parasitism greatly varies both geographically (Ardia 2007; Freeman-Gallant et al. 2001; Martin II et al. 2004; Møller et al. 2006; 2011) and temporally (i.e., laying date) (Sorci et al. 1997; Merino et al. 2000) in association with level of immune response and related variables. Moreover, immunocompetence of nestlings is traded off against nestling growth (Soler et al. 2003), and the resolution of the

trade-off would depend on environmental conditions (i.e., resource availability and parasitism) with important temporal and geographical variation (Roff & Fairbairn 2007). Thus, nest building effort should be adjusted to environmental characteristics to produce net fitness benefits. In this scenario, experimental manipulations of nest material will not always result in detection of the expected benefits, which would vary depending of geographical and temporal variation of environmental conditions. In fact, the expected beneficial effects of nests materials in terms of probability of nestling recruitment (e.g. body mass, immunocompetence) have not been detected in several experimental studies (see review in Dubiec et al. 2013).

Recently, telomere length and dynamics have been proposed as measures that encapsulate the effects of stressful environmental conditions on nestlings' development and survival prospects (Monaghan & Haussmann 2006; Monaghan 2014). Telomeres are specialized structures at the end of the chromosome, which consist on shorts repeats of the non-coding DNA sequence TTAGGG that protect wholeness of genetic information during cell division (Blackburn 1991). Recent studies have reported negative associations between telomere length and baseline corticosterone levels (Quirici et al. 2016), oxidative stress (Badás et al. 2015; Kim & Velando 2015), and parasite infection (Asghar et al. 2015a). Moreover, early life telomere length has strong maternal effects (Asghar et al. 2015b) and telomere dynamics depend on abiotic (e.g. altitude, laying date (Stier et al. 2016; Soler et al. 2015)) and biotic environmental conditions (e.g. nestling competition for food (Reichert et al. 2014; Nettle et al. 2015; Soler et al. 2015)), including those related to parental behaviour (Sudyka et al. 2014). Thus, telomere length and dynamics in nestlings appear as appropriate target variables for testing the effects of nest building behaviour of adults (e.g. nest material used) on nestling development.

Here, we experimentally explored the effects of nest material on telomere length and dynamics in spotless starling nestlings during the nesting phase, while considering temporal and geographical variation. Briefly, we removed or added green plants and/or feathers in nests with recently hatched nestlings, and explored the effects on telomere length and telomere attrition of nestlings close to fledging. We performed this experiment in two different populations differing in ectoparasitism level but not in bacterial loads of nestlings (see Results), while controlling the expected experimental effects for hatching date. Hatching date is usually related to phenotypic condition of nestlings and is considered to reflect availability of resources for developing nestlings (Verhulst & Nilsson 2008) and, consequently, should affect telomere length and dynamics. We expected to detect the beneficial effects of these nest materials in terms of telomere length and reduced telomere attrition in nestlings that grew in nests with experimentally added plants and/or feathers, especially in the area with higher ectoparasitism. Furthermore, we also quantified ectoparasitism and bacterial loads on the skin of nestlings, and explored the expected negative relationship with telomere length and positive relationship with telomere shortening in nestlings close to fledging.

Materials and methods

Study area and species

The study was performed in Hoya de Guadix, southeast of Spain, a high-altitude plateau 1000 m a.s.l. with a semi-arid climate, during the 2012 breeding season. The spotless starling populations under study breed in cork-made nest boxes (internal height * width * depth: 350 * 180 * 210 mm, bottom-to-hole height: 240 mm) attached to tree trunks or walls at 3-4 m above ground. The two studied populations are located in the old railway stations of La Calahorra (37º15’N, 3º01’W) and Hueneja (37º13’N, 2º56’W),

20 km apart. Ecological conditions of the study areas are apparently quite similar except for colony size and ectoparasitism level (see Results). Approximately 80 pairs breed per year at La Calahorra, while 35 pairs reproduce in Hueneja. Ectoparasitism by *Carnus hemapterus* and occupation of nest boxes was higher in La Calahorra than in Hueneja, although empty nest boxes were available in both populations during the study. Only first breeding attempts were considered in this study.

The hole-nesting spotless starling mostly breeds in colonies and use a variety of nesting materials, including feathers and green plants that are used both for the structural and the lining layer of the nest (Veiga and Polo 2016; Peralta-Sanchez et al. 2012; Ruiz-Castellano et al. 2016). In the studied population, starlings start to lay its typical 4-5 egg clutches at mid-April. Full incubation starts with the penultimate egg resulting in asynchronous hatching, which usually takes place at early May onwards (Soler et al. 2008). At the beginning of April, nest boxes were checked every second-third day until eggs were detected. Hatching date (age 0), defined as the day when half or more of the brood is hatched (Tomás 2015), was established by daily visiting nest boxes close to the expected dates (i.e., considering that incubation period lasts for 7-12 days after clutch completion). Nestling period range from 18 to 25 days (Veiga and Polo 2016).

Experimental design

Our experiment followed a full-factorial design with treatments of feathers and green plants as nest materials starting on day 3 of nestling age. We first recorded number of feathers and whether plants were present in nests and, subsequently, all plants and feathers were removed. Each nest was randomly assigned to one of the feathers and plants treatments. Since feathers of different colours may also differ in antimicrobial properties (Peralta-Sánchez et al. 2010), feathers' treatment consisted on allocating (i) 15 pigmented, or

(ii) 15 unpigmented feathers to the nest, or (iii) leaving the nest without feathers. This number of feathers is within the range and close to the modal number of feathers found in starling nests in our study area. Feathers were marked on the quill with a permanent marker to distinguish them from feathers introduced by adult birds. The plant treatments consisted in (i) introducing 1.6 g of a mixture of aromatic plants (the maximum of the quantity that starlings introduce in the nests during the nestling stage) or (ii) leaving the nest without plants (see details below).

All nestlings in the nest were individually marked by cutting some of their down feathers from the head, back or wings, weighed to the nearest 0.1g and their tarsus length measured to the nearest 0.01mm. Moreover, the belly of one randomly selected chick was sampled to characterize the bacterial environment of the nest (see below) before the experimental treatment. In addition, from each hatchling we collected a drop of blood by brachial venipuncture with the aid of a needle for estimating telomere length. Because of difficulties and risks associated with blooding recently hatched nestlings, we just punctured their brachial vein and collected a small drop of blood in a blotting paper, which was kept dry at 4 ºC until DNA isolation in the following months (see below).

Two days after the first visit (day 5 of nestling age), we removed all green plants and any feather added by birds, and refreshed green plants in the corresponding nests. On day 8 of nestling age, all nestlings were ringed and we sampled one chick belly per nest (not sampled during the first visit) to estimate bacterial load. Moreover, we quantified parasitism by *C. hemapterus* flies, a 2 mm blood-sucking fly found in nests of an extremely wide diversity of birds (Grimaldi 1997; Brake 2011). *Carnus* feeds exclusively on birds while at the nests, mainly on nestlings (Václav et al. 2016), but also on incubating birds (López-Rull et al. 2007; Avilés et al. 2009). Briefly, on day

8, nestlings were carefully taken from the nest and put inside a white cloth bag thus minimizing the possibility that flies jumped into the nest material. We counted the number of parasites on the body surface of each chick as well as the remaining flies in the bag to estimate parasite load for every brood (Avilés et al. 2009).

On day 14 of nestling age, we collected a second blood sample from each nestling for estimating telomere length in 75 µL heparinized capillary tubes after puncturing the brachial vein. Blood was later stored in an Eppendorf tube with absolute ethanol and maintained at 4 ºC until DNA isolation three months later (i.e. September 2012). During this visit, we also recorded body mass, tarsus and wing length of all nestlings.

Preparation of experimental nest lining feathers and aromatic plants has been explained previously (Ruiz-Castellano et al. 2016). Briefly, experimental unpigmented and pigmented body feathers were collected from chickens that grew in small farms close to the study area. In the laboratory, all experimental feathers were sterilized using a UV sterilizer chamber (Burdinola BV-100), and sprayed with approximately 84 mL of an overnight culture of *Bacillus licheniformis* D13 per m2. *Bacillus licheniformis* is one of the most common feather degrading bacteria (Burtt & Ichida 1999) which also produce antimicrobials (Callow & Work 1952; Gálvez et al. 1994; Lebbadi et al. 1994) and, thus, we assure our experimental feathers harboured similar amounts of antimicrobial producing bacteria. Plants introduced in nests were 1.6 g of a mixture of fragments of the four plant species most used by starlings in the studied population (*Marrubium vulgare*, *Artemisia barrelieri*, *Lamium amplexicaule* and *Anacyclus clavatus*). These species produce volatile compounds or essential oils with known antimicrobial activity (Ruiz-Castellano et al. 2016). Sterile gloves were employed to collect and

manipulate feathers and plants used in nests. For further information on experimental protocols see Ruiz-Castellano et al. (2016).

Bacterial sampling and laboratory work

For each nest visit and sampling we wore new gloves sterilized with 96% ethanol to prevent contamination of bacterial samples among nests. For bacterial sampling of nestlings we cleaned the complete belly surface of nestlings with a sterile rayon swab (EUROTUBO® DeltaLab) slightly wet with sterile sodium phosphate buffer (0.2 M; pH = 7.2). The swab was kept in an Eppendorf tube with the buffer solution and preserved at 4 ºC in a portable refrigerator until being processed in the laboratory within 24 h after collection. Once in the laboratory, 100 µL of solution containing bacteria were cultivated in four different solid media (Scharlau Chemie S.A. Barcelona), respectively for aerobic mesophilic bacteria (Tryptic Soy Agar), *Enterobacteriaceae* (Hektoen Enteric Agar), *Staphylococcus* (Vogel-Johnson Agar), and *Enterococcus* (Kenner Fecal Agar). Plates were incubated at 37 ºC for 72 h, when the number of colonies on each plate was counted. For more details see Peralta-Sánchez et al. (2010).

Belly bacterial density was estimated by standardization of the number of colonies per cm2 of sampled surface (CFU, Colony Forming Units). Belly surface was estimated from measurements of length and breadth of nestling belly, obtained with a digital calliper to the nearest 0.01 mm, assuming that nestling belly is half of an ovoid (Narushin 2005). We measured the surface area of 12 and 7 nestlings respectively sampled at the age of 3 and 8 days. Bacterial counts of these samples did depend on nestling age, but the relationships with belly area sampled at day 3 (mesophilic bacteria: $F_{1,10}$ = 1.38, P = 0.266, *Enterococcus*: $F_{1,10}$ = 1.56, P = 0.240) or at day 8 (mesophilic bacteria: $F_{1,3}$ = 2.74, P = 0.454, *Enterococcus*: $F_{1,3}$ = 2.74, P = 0.666) were far from statistical significance. Thus, we standardized bacterial

density to average belly area for different nestling ages (3 day old nestlings: 7.24 cm2; 8 day old nestlings: 9.36 cm2).

Characterization of bacterial environments by traditional culture techniques produces a relatively narrow picture of bacterial communities (Lee et al. 2013), but it has been demonstrated as an appropriate method for exploring effects of skin bacterial density on nestlings (Gwinner & Berger 2005; Mennerat 2009b; González-Braojos et al. 2012a; 2012b; 2012c) and thus, for our purposes.

Telomere length estimations

DNA was extracted from blood samples using a standard chloroform-isoamyl alcohol based protocol (see Ferraguti et al. 2013; Soler et al. 2015). DNA concentration was adjusted to 20 ng/µL using distilled water and conserved frozen (-20ºC) until further analyses. Relative telomere length (hereafter telomere length) was estimated by q-PCR following Criscuolo et al. (2009). We used the single copy gene glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) as control to normalize the quantity of telomere sequence to the amount of DNA in the q-PCR reaction. The final PCR volume was 20 µL containing 10 µL of LightCycler 480 SYBR Green I Master (Roche) and 1 µL of DNA at 20 ng/µL 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 than 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 1.858 and 2.143 and for the GAPDH product between 1.893 and 2.007. The slope of the calibration curve ranged between -3.718 and -3.021 for the telomere product and -3.608 and -3.163 for the GAPDH product. The melting curves of the control gene cycles confirmed no evidence of primer dimmer or non-specific amplification. Different techniques are available for measuring telomere length in wildlife (reviewed in Nussey et al. 2014). However, the method employed here is adequate to compare patterns of variation within species based on repeated measures of the same individuals across time, as we have done here (for a similar approach see Asghar et al. 2015a).

Estimated values of telomere length by the techniques explained above largely depend on the method of blood conservation and DNA isolation (Tolios et al. 2015). Mainly because of difficulties and risks associated to bleeding recently hatched nestlings, and the amount of blood needed to collect samples within capillaries, methods for conservation of samples from recently hatched nestlings and from nestlings close to fledging differed. Thus, although estimates of telomere length of hatchlings were much lower (NQR = 0.776, SE = 0.020, N = 131 (only nestlings with information in both nestling stages) than those of fledglings (NQR = 1.00, SE = 0.039, N = 131)), differences can be due to different methodologies of blood conservation. We statistically account for differences due to different protocols by using ranked values (i.e. rank 1 was assigned to the smallest value, while ranks for ties were the mean) of NRQs for hatchlings and for fledglings (see below).

Statistical analyses

Tarsus length, body mass, hatching date and log10-transformed numbers of feathers in the nests previous to the experimental addition approached normal distributions (Kolmogorov-Smirnov tests for continuous variables $P > 0.2$). Abundance of *Carnus* flies were log10-transformed and bacterial loads estimated for mesophilic bacteria and for Enterococci were log10-transformed before the analyses to approach normal distributions. Estimates for Enterobacteria and Staphylococci included many zero values and thus were analysed as binomial distributed variables (i.e. presence / absence) in the analyses. The presence of green material in the nests was included in the models as a binomial independent factor. We managed to collect information from 52 nests, 137 hatchlings and 135 fledglings. For 131 nestlings that grew in 52 nests we obtained information from both stages, as hatchlings and as fledglings. Differences in ranked values among fledglings and hatchlings were used as estimation of changes in telomere length. These values did not differ from normal distributions (Kolmogorov-Smirnov tests for continuous variables: $P> 0.2$). Statistically significant associations between independent factors and telomere length of hatchlings or fledglings were detected independently of whether ranked or raw values of telomere length were analysed (results not shown). However, because telomere dynamic along the nestling period was estimated as the difference in ranked values for each nestling, for consistency we only show results using ranked values.

Trying to reduce the number of independent factors included in models exploring relationships with telomere lengths or dynamics, we separately analysed the effect of variables describing nest materials and nestling measurements on the one hand, and bacterial loads on the other hand. One of the models explaining telomere length of hatchlings therefore included information of hatching date, tarsus length, body mass and number of feathers

as continuous predictors, and study area and presence of plants in the nest as categorical predictors. The other model included information on hatching date and bacterial loads as continuous predictors, and study area as a categorical predictor. Nestlings sharing the same nest have identical values for some of these variables (e.g. nest materials, hatching dates) and, to account for non-independence of data, we adjusted degrees of freedom to number of sampled nests.

Experimental effects on body mass, tarsus length, *Carnus* flies, bacterial loads, and telomere length and dynamics in fledglings were analysed by means of general linear mixed models (GLMM) with study area, plant and feather treatments and their interactions as fixed categorical factors, and nest identity nested within the major order interaction among fixed factors as the random factor. The experimental effects on prevalence of *Enterobacteriaceae* and of *Staphylococcus* were analysed in generalized linear models with binomial distribution and logit link function while correcting for overdispersion. Feather and plant treatments, study area and their interactions were included as fixed effects in the models. Experimental effects of feathers were analysed in two different sets of models. First, we explored the effects of feathers as a whole (i.e., factor with two-levels: with vs without feathers) and thus considered together nests under pigmented and unpigmented feather treatments. Secondly, we explored the effects of feather pigmentation (two-level factor: pigmented vs unpigmented feathers) and thus considered only nests with experimentally added feathers. The rationale for this second approach is based on previous results suggesting differential effects of pigmented and unpigmented feathers in barn swallow (Peralta-Sánchez et al. 2010) and spotless starling nests (Ruiz-Castellano et al. 2016). Since telomere length and attrition experienced by nestlings were related to hatching date in a non-linear manner (see Results), we used residuals of these trends to explore the experimental effects exposed above.

Associations between bacterial or *Carnus* loads and telomere length and dynamics of nestlings at the age of fledging were also explored in separate models. We used residuals of telomere length and attrition on hatching date as dependent variables and bacterial loads of nestlings three and eight days after hatching as continuous predictors, and study area as categorical predictor. Again, the degrees of freedom were adjusted to the number of sampled nests.

For final model selection, fixed factors with the largest p-values were removed one by one up to p-values = 0.1, starting for the two-level interactions. Full and reduced models are shown except for those showing bacterial influence for which only final models are discussed. Statistically non-significant main effects were retained in the models when the interaction with other factors reached statistical significance.

Results

Experimental manipulation of nest materials and biometry, bacterial loads and parasitism of nestlings

Body mass (linear terms: Beta(SE) = -0.860(1.225), F = 0.492, df_{adj} = 1,51, P = 0.486; quadratic term: Beta(SE) = 1.029(0.225), F = 0.705, df_{adj} = 1,51, P = 0.405) or tarsus length (linear terms: Beta(SE) = 0.086(1.241), F = 0.005, df_{adj} = 1,51, P = 0.944; quadratic term: Beta(SE) = 0.005(1.241), F = 0.001, df_{adj} = 1,51 , P = 0.996) of 14 days old nestlings did not depend on hatching date. Experimental plant treatments and study area affected tarsus length of 14 days old nestlings (Table 1). Nestlings in nests in La Calahorra were those with the larger tarsi, and, within each study area, nestlings that grew in nests that received experimental plants were those with the shorter tarsi (Fig. 1A). The statistical model considering experimental nests that received pigmented or unpigmented feathers failed to detect significant associations with tarsus

length (P > 0.33). Experimental treatments, study area, or their interactions failed to explain significant proportion of variance in nestling body mass (P > 0.126). However, when only considering nests that received experimental feathers, the interaction between feathers (i.e. pigmented vs unpigmented) and plant treatments explained nestling body masses (GLM, final model included feather (F = 0.063, df = 1,39.7, P = 0.804) and plant treatments (F = 0.043, df = 1,39.7, P = 0.837), their interaction (F = 6.705, df = 1,39.7, P = 0.013) and the random effect of nest identity (F = 1.226, df = 32,59, P = 0.245)). Unpigmented feathers in nests with plants added had positive effects on nestling body mass, while a positive effect of pigmented feathers was detected in nests with green plants removed (Fig. 1B).

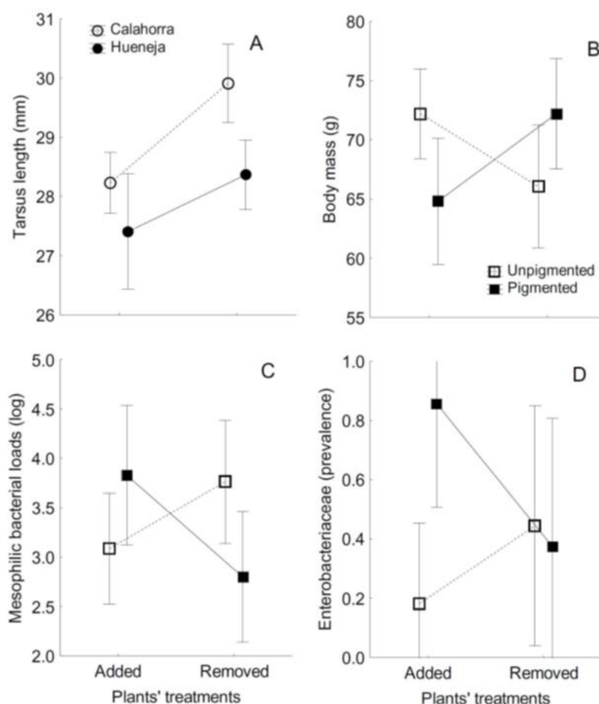

Figure 1: Effects of experimental green plants (added vs removed) as nest material on tarsus length (A), body mass (B), mesophilic bacterial loads (C), and prevalence of *Enterobacteriaceae* (D), in relation to study area (Calahorra and Hueneja, A), and feather treatment, (unpigmented and pigmented, B, C and D). Average values (± 95% CI) are shown.

Table 1: Results from full and reduced Generalized Linear Mixed Models explaining experimental effects of feathers and plants as nest materials on tarsus length, and telomere length and dynamics of starling nestlings close to fledging (residuals after controlling for the effects of hatching date, see text). Study area, treatments and their interactions were included as fixed factors and nest identity nested within the interaction between the three factors was included as the random effect. Reduced model resulted from backward stepwise selection up to P-values = 0.1. Significant values are in bold.

		Tarsus length			Telomere length			Telomere dynamics		
	Effect	F	df	P	F	df	P	F	df	P
Study area (1)	Fixed	**6.71**	**1,44.8**	**0.013**	**12.96**	**1,43.9**	**0.001**	**8.87**	**1,43.0**	**0.005**
Plant treatment (2)	Fixed	**4.87**	**1,45.6**	**0.032**	0.00	1,45.0	0.965	0.30	1,43.7	0.589
Feather treatment (3)	Fixed	1.46	1,45.5	0.233	1.67	1,45.0	0.202	**10.20**	**1,43.6**	**0.003**
(1)*(2)	Fixed	0.05	1,45.4	0.816	**4.21**	**1,44.7**	**0.046**	0.40	1,43.5	0.532
(1)*(3)	Fixed	2.30	1,45.6	0.136	0.28	1,44.9	0.600	2.72	1,43.5	0.107
(2)*(3)	Fixed	**5.04**	**1,46.3**	**0.030**	0.28	1,46.3	0.596	1.42	1,44.5	0.240
(1)*(2)*(3)	Fixed	1.17	1,46.2	0.285	0.91	1,45.9	0.345	0.00	1,44.2	0.978
Nest ((1)*(2)*(3))	Random	**2.23**	**43,90.0**	**0.001**	1.29	43,83.0	0.157	**1.92**	**44,79.0**	**0.006**
Reduced model										
Study area (1)	Fixed	**5.82**	**1,43.3**	**0.020**	**14.73**	**1,41.9**	**<0.001**	**8.87**	**1,43.6**	**0.005**
Plant treatment (2)	Fixed	**8.47**	**1,49.6**	**0.005**	0.24	1,42.8	0.628			
Feather treatment (3)		1.98	1,44.1	0.166				**13.19**	**1,43.6**	**0.0007**
(1)*(2)	Fixed				10.35	1,41.8	0.003			
(1)*(3)	Fixed	3.06	1,43.3	0.087				3.55	1,43.9	0.066
(2)*(3)	Fixed	3.76	1,49.5	0.058						
Nest ((1)*(2))	Random	**2.24**	**46,90.0**	**0.001**	1.23	48,83.0	0.200	**1.89**	**1,43.4**	**0.006**

Hatching date was not associated with bacterial loads of 8 days old nestlings (MANOVA, linear term: Wilks = 0.925, F = 0.938, df = 4,46, P = 0.451; quadratic term: Wilks = 0.923, F = 0.961, df = 4,46, P = 0.438). Experimental treatments, study area or their interactions failed to explain loads of mesophilic and Enterococci bacteria (GLM, F < 2.49, df = 1,49, P > 0.121), and prevalence of *Enterobacteriaceae* and Staphylococci (GLZ, Chi-square < 2.75, P > 0.097). When considering experimental nests with added feathers, feather treatments (pigmented vs unpigmented) explained load of mesophilic bacteria (i.e., in interaction with plant treatment, GLM, final model: F = 7.37, df = 1,31, P = 0.011) and prevalence of *Enterobacteriaceae* (GLZ, final model: Chi-square = 3.235, P = 0.072; interaction with plant treatment: GLZ, final model: Chi-square = 4.758, P = 0.029). In nests with experimental plants added, mesophilic bacterial density and *Enterobacteriaceae* prevalence were lower in nests with unpigmented feathers (Fig. 1C and Fig. 1D). In experimental nests with plants removed the opposite trend was detected for the density of mesophilic bacteria, while prevalence of Enterobacteria did not differ between nests with pigmented and unpigmented experimental feathers (Fig. 1C and Fig. 1D).

Abundance of *Carnus* flies on 8 days old nestlings tended to decrease as the season progressed (linear term: Beta(SE) = -3.634 (1.999), F = 3.306, df = 1,48, P = 0.075; quadratic term: Beta(SE) = 3.219(1.999), F = 2.594, df = 1,48 , P = 0.114). Abundance of *Carnus* in nests was not significantly affected by experimental treatments or interactions among them (GLM, F < 2.302, P > 0.136), but was larger in La Calahorra (log-transformed average parasite abundances (SE) = 2.53(0.18)) than in Hueneja (1.45(0.36)) (GLM, F = 8.13, df = 1,49, P = 0.006). When only considering nests with experimental feathers, experimental treatments also failed to explain a significant proportion of variance in parasite loads (GLM, F < 0.247, df =1, 33, P > 0.622).

Telomere length and dynamics

Telomere length of hatchlings

Telomere length of hatchlings (i.e., before the experiment) did not associate with biometrical variables (tarsus length and body mass) neither differed between study areas, but did positively associate with number of feathers in the nest and with hatching date (Table 2). Nestlings that hatched at intermediate-late dates in nests harbouring a higher number of feathers prior to the experimental removal had longer telomeres (Fig. 2). In addition, when considering bacterial loads of nestlings, those that harboured *Enterococcus* on their belly skin at a higher density also had longer telomeres (Beta (SE) = 0.17 (0.08), F = 4.13, df_{adj} = 1,49, P = 0.048) even after controlling for the significant linear and negative quadratic effects of hatching date and total nest lining feathers in their nests (F > 5.23, df_{adj} = 1,45, P < 0.024; sign of the estimates are identical to those showed in Table 2).

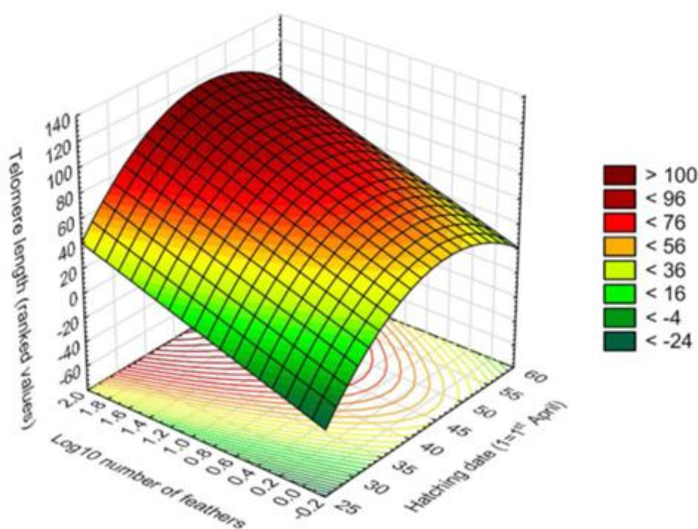

Figure 2: Surface plot showing the relationship between telomere length of spotless starling hatchlings with hatching date and number of feathers in the nest (log10-transformed). Different colours indicate different values for telomere length.

Table 2: Results from full and reduced models explaining telomere length of hatchlings depending on characteristics of nestlings (hatching date, tarsus length and body mass), nest materials (log10-transformed number of feathers, and presence or absence of green plants), and study area. Final model resulted from backward stepwise selection up to P-values = 0.1. Corrected P-values by adjusting degrees of freedom (df = 128) to number of sampled nests (df = 48 and 50 for full and final model respectively) are also shown. Significant results are in bold.

	Beta	SE	F	P	Corrected P
Hatching date	**2.645**	**1.307**	**4.10**	**0.045**	**0.049**
Hatching date^{2}	-2.363	1.303	3.29	0.072	0.076
Tarsus length	0.274	0.177	2.39	0.124	0.129
Body mass	-0.218	0.181	1.45	0.231	0.235
Number of feathers	**0.265**	**0.102**	**6.69**	**0.011**	**0.013**
Green plants	-0.058	0.087	0.45	0.503	0.505
Study area	0.113	0.112	1.01	0.316	0.576
Reduced model					
Hatching date	**2.638**	**1.245**	**4.49**	**0.036**	**0.039**
Hatching date^{2}	-2.461	1.238	3.95	0.049	0.052
Number of feathers	**0.244**	**0.096**	**6.48**	**0.012**	**0.014**

Telomere length of fledglings and experimental manipulation of nest materials

Telomere length of fledglings was significantly related to hatching date in linear (Beta (SE) = -3.01 (1.26), F = 5.70, df = 1,132, P = 0.018) and quadratic (Beta (SE) = 0.19 (0.08), F = 5.44, df = 1,132, P = 0.021) manners. Minimum values of telomere length therefore appeared in nestlings of intermediate hatching date (Fig. 3A). After controlling for this effect, telomere length of fledglings differed among study areas (Table 1, Fig. 4A). Furthermore, the experimental effects of addition of green plants to the nest during nestling growth greatly depended on study area (Table 1). In the area with nestlings with the longest telomeres at the fledging age (Hueneja), the

experimental group with added green plants presented shorter telomeres than controls, while the opposite effect was detected in the area with nestlings with the shortest telomeres at this age (La Calahorra) (Fig. 4A). The manipulation of number of feathers did not affect final telomere length (Table 1). The inclusion of biometrical information (body mass and tarsus length) in the model did not affect the detected experimental effects of plant treatments on telomere length (interaction between areas and plant experimental treatments, $F = 9.09$, $df = 1,52.9$, $P = 0.004$), while none of the biometrical variables reached statistical significance (all $F < 0.44$, $df = 1,79$, $P > 0.50$).

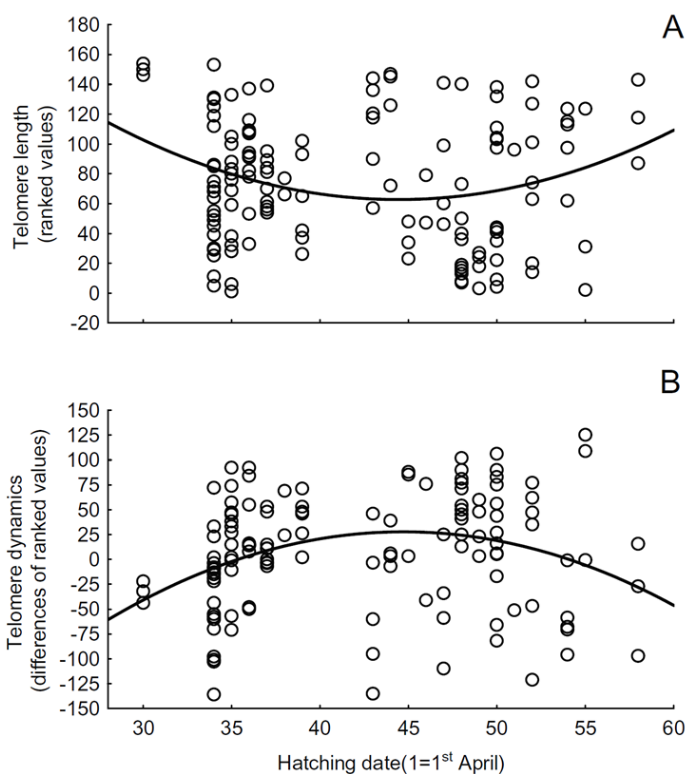

Figure 3: Relationship between hatching date and telomere length (A) and dynamic (B) of 14 day old spotless starling nestlings.

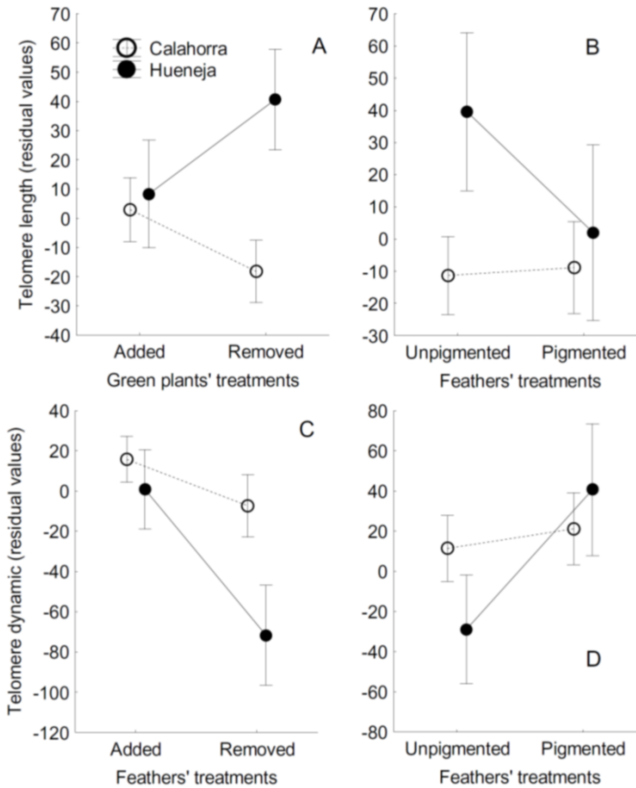

Figure 4: Effects of experimental green plants and feathers (added vs removed or pigmented vs unpigmented) as nest material on telomere length and dynamics (i.e. rank values of telomere length at hatching minus those of telomere length at fledging times) of spotless starling fledglings in relation to study area (Calahorra and Hueneja). Telomere length and dynamics show average (± 95% CI) residual of ranked values after controlling for the curvilinear association with hatching date.

In nests with experimentally added feathers, nestlings from nests with unpigmented feathers tended to have longer telomeres that nestlings from nests with pigmented feathers, but only in the Hueneja population (final GLM model, interaction between study area and feather treatment, F = 3.61, df = 1,22.3, P = 0.068, Fig. 4B). Study area (F = 6.36, df = 1,22.3, P = 0.019) and the interaction with plant treatment (F = 8.59, df = 1,21.3, P = 0.008) also reached statistical significance for this subset of nests.

Telomere dynamics of nestlings and experimental manipulation of nest materials

Telomere dynamics of nestlings along their stay in the nest was related with hatching date in linear (Beta (SE) = 28.36 (9.40), F = 9.09, df = 1,128, P = 0.003) and quadratic (Beta (SE) = -0.32 (0.11), F = 8.57, df = 1,128, P = 0.004) manners. Nestlings of intermediate hatching date experienced the highest reduction in ranked values of telomere length (Fig. 3B). After controlling for this effect, telomere dynamics differed between study areas (Table 1). Nestlings from La Calahorra population showed the largest reduction in ranked values of telomere length from hatching to fledging age (Fig. 4C). Furthermore, reduction in these ranked values was significantly higher in those nestlings growing in nests with experimentally added feathers (Table 1, Fig. 4C). The inclusion of biometrical information (growth in body mass and tarsus length) in the model did not affect the detected effects of study area (F = 7.68, df = 1, 45.4, P = 0.008) neither of feather treatment (F = 13.60, df = 1, 48.6, P = 0.0006). None of the biometrical variables nor the addition of plants reached statistical significance (F < 0.42, df = 1,76, P > 0.51).

Results from statistical models exploring possible differential effects of feather colours indicate that telomeres of nestlings that developed in nests with unpigmented feathers shortened at a lower rate than those of nestlings in nests with pigmented feathers (final GLM model, F = 9.078, df = 1,29.6, P = 0.005), but mainly in the Hueneja population (final GLM model, interaction between feather treatment and population, F = 5.442, df = 1,28.5, P = 0.027) (Fig. 4D).

Telomeres and biometry, parasitism and bacterial loads of nestlings

Body mass or tarsus length failed to explain telomere length of 14 days old nestlings (i.e. residual values after controlling for hatching date, see above) (body mass: F = 0.61, df_{adj} = 1,50, P = 0.434; tarsus length: F = 0.01, df_{adj} = 1,50, P = 0.959), after controlling for the effect of study area (F = 9.99, df_{adj} = 1,50, P = 0.003) and the random effect of nest identity nested within study area (F = 1.43, df_{adj} = 50,81, P = 0.075). Furthermore, abundance of *Carnus* also failed to predict telomere length of nestlings after controlling for the effect of study area (F = 0.826, df = 36, P = 0.826). Finally, the final model exploring the association between bacterial loads and telomere length of fledglings showed a negative effect of the presence of Staphylococci on the skin of nestlings in the second sampling (Beta(SE) = -20.68(7.56), F = 7.48, df_{adj} = 1,48, P = 0.009, Fig.5). This was the case after controlling for the significant effect of study area (F = 18.91, df_{adj} = 1,48, P < 0.0001), and the non-significant effects of the presence of *Enterobacteriaceae* in first (Beta(SE) = -13.03(7.52), F = 3.01, df_{adj} = 1,48, P = 0.089) and second sampling (Beta(SE) = 13.80(7.44), F = 3.44, df_{adj} = 1,48, P = 0.070).

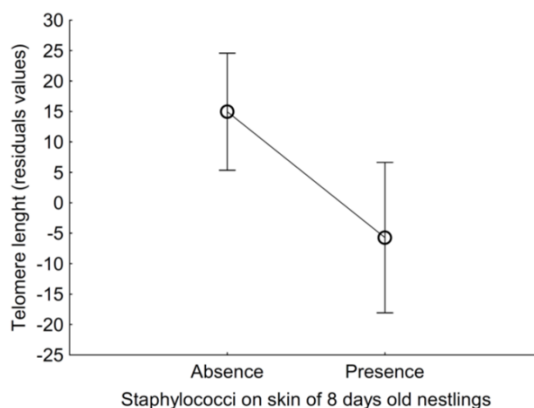

Figure 5: Effect of *Staphylococcus* presence on the skin of 8 day old spotless starling nestlings on telomere length. Telomere length shows average (± 95% CI) residual of ranked values close to fledging after controlling for the curvilinear association with hatching date.

Body mass or tarsus length did not explain telomere dynamics experienced by nestlings during the nesting stage (after controlling for the effect of hatching date) (body mass: F = 0.12, df_{adj} = 1,50, P = 0.731; tarsus length: F = 0.29, df_{adj} = 1,50, P = 0.591), after controlling for the effect of study area (F = 6.25, df_{adj} = 1,50, P = 0.016) and the random effect of nest identity nested within study area (F = 2.00, df_{adj} = 50,77, P = 0.003). Abundance of *Carnus* also failed to predict telomere length of nestlings after controlling for the effect of study area (F = 0.38, df = 1,36, P = 0.541). Finally, bacterial loads harboured by nestlings soon after hatching explained telomere dynamics. Nestlings with lower density of mesophilic bacteria (Beta(SE) = -16.03(6.32), F = 9.06, df_{adj} = 1,48, P = 0.004) and higher density of *Enterococcus* (Beta(SE) = 17.12(6.26), F = 7.49, df_{adj} = 1,48, P = 0.009) before the experimental treatment experienced the larger reduction in ranked values of telomere length from hatching to fledging ages. That was the case after controlling for the effects of study area (F = 15.90, df_{adj} = 1,48, P = 0.0002) and the non-significant positive effects of prevalence of *Enterobacteriaceae* (Beta(SE) = 19.40(9.83), F = 3.89, df_{adj} = 1,48, P = 0.054) and of *Staphylococcus* (Beta(SE) = 28.27(15.33), F = 3.40, df_{adj} = 1,48, P = 0.07). Final models did not retain any of the variables reflecting bacterial loads of nestlings in the second sampling.

Discussion

We have found experimental and empirical evidence supporting the hypothesis that nest materials affect growth and telomere length and dynamics of spotless starling nestlings. These results point to direct effects of nest materials on survival prospects of nestlings, mainly due to the known association of telomere length with survival (Asghar et al. 2015a). However, interestingly, the strength of such relationships, and even the sign of some of these effects, depends on study area. Nestlings with the shortest final telomere

length and the largest telomere attrition grew in the area with the highest ectoparasite abundance and nest density, which suggests a link between parasitism and nestling telomeres. We also detected curvilinear associations between hatching date and telomere length and dynamics, which may reflect the effects of territory and/or parental quality on telomere characteristics of nestlings. Finally, we also discovered a negative relationship between presence of Staphylococci on the skin of 8 days old nestlings and telomere length of fledglings, which suggests a role for bacteria determining telomere dynamics during the nestling phase. Below we discuss possible mechanisms that could explain these relationships and, therefore, the hypothesis that nest-building behaviour affects cellular senescence and offspring development.

Evidence supporting the assumed close relationship between telomere length and/or dynamics and survival prospects of nestlings is accumulating during recent years. Laying or hatching dates are usually considered as variables reflecting territory or parental quality (Brinkhof et al. 1993; Moreno 1998; De Neve et al. 2004; Verhulst & Nilsson 2008; Tomás et al. 2012) and, thereby, availability of resources for developing offspring. Thus, laying or hatching dates should be related to telomere dynamics of nestlings (Foote et al. 2011; Soler et al. 2015). We detected such clear linear and quadratic patterns of association between telomere dynamics of nestlings and hatching date. Telomere length of hatchlings and telomere attrition experienced during development increased as the season progressed with maximum values at intermediate dates. However, telomere length of fledglings decreased as the season progressed, reaching minimum values at intermediate dates. These results suggest some kind of adjustment between telomere length at hatching and telomere attrition during development. This may be for instance explained by differential maternal investment or genetic quality of offspring of intermediate hatching dates. Mechanisms underlying such relationship should be further explored. For the hypothesis tested, it highlights the importance of

considering hatching dates when exploring associations between telomere dynamics and life history traits. Accordingly, we used residuals of telomere length and attrition after correcting for phenology.

We also found a strong influence of study area on telomere length and dynamics. Nestlings from the area with larger colony size and ectoparasite abundance (La Calahorra) had shorter initial telomeres and experienced more attrition than those from the area of smaller colony size and lower *Carnus* abundance (Hueneja). These results may suggest a link between risk of parasitism and telomere dynamics. However, we failed to identify any significant direct association between *Carnus* load and telomere dynamics. We know very little on geographical variation in telomere length and dynamics, but geographic variations in factors affecting growth or oxidative stress of nestlings, including any parasites or pathogens not considered here, may be the cause of the detected differences (Ilmonen et al. 2008; Asghar et al. 2015a). Further research is in any case necessary to elucidate factors explaining the detected differences between study areas.

Several mechanisms including direct effects of nest material, or indirect genetic effects, related to sexual selection processes or maternal effects, could explain the detected associations between nest materials and telomere length and dynamics. The detected positive association between number of feathers in the nest at the time of hatching and telomere length of hatchlings would be hardly explained by the hypothetical direct beneficial effects of feather materials on developing nestlings. Although we cannot rule out the possibility that thermoregulatory or antimicrobial properties of feathers during the incubation period explained such relationship, indirect effects due to sexual selection seem a more plausible explanation. In spotless starlings, feathers are carried to the nests mainly by females as a post-mating sexually selected signal of quality (Veiga & Polo 2005; Polo & Veiga 2006)

in response to the green plants carried by males, which potentially are another sexually selected trait (Veiga et al. 2006; Tomás et al. 2013). Telomere length is a heritable character, but only on the maternal side (Reichert et al. 2015; Asghar et al. 2015b). Thus, hatchlings with longer telomeres may be those from high quality mothers that carried to the nests a large number of feathers. The fact that presence of green plants in the nest, which are mainly carried by males as stated above, did not predict telomere length of hatchlings, is in agreement with the hypothetical stronger influence of females determining telomere length of hatchlings. Further studies are however necessary to confirm this possibility.

Indirect genetic associations seem less likely to account for the detected experimental effects of nest materials on telomere length and dynamics of fledglings. The experimental results could only be explained by direct effects of nest materials on nestling growth, and/or by differential parental effort in response to the experimental manipulations. Interestingly, we know that both green plants (Clark & Mason 1985; Dubiec et al. 2013) and feathers (Mennerat et al. 2009b; Peralta-Sánchez et al. 2010; Ruiz-Castellano et al. 2016) employed as nest materials influence probability of ectoparasitic and bacterial infections respectively with potential benefits in nestling development (Mennerat et al. 2009a; Sanz & García-Navas 2011). These effects however may vary between populations and or seasons (Mennerat et al. 2009a), and are expected to be larger under higher selection pressures. Thus, the predicted positive effects of green plants should be mainly detected in nestlings that developed in the population that experienced the highest parasite loads by *C. hemapterus* (see above), which is in accordance with our results. We however failed to detect a direct effect of experimental green plants on *Carnus* load, although other blood sucking insects (i.e., biting midges, blackflies) not considered in this study could play a role in this respect.

The negative effect of experimental green plants on telomere length of nestlings growing in Hueneja population is more difficult to explain. Interestingly, telomere length of nestlings that grew in nests with added plants did not differ between populations. We could speculate with the possibility that because green plants apparently boost nestling growth (Mennerat et al. 2009a), the reduced telomere length of nestlings that grew in nests with added green plants at Hueneja was just the result of the higher rates of cell divisions experienced by these nestlings. Adult birds may transport green plants to the nests mainly when risk of parasitism at the nest is high, and offspring may adjust growth rate to risk of parasitic infections or related cues (Saino et al. 1998). In agreement with a possible adjustment of growth to risk of parasitic infection, nestlings from the area with the highest parasite loads and shorter telomeres had longer tarsi than those from the area with longer telomeres. Enhancing growth as a response to parasitism is however costly in terms of poor development of the immune system (Saino et al. 1998; Soler et al. 2003). Immunity is a good predictor of nestling recruitment (Cichon & Dubiec 2005; Moreno et al. 2005) and, consequently, if nestlings use abundance of green material as a cue to adjust differential investment in development at the cost of immunity, the experimental addition of green plants would result in reduced survival prospects, at least in areas experiencing lower levels of selection pressure by parasites. Recently published experimental results by Polo et al. (2015) support this prediction in our species model. Although in our opinion this is the most likely explanation, further research is however necessary to determine factors explaining population differences in growth trajectories of nestlings depending on green nest materials.

Experimental feathers also influenced telomere length of nestlings. In contrast to pigmented feathers, the experimental addition of unpigmented feathers resulted in nestlings with telomeres of relative longer size and lower attrition rates, but it occurs only in nests of the Hueneja population.

Unpigmented feathers collected from barn swallow nests have superior antimicrobial properties than pigmented feathers (Peralta-Sánchez et al. 2014) and are preferred as nest materials by swallows (Peralta-Sánchez et al. 2010) and starlings (Ruiz-Castellano et al. unpublished). Thus, the detected effects may be the consequence of antimicrobial properties of unpigmented feathers. In fact, for nestlings in experimental nests with green plants added, those in nests with unpigmented feathers tended to be heavier and experienced lower bacterial loads than nestlings in nests with experimental pigmented feathers. Thus, it is possible that the differential antimicrobial capabilities of unpigmented feathers explained the detected effects in telomere length. However, if that was the case, the effects would be more evident in the area with the highest risk of infection; a prediction that our result do not support since the experimental effect was detected in the population possibly experiencing the lowest risk of infection (i.e., smaller colony size). Another possible explanation is that unpigmented feathers were more attractive for males who may have invested in feeding the offspring differentially in nests with the unpigmented, most conspicuous, experimental feathers (Veiga & Polo 2005) which may have direct effects on telomere dynamics in nestlings (Badás et al. 2015; Kim & Velando 2015). Any explanation need however of further investigation directed to explore mechanisms explaining the detected links.

When considering telomere attrition, we detected significant effects of feather, but not of green plant treatment. Contrary to our prediction, we found that telomere attrition was higher in nestlings from nests with added feathers. However, it was mainly due to the effects of pigmented feathers because telomere attrition of nestlings under this treatment was significantly higher than that of nestlings in nests with unpigmented feathers. We did not find significant associations between telomere attrition and body mass or tarsus length of nestlings, but bacterial load in nestlings was lower in nests with

experimental unpigmented feathers than in nests with experimental pigmented feathers. In addition, recent research has detected a positive effect of feather material on nestling immune response (Ruiz-Castellano et al. unpublished). Here we also found a negative association between prevalence of Staphylococci on nestling skin and telomere length, which suggests a role of bacteria driving telomere dynamics, but also that nestlings with weaker immune defence are those with shorter telomere length as suggested above. Thus, it is possible that the differential level of telomere attrition experienced by nestlings in nests with added feathers was due to differential investment in immunity, mainly in nests with pigmented feathers. Experimental manipulation of developing immune system is however necessary to further support this idea.

Summarising, whatever the relative importance of bacteria, parasites, immune system and resource availability in telomere length and dynamics, our results show a direct link between nest materials and telomere dynamics in developing offspring, which was the main purpose of our experiment. The strength and even the sign of the experimental effects differ for populations that varied in ectoparasitism loads and telomere length and dynamics of nestlings, which suggest that the effects of nest material on predictors of survival prospects of nestlings would depend on ecological characteristics of birds' populations. We hope these results encourage further research to clarify mechanisms and ecological conditions underlying the detected associations.

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Statement of authorship

JJS, GT, MMV, and JF designed the study with considerable assistance from JMP and CRC. CRC performed all bacterial and molecular analyses with considerable assistance from JMP. GT and CRC performed most of the field work with assistance by JJS, MMV, MRR. JJS performed all the statistical analyses and wrote the manuscript with substantial contribution from all authors.

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CAPÍTULO 4

Parental activity and nest-lining feathers enhance antimicrobial capabilities of bacterial communities in avian nests

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Abstract

The use of feathers as nest material has been recently proposed as a kind of self-medication strategy, since antimicrobial-producing microorganisms living on them may defend offspring against pathogenic infections. Here we analysed antimicrobial activity of bacterial colonies isolated from bird eggshells and nest-lining feathers against 18 bacterial strains, comprising potential pathogens. Samples were collected from spotless starling (*Sturnus unicolor*) nests, and also from artificial nests to isolate the effects of breeding activity on bacterial communities. We expect that antimicrobial activity should be higher for colonies isolated from feathers than from eggshells, and that it should be higher in starling nests than in artificial nests without parental influence. According to expectations, we found that bacterial colonies isolated from feathers were more frequently active against tested bacterial strains than those isolated from eggshells. Moreover, bacterial density on feathers and keratinolytic bacteria on eggshells were higher in starling nests than in artificial nests, and colonies isolated from the formers demonstrated higher antimicrobial capabilities than those isolated from the latter. These results suggest that antimicrobial capabilities of bacteria growing on nest-lining feathers are one of the mechanisms explaining the previously detected antimicrobial effects of this material on avian nests, and that incubation activity selects for bacteria with important antimicrobial activity. Therefore, results confirm that usage of feathers as nest material is a self-medication behaviour, which prevents proliferation of pathogenic micro-organisms in nests, and suggest that particular breeding activities have a role boosting growth of especially active antibiotic producing bacteria.

Keywords: Antimicrobial activity, eggshell bacterial load, feather nest material, incubation behaviour, keratinolytic bacteria, Spotless Starling.

Introduction

Animals are infected by numerous parasites of different taxa including arthropods, helminths, bacteria, fungi, etc. (Loye & Zuk 1991; Goater et al. 2001; Benskin et al. 2009). By definition, parasites reduce survival prospects and fitness of their hosts (Price 1980) and select for a plethora of defensive strategies in hosts that reduce the probability of infection and/or the negative effects of parasitism (Schmid-Hempel 2011). The immune system is with no doubt the main evolutionary response to parasitism selection pressures (Playfair & Bancroft 2004). However, the use of compounds produced by other organisms for protection against pathogens, called self-medication (Clayton & Wolfe 1993), is widespread in nature and has recently received special attention, not only because of its effects on the evolution of parasites and hosts, but also because of its promising implications regarding animal and human health (De Roode et al. 2013).

Self-medication behaviours have prophylactic and/or therapeutic functions (Lozano 1998; Hart 2005; De Roode et al. 2013). Green plants are one of the materials more commonly used by animals for self-medication, mainly because of the abundance and diversity of secondary metabolites present in plants, including volatile compounds that repel and/or kill parasites (Jacobson 1982; Clark & Mason 1985; Scott-Baumann & Morgan 2015). It is known for instance that chimpanzees (*Pan troglodytes*) reduce helminth parasitic loads by consuming *Vernonia amygdalina* (Huffman 2001), and that fruit flies (*Drosophila melanogaster*) reduce parasitoid attacks to their eggs by laying in high ethanol fruits (Milan et al. 2012). Birds also use green materials in their nests to reduce infections by ectoparasites (Dubiec et al. 2013) and microorganisms (Clark & Mason 1985; Gwinner & Berger 2005; Mennerat et al. 2009).

An even more sophisticate method of self-medication is the use of antibiotic producing microorganisms. It is for instance well known that fungus-growing ants (Genera: *Attine*) cultivate antibiotic producing *Streptomyces* bacteria in special cuticular crypts connected to exocrine glands (Currie et al. 2006) that prevent infection of their fungal cultivars by specialized parasitic fungi (Currie et al. 1999). This type of self-medication has also been observed in other taxa including birds (Flórez et al. 2015). This is for instance the case of common hoopoes (*Upupa epops*) that host antibiotic producing bacteria in their uropygial gland that prevent trans-shell bacterial infection of their eggs (Soler et al. 2008a; Martín-Vivaldi et al. 2010; 2014) and feather degradation by pathogenic microorganisms (Ruiz-Rodríguez et al. 2009).

The use of antibiotic-producing bacteria may be widespread in birds, for instance by using nest materials that differentially favours growth of microorganisms with antimicrobial properties (Soler et al. 2010). Particular attention has received the possible antibacterial function of feathers in avian nests (Peralta-Sánchez et al. 2010; 2011; 2014; Ruiz-Castellano et al. 2016), which was predicted due to known antimicrobial properties of most bacterial strains that are able to degrade keratin (Soler et al. 2010), the main component of feathers. The microbiome of feathers is relatively complex (Shawkey et al. 2005; Bisson et al. 2007; Dille et al. 2016). One of the most common bacteria found in feathers of wild birds are those belonging to the genus *Bacillus* (Burtt & Ichida 1999; Whitaker et al. 2005; Kent & Burtt 2016), for which antimicrobial properties are well known (Abriouel et al. 2011). Thus, by using feathers as nest lining material, birds will influence bacterial environments where offspring develop due to antagonistic activity of keratinolytic microbiota, which could be directed towards potentially pathogenic bacteria (Soler et al. 2010). There is experimental and correlational evidence of beneficial effects of the use of feathers as nest lining material in terms of

reducing bacterial loads in eggshells of barn swallows (*Hirundo rustica*) (Peralta-Sánchez et al. 2010; 2011) and spotless starlings (*Sturnus unicolor*) (Ruiz-Castellano et al. 2016). However, antimicrobial capability of bacteria growing in nest-feathers is not the only hypothetical mechanism explaining such results. Differential investment in nest sanitation activity in relation with the experimental addition of attractive feathers to avian nests might also account for the detected effects on eggshell bacterial load (Veiga & Polo 2011b). Although these two hypothetical mechanisms are not mutually exclusive, we here explore exclusive predictions of the hypothesis that particular antimicrobial capabilities of bacteria are one of the underlying mechanisms.

We analysed antimicrobial activity of bacterial colonies isolated from eggshells and feathers in bird nests, both with and without experimental feathers added, with the expectation that those from experimental feathers should demonstrate higher antimicrobial activity than those from eggshells. The reasoning is that feathers would harbour keratinolytic bacteria at a higher density than the eggshells. Furthermore, it is known from laboratory studies that growth rates of keratinolytic bacteria are lower in pigmented than in white feathers (Goldstein et al. 2004; Gunderson et al. 2008) which, in theory, may result in different bacterial communities. Consequently, different selective pressures due to bacterial interference (Riley & Wertz 2002) could be expected, which will originate different antimicrobial capabilities depending on feather pigmentation. Thus, antimicrobial effects of bacterial colonies isolated from swallow nest-feathers differ among unpigmented and pigmented ones (Peralta-Sánchez et al. 2014). Here, to explore these effects, our experimental treatments consisted in adding to nests: (i) unpigmented feathers, (ii) pigmented feathers, or (iii) both. Finally, there was a group of nests (iv) with no feathers added as control.

Even in the case that antimicrobial capabilities of bacteria growing on feathers were particularly high, some other environmental factors are likely modulating it such as the strength of bacterial interference (Riley & Wertz 2002). Particularly interesting is the environmental conditions linked to parental activity (i.e., incubation) that has been hypothesized to favour the growth of bacterial colonies with antimicrobial properties on the eggshells (Grizard et al. 2014). Incubation activity reduces microbial load and diversity on eggshells (Cook et al. 2005a; 2005b; Shawkey et al. 2009; Ruiz-De-Castañeda et al. 2012), mainly due to a reduction in eggshell humidity (D'Alba et al. 2010; Ruiz-De-Castañeda et al. 2011). In addition, because parental activity would influence environmental conditions, it is also possible that it affects antimicrobial capabilities of growing bacteria because, for instance, the production and activity of secondary metabolites of bacteria depends on temperature (Biswas et al. 1991; Mataragas et al. 2003). Here, we explore this hypothesis by quantifying antimicrobial activity of colonies isolated from experimental nest-feathers and eggshells in nests with and without breeding activity. For this purpose, we used active nests of spotless starlings and nest-boxes containing quail (*Coturnix japonica*) eggs, as well as pigmented and/or unpigmented feathers.

Materials and Methods

Study area and species

The study was performed during the breeding season of 2014 in the Hoya de Guadix, southeast Spain (37º18'N, 3º11'W), a high-altitude plateau 1000 m a.s.l, with a semi-arid climate. There were 80 cork-made nest-boxes (internal height * width * depth: 350 * 180 * 210 mm, bottom-to-hole height: 240 mm) available for spotless starlings attached to tree trunks or walls at 3-4 m above ground. Starlings in our population usually commence to build their nests in

March with straws, green plants and feathers as nest material. Egg laying is quite synchronous in our population and starts at mid-April (Soler et al. 2008b). Clutches are of 4-5 eggs on average, with incubation typically starting with the penultimate egg, mainly performed by females (Navarro et al. 2011; Veiga & Polo 2016).

Field work and Experimental procedures

Natural nests

Since April 10th, approximately one week before the start of egg laying, we visited nest-boxes every three days. Once a nest was detected as complete, we removed any feather that starlings had in their nests and randomly assigned each nest to one of the four experimental treatments. Treatments consisted of allocating (i) 20 pigmented, or (ii) 20 unpigmented experimental (see below) feathers to the nests, or (iii) 10 pigmented plus 10 unpigmented feathers (hereafter mixed nests), or (iv) leave the nest without feathers. Seven days later (i.e., the day with three or less eggs in the nest, before incubation started) we refreshed feather treatments by removing feathers incorporated by adults and adding new experimental feathers when necessary. Ten days later (i.e., at the end of incubation), we measured length and width of all eggs with a digital calliper to the nearest 0.01mm. On this day, we also sampled bacteria on the eggshells (see below), and collected one experimental feather from nests under pigmented and unpigmented feathers experimental treatments. From nests under the mixed experimental treatment, we collected one pigmented and one unpigmented experimental feather, and from nests without experimental feathers, we collected one pigmented and one unpigmented non-experimental feather if present.

Artificial nests

The experimental treatments for artificial nests were the same than for natural ones. Forty new nest-boxes were placed in the study area for this experiment (10 nest-boxes per feather treatment). The entrance of these nest-boxes was closed with a plastic mesh to prevent animals' access. On the same day (hereafter day 0), they were filled to one fourth of the volume with UV-sterilized polyester fiberfill, on top of which experimental feathers were placed in a hollow simulating a nest cup. Seven days later, one quail egg (Huevos Guillén S.L., Quart de Poblet, Valencia, Spain) previously cleaned with disinfectant wipes (Aseptonet, Laboratoires Sarbec, Cod.998077-51EN) was laid on top of the experimental nest material. Afterwards, nest-boxes were visited every second day to gently move the eggs ensuring contact of the whole eggshell surface with nest lining material. Ten days after placing the eggs in nests, we sampled bacteria from eggshells and collected one feather per nest (from mixed nests we collected one pigmented and one unpigmented feather).

The use of quail eggs in artificial nest is an approach that has been successfully employed in previous studies (Soler et al. 2015; Ruiz-Castellano et al. 2016) found that bacterial load of quail eggs did not differ from that of natural magpie (*Pica pica*) eggs before incubation started, which supports the use of quail eggs for our purposes.

Bacterial sampling

For each nest visit and sampling we wore new gloves sterilized with 96% ethanol to prevent contamination of eggshell bacterial samples among nests. Eggshells were sampled by cleaning the complete egg surface with a sterile rayon swab (EUROTUBO® DeltaLab) slightly wet with sterile sodium phosphate buffer (0.2 M; pH = 7.2). In starling nests, an egg not previously sampled was randomly selected. After cleaning, we introduced the swab in an

Eppendorf tube with the buffer solution to preserve it. Feather samples were collected in individual hermetic bags. Eggshell and feather samples were preserved at 4-6 ºC in a refrigerator until being processed in the laboratory within 24 h and a month after collection, respectively.

Laboratory work

Preparation of experimental feathers

From chickens that grew in small farms close to the study area, we collected white (i.e., unpigmented) and non-white (i.e., pigmented) body feathers of similar size as those used by starlings from our population as lining material. Experimental feathers were marked on the quill with a permanent marker allowing distinguishing them from those carried by adults to the nests. Subsequently, feathers were sterilized in the laboratory using a UV sterilizer chamber (Burdinola BV-100) during 10 minutes on each feather side. Afterwards, we stored 10 pigmented or unpigmented feathers (i.e., the average number of feathers found in starling nests in previous years in the study area) in separate hermetic bags and stored them at 4 ºC until its use in experimental nests.

Cultivation of eggshell and feather samples

Feather samples were processed in the sterilizer chamber. From each feather, 1 cm2 was cut with sterilized scissors and placed in an Eppendorf tube previously weighed in a precision balance (Metler Toledo, AB135-S/FACT Classic Plus; precision 0.00001 g). Tubes containing feather samples were weighed again to obtain the feather mass. Subsequently, 1 mL of sterile sodium phosphate buffer (0.2 M; pH 7.2) was added and vigorously shaken in a vortex (Boeco V1 Plus) favouring bacterial release from feathers. Similarly, bacterial samples of starling and quail eggshells were shaken in a vortex. 100 µl of buffer containing bacteria homogeneously were used for cultivation of

serially diluted samples (until 10-4) in the following three different solid media: Tryptic Soy Agar (TSA), a broadly used general medium to grow aerobic mesophilic bacteria (Scharlau Chemie S.A., Barcelona), and two specific media; feather meal agar made with unpigmented feathers meal (UP-FMA), and feather meal agar made with pigmented feathers meal (P-FMA). These two last media mainly contain keratin, and therefore are useful for isolating keratinolytic bacteria. FMA media was made with 15 g L-1 feather meal, 0.5 g NaCl, 0.30 g L-1 K2HPO4, 0.40 g L-1 KH2PO4, 15 g L-1 agar (Sangali & Brandelli 2000) and 0.1 mg L-1 cycloheximid to avoid fungi growth (Shawkey et al. 2005; Møller et al. 2009; Czirják et al. 2010). Plates were incubated at 37 ºC, and after 72 h, the number of colonies on each plate was counted.

Eggshell bacterial density was estimated by standardization of the number of colonies per cm2 of sampled eggshell (CFU, Colony Forming Units). Eggshell surface was estimated following Narushin (2005) from length and width of each egg: $S = (3.155 - 0.0136*L + 0.0115*W)*L*W$, where S is the egg surface in cm2, W is the egg width and L is the egg length. Feather bacterial density was estimated by the number of colonies per mg of feather.

Antimicrobial activity of colonies isolated from eggshells and feathers

From each cultivated plate, a maximum of five colonies of different morphology were isolated and transferred to 6 mL of Brain Heart Infusion (BHI, Scharlau Chemie S.A., Barcelona), and incubated at 37 ºC during 24 h. Then, BHI containing bacteria was spread onto TSA plates by streaking technique, incubated at 37 ºC during 24 h, and preserved at 4 ºC until the antimicrobial assay within the following 72 h. This step was repeated twice to minimize possibilities of contamination of each isolate. For antimicrobial assays, each colony was replicated by spotting onto 18 TSA plates (35 colonies per plate), and incubated for 24 h at 37 ºC. After growing, plates

were covered with 6 mL of soft agar (Brain Heart Infusion added 0.8% agar, Scharlau Chemie S.A., Barcelona), inoculated with 100 µL of a 24 h culture of the indicator strains, and then incubated for 24 h at 37 ºC. The antimicrobial activity of each isolated colony was revealed by the presence of clear growth-inhibition halos around the spot of isolates (for more details see Ruiz-Rodríguez et al. 2012).

Tests of antimicrobial activity were performed against 18 typified bacterial strains of a wide range of bacterial taxa from the Spanish Type Culture Collection (CECT) and from our laboratory collection. We used potentially pathogenic (*Enterococcus faecalis* MRR-103, *Escherichia coli CECT774*, *Listeria monocytogenes* CECT4032, *Salmonella choleraesuis* CECT443, *Staphylococcus aureus* CECT240) and non-pathogenic bacteria (*Bacillus licheniformis* D13, *Bacillus megatherium*, *Bacillus thuringiensis*, *Enterococcus faecium* UJA34, *Klebsiella* sp., *Lactobacillus lactis lactis* LM2301, *Lactobacillus paracasei* 11-2, *Lactobacillus plantarum* CECT784, *Listeria inocua* CECT340, *Micrococcus luteus* 241, *Mycobacterium* sp., *Proteus* sp., *Pseudomonas putida*.) (Pinowski et al. 1994; Hubálek 2004; Benskin et al. 2009). We did not include keratinolytic bacteria as a pathogenic bacterial strain because of the antimicrobial properties of some of these bacteria (Abriouel et al. 2011).

Statistical analyses

Estimated bacterial densities were log10 transformed to approach Gaussian distributions. We used MCMCglmm models as implemented in R (R Core Teem 2015) with the appropriate libraries ("MCMCglmm" (Hadfield 2010), "MASS" (Venables & Ripley 2002) and "mvtnorm" (Genz & Bretz 2009)) to explore predictors of bacterial loads of feathers and eggshells and antimicrobial activity of bacterial colonies. Models explaining eggshell bacterial load included density of mesophilic and of keratinolytic (both UN-

FMA and P-FMA) bacteria as dependent variables and experimental treatments (i.e., four levels of feather manipulations) and nest type (i.e., natural or artificial) as fixed factors. MCMCglmm models exploring bacterial density of feathers also included feather colour as independent fixed factor. Because feathers' colour and two of the experimental treatments provided the same information (i.e., in experimental nests with only unpigmented or pigmented feathers we could sample just one type of feather), treatment in these models refers to unicolor or mixed colour. However, since two feathers were sampled in nests with mixed treatments, we included nest identity as random factor in these models. It should be noted here that estimated bacterial density of feathers and of eggshells cannot be compared because the former is relative to feather weight and the latter to eggshell surface.

Frequencies of positive or negative antimicrobial activities in colonies isolated from feathers and eggs were included in the statistical models as a multi-response variable. Thus, probability of detecting antimicrobial activity against pathogenic bacteria or non-pathogenic bacteria was used as binary response in separate models, by assuming multinomial distribution of our statistical MCMCglmm models. In a first model we explored the effects of bacterial origin (i.e., feathers or eggshells), nest type (i.e., artificial or natural) and experimental treatments (four levels) on the antimicrobial activity of isolates. This model also included nest identity as random factor because more than one sample was collected from some of the experimental nests. Furthermore, we explored the effect of feather colour in which bacteria were isolated on the antimicrobial activity. To do this, we separately analysed microbial activity of colonies isolated from feathers in models that included nest type (natural vs artificial), experimental treatment (unicolor or mixed) and feather colour as fixed factors, and nest identity as random effects. Full models included interactions, but main effect probabilities were estimated in models without interactions. Models were reduced starting from the higher

order interaction by removing one by one independent factors with higher probability up to P < 0.1. Results from full and reduced models are shown in results.

Used priors for models that did or did not include random factors were [prior1 = list(R = list(V = 1, nu = 0.002), G = list(G1 = list(V = 1, nu = 0.002)))] and [prior2 = list(R = list(V = 1, nu = 0.002))], respectively. Estimates and probabilities were calculated with 100000 interactions (nitt=), thining intervals (thin=) of 10, and with a burn in period of (burnin=) 10000. Inferences were based on posterior means of the estimate (post.mean) and 95% credibility intervals as well as on the probability of parameters being different from zero (pMCMC).

Results

Bacterial loads of feathers and eggshells

Feathers from natural nests harboured bacteria at a higher density than feathers from artificial nests, while experimental treatments, feather pigmentation, and the interaction among factors did not explain additional variance (Table 1). This was the case independently of using culture medium for mesophilic bacteria or restrictive media for keratinolytic bacteria (Fig. 1). However, bacterial loads of eggshells in natural nests were similar to that of artificial nests and neither the experimental treatment nor the interaction with artificial-natural nests explained additional variance of bacterial loads significantly (Table 1). Interestingly, independently of experimental treatment, eggshells in natural nests harboured keratinolytic bacteria at a higher density than eggshells in artificial nests (Table 1, Fig. 1), which suggests a link between the high density of bacteria in feathers of natural nests and density of keratinolytic bacteria on the eggshells.

Table 1: Results from MCMCglmm models explaining bacterial loads on feathers and eggshells cultured in TSA, UP-FMA and P-FMA media. We report posterior mean of the estimate (post.mean) and 95% credibility intervals as well as the probability of parameters being different from zero (pMCMC). Main effect probabilities were estimated in models without interaction and interaction probabilities in the complete model. M = Mixed colours; NF = No feathers; P = Pigmented feathers. Associations with a P < 0.05 are in bold.

	TSA		UP-FMA		P-FMA	
	post.mean (95% CI)	pMCMC	post.mean (95% CI)	pMCMC	post.mean (95% CI)	pMCMC
FEATHERS						
Complete model						
Nest type	**0.647 (0.335-0.930)**	**<0.001**	**0.550 (0.147-0.947)**	**0.008**	**0.580 (0.215-0.930)**	**0.015**
F colour	-0.094 (-0.344- 0.152)	0.458	0.086 (-0.274-0.405)	0.618	0.048 (-0.245- 0.322)	0.737
F treatment (1)	-0.031 (-0.328-0.279)	0.836	0.216 (-0.186-0.623)	0.291	0.133 (-0.235- 0.495)	0.462
Interactions		> 0.230		> 0.069		> 0.151
Final model						
Nest type	**0.655 (0.359-0.942)**	**0.001**	**0.503 (0.123-0.902)**	**0.013**	**0.551 (0.197-0.906)**	**0.003**
EGGSHELLS						
Complete model						
Nest type	0.033 (-0.385-0.441)	0.866	**0.167 (0.024-0.306)**	**0.025**	**0.097 (0.007-0.182)**	**0.029**
F treatment (2) M	0.012 (-0.584-0.572)	0.975	0.045 (-0.148-0.235)	0.636	-0.050 (-0.167-0.064)	0.398
F treatment (2) NF	0.165 (-0.399-0.720)	0.564	0.075 (-0.102-0.265)	0.418	-0.060 (-0.174-0.052)	0.290
F treatment (2) P	0.014 (-0.536-0.588)	0.964	0.057 (-0.132-0.245)	0.548	0.050 (-0.062-0.167)	0.395
Interactions		> 0.120		>0.544		>0.440
Final model						
Nest type			**0.169 (0.030-0.302)**	**0.016**	**0.097 (0.012-0.186)**	**0.027**

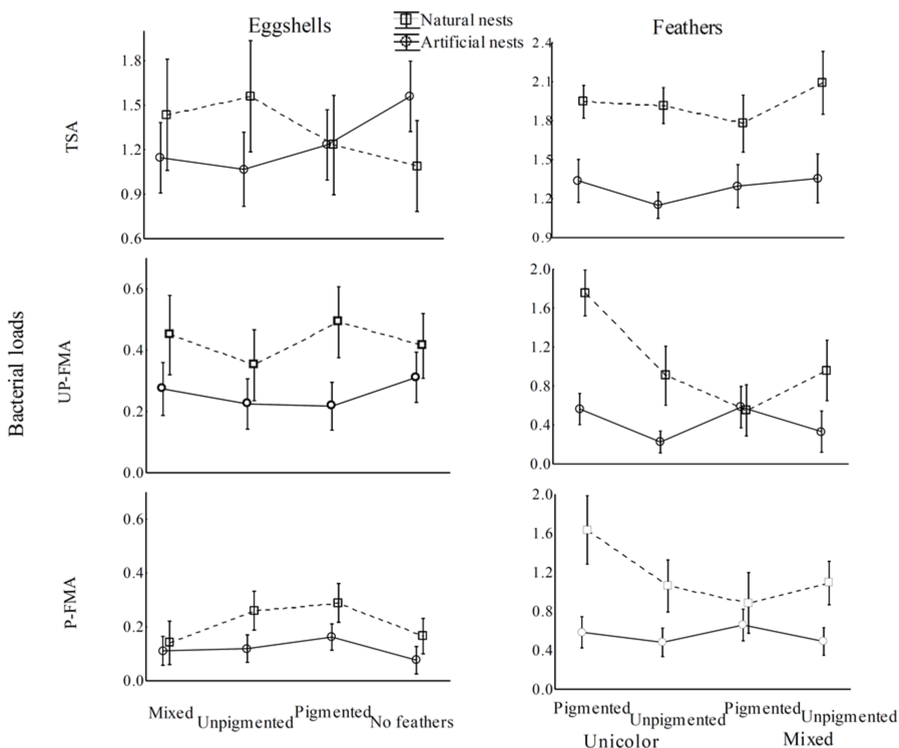

Figure 1: Average bacterial density of feathers (colonies/mg of feather) and eggshells (colonies/mm^2) estimated in TSA, UP-FMA and P-FMA media. Eggshell bacterial density in artificial and natural nests under four different experimental feather treatments (mixed, unpigmented, pigmented, and no feathers) are shown. Values for pigmented and unpigmented feathers that were in artificial or natural nests, with (mixed) or without (unicolor) feathers of other pigmentation are shown.

Antimicrobial activity of bacteria isolated from artificial and natural starling nests

We performed a total of 9719 tests of antimicrobial activity against 18 indicator bacterial strains. 5145 of these tests were performed with bacterial colonies isolated from feathers (N = 285), and 4574 with colonies isolated from eggshells (N = 253) (Table 2). Antimicrobial activity was more frequently detected in colonies from feathers and from natural starling nests, while the effect of nest type (artificial vs natural) did not depend on the origin

of the bacterial colonies (feathers vs eggshells). This was the case independently of considering potentially pathogenic or non-pathogenic indicator bacterial strains for avian embryos (Table 3, Fig. 2).

Table 2: Average antimicrobial activity (Mean % (SE)) of colonies isolated from feathers and eggshells of natural and artificial nests against each of the pathogenic and non-pathogenic bacteria strains tested.

	Artificial nests		Starling nests	
	Eggs	Feathers	Eggs	Feathers
Pathogenic strains				
E. faecalis	14.49 (2.98)	17.06 (4.56)	19.92 (4.34)	25.32 (3.79)
E. Coli	9.83 (2.32)	18.33 (3.84)	15.75 (3.47)	24.52 (3.46)
L. monocytogenes	17.91 (3.42)	17.60 (2.86)	24.00 (3.78)	25.32 (4.24)
Salmonella	9.40 (2.36)	15.78 (3.64)	16.75 (2.84)	23.39 (3.60)
S. aureus	21.07 (3.53)	26.23 (4.57)	32.75 (5.60)	34.95 (4.11)
Non-pathogenic strains				
B. licheniformis	21.62 (3.59)	25.10 (4.40)	37.25 (5.17)	40.91 (4.65)
B. megatherium	26.75 (3.29)	26.47 (3.98)	50.00 (5.51)	40.59 (4.02)
B. thuringiensis	15.64 (2.98)	17.79 (3.94)	17.75 (3.62)	22.42 (3.97)
E. faecium	16.28 (3.10)	20.54 (4.24)	18.75 (4.37)	26.13 (4.55)
Klebsiella	11.24 (2.49)	17.01 (3.26)	21.50 (3.70)	26.94 (4.13)
L. lactic	8.93 (2.01)	19.85 (3.97)	17.75 (3.85)	25.00 (3.78)
L. paracasei	16.67 (3.35)	25.44 (4.24)	21.00 (4.07)	25.81 (4.39)
L. plantarum	13.89 (3.27)	21.08 (3.70)	19.50 (4.18)	22.74 (4.32)
L. inocua	17.82 (2.69)	27.75 (4.72)	17.75 (3.78)	25.81 (4.19)
M. luteus	9.32 (2.43)	19.31 (3.51)	25.67 (6.39)	25.97 (4.18)
Mycobacterium	11.58 (2.94)	19.61 (4.12)	16.75 (6.39)	30.16 (4.14)
Proteus	10.34 (2.51)	22.45 (3.97)	25.42 (4.19)	27.90 (4.02)
P. putida	8.08 (2.25)	15.10 (2.89)	17.67 (3.78)	22.26 (3.86)

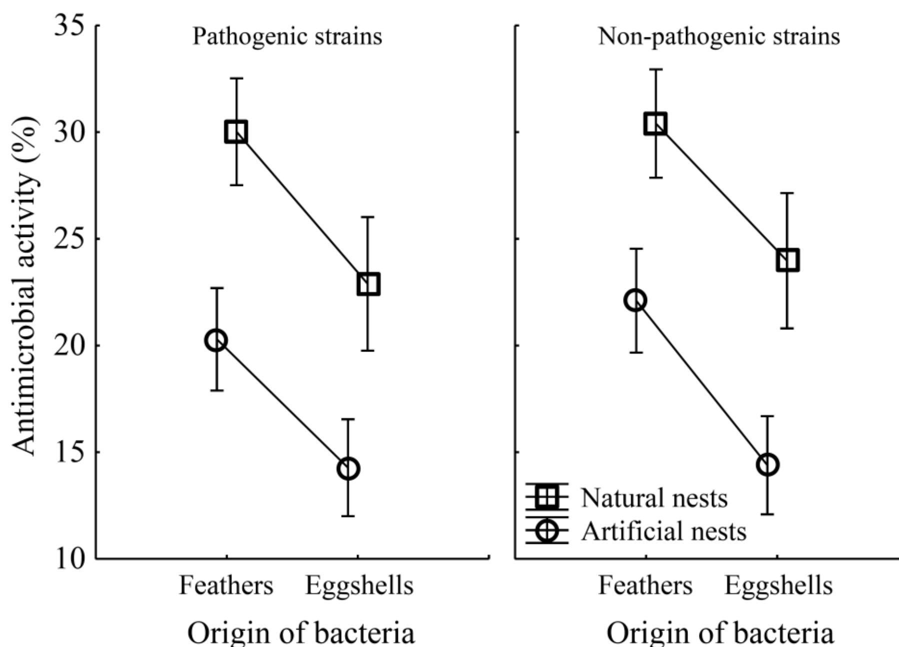

Figure 2: Average antimicrobial activity (%) ± SE of colonies isolated from feathers and eggshells of natural and artificial nests against pathogenic and non-pathogenic bacteria strains.

We separately explored the effects of experimental treatments on antimicrobial activity of bacteria isolated from feathers and from eggshells. On the one hand, feather bacteria from natural nests demonstrated higher antimicrobial activity than those from artificial nests, with a tendency for feather colour explaining additional variance in antimicrobial activity against pathogenic strains: bacteria from unpigmented feathers had more antimicrobial activity (Table 3). On the other hand, antimicrobial activity from eggshell bacteria was higher in natural nests than in artificial nests (Table 3).

Table 3: Results from MCMCglmm models explaining antimicrobial activity of bacterial colonies isolated from feathers and eggshells. Frequencies of antimicrobial activity (positive or negative) against pathogenic or non-pathogenic strains were included in the models as multi-response variable. We report posterior mean of the estimate (post.mean) and 95% credibility intervals as well as the probability of parameters being different from zero (pMCMC). Main effect probabilities were estimated in models without interaction and interaction probabilities in the complete model. M = Mixed colours; NF = No feathers; P = Pigmented feathers. Associations with a $P < 0.05$ are in bold.

	Pathogenic strains		Non-pathogenic strains	
	post.mean (95% CI)	pMCMC	post.mean (95% CI)	pMCMC
Bacteria from feathers and eggshells considered together				
Complete model				
Nest type	**0.672 (0.117 – 1.215)**	**0.016**	**0.755 (0.176 – 1.301)**	**0.011**
Origin	0.374 (-0.081 - 0.871)	0.123	**0.483 (0.011 - 0.977)**	**0.050**
Interaction	-0.063 (-0.795 - 0.657)	0.864	-0.227 (-0.994 - 0.489)	0.540
Final model				
Nest type	**0.641 (0.262 – 1.001)**	**0.002**	**0.628 (0.234 – 1.024)**	**0.005**
Origin	**0.346 (0.021 – 0.707)**	**0.062**	**0.390 (0.030 - 0.755)**	**0.037**

	Pathogenic strains		Non-pathogenic strains	
	post.mean (95% CI)	pMCMC	post.mean (95% CI)	pMCMC
Bacteria from feathers				
Complete model				
Nest type	**1.122 (0.012 - 2.218)**	**0.041**	**1.123 (0.077 - 2.249)**	**0.044**
F treatment (2)	0.319 (-0.954 - 1.538)	0.613	0.434 (-0.701 - 1.636)	0.459
Feather colour	0.930 (-0.142 - 2.071)	0.094	0.835 (-0.278 - 1.921)	0.129
Interactions		> 0.301		> 0.400
Final model				
Nest type	**0.657 (0.111 - 1.196)**	**0.016**	0.511 (-0.068 - 1.081)	0.083
Feather colour	0.4008 (-0.089 - 0.877)	0.095		
Bacteria from eggshells				
Complete model				
Nest type	0.920 (-0.426 - 2.214)	0.153	0.717 (-0.529 - 2.051)	0.268
F treatment (2) M	0.206 (-0.800 - 1.270)	0.702	0.020 (-1.023 - 1.014)	0.969
F treatment (2) NF	0.187 (-0.940 - 1.460)	0.752	0.391 (-0.879 - 1.567)	0.526
F treatment (2) P	0.590 (-0.607 - 1.677)	0.309	0.740 (-0.368 - 1.880)	0.190
Interactions		> 0.533		> 0.291
Final model				
Nest type	**0.621 (0.078 - 1.146)**	**0.024**	0.533 (-0.017 - 1.111)	0.063

Discussion

Our main aim was to explore predictions of one of the mechanisms potentially explaining the detected antimicrobial effects of feathers in avian nests (Peralta-Sánchez et al. 2010; Ruiz-Castellano et al. 2016) that deal with the antimicrobial potential of bacteria growing on nest feather material. We found that colonies isolated from feathers were more frequently active against tested bacterial strains than those isolated from eggshells. Moreover, bacterial densities on eggshells and feathers were higher in natural than in artificial nests, and colonies isolated from the former demonstrated higher antimicrobial capabilities than those from the latter. This suggests that environmental conditions created by breeding activity enhance antimicrobial capabilities of the nest bacterial community. Below we discuss alternative explanations, possible biases of our experimental approach, and the importance of nest building behaviour determining bacterial environments and risk of infection for developing offspring.

The effects of breeding activity were explored by using quail eggs in artificial nests, an approach that has been successfully employed in previous studies experimentally exploring hypotheses to explain variation in avian eggshell bacterial loads (Soler et al. 2015; Ruiz-Castellano et al. 2016). Since eggshells of different species may vary in their antimicrobial properties, and therefore in probability of bacteria colonization and growth (Wellman-Labadie et al. 2008a; 2008b; 2010; Soler et al. 2011; D'Alba et al. 2014; Martín-Vivaldi et al. 2014), comparisons of the effects of experimental treatments on quail eggs and on starling eggs may suffer from some kind of bias. This possibility however would hardly explain the detected higher bacterial loads on starling eggshells in natural nests because the same effects were detected in experimental feathers (i.e., feathers added in natural and artificial nests were prepared in our laboratory). Moreover, although it is known that incubation reduces eggshell bacterial loads and diversity (Cook et

al. 2005a; Shawkey et al. 2009; Soler et al. 2015; Ruiz-Castellano et al. 2016), density of bacteria on the eggshells in natural nests with incubation activity is usually higher than in artificial nests (Soler et al. 2015; Ruiz-Castellano et al. 2016). Thus, it is likely that environmental factors linked to breeding activity such as temperature and humidity were responsible for the higher bacterial density detected in sampled material from natural nests.

Previous research has shown that incubation differentially favours growth of some bacterial strains (Grizard et al. 2014). Interestingly, some of the detected bacteria that increased in density on the eggshells during incubation were keratinolytic bacteria of the genus Pseudomonas or Bacillus (Wang et al. 2011; Potter et al. 2013; Grizard et al. 2015). Because of the antimicrobial properties of some of these bacteria, these results opened the possibility that avian incubation behaviour selects for beneficial-antibiotic producing bacteria on the eggshell that would protect embryos from pathogenic infections (Wang et al. 2011). In accordance with these studies, we found that the expected higher bacterial density of eggs from natural nests, with incubation activity, was only true for keratinolytic bacteria.

In addition, we also found that antimicrobial capabilities of colonies isolated from natural nests were higher than that of colonies isolated from artificial nests. We know that bacterial production of secondary metabolites depends on several environmental factors like temperature or nutrient availability (Biswas et al. 1991; Mataragas et al. 2003). Thus, it is possible that the modification of nest environmental conditions (Cook et al. 2005b; Ruiz-De-Castañeda et al. 2011) and nutrients for bacterial growth (Ibáñez-Álamo et al. 2014), which are likely associated with breeding activity, enhance bacterial segregation of antimicrobial secondary metabolites (see Introduction for rationality of this expected association).

Independently of the effects of breeding activity, and in accordance with the hypothesis tested, antimicrobial activity was more frequently detected in colonies isolated from feathers than from eggshells. We failed to detect the predicted differential effects of feather colour on the antimicrobial activity of the colonies isolated from them. In a previous study, colonies isolated from unpigmented feathers of barn swallow nests had higher antimicrobial potential than those from pigmented feathers (Peralta-Sánchez et al. 2014). However, in starlings, we detected stronger antimicrobial beneficial effects for pigmented feathers (Ruiz-Castellano et al. 2016), and the higher antimicrobial activity of colonies isolated from pigmented feathers detected here did not reach statistical significance. A larger sample size may be necessary to detect a differential effect of feather pigmentation on antimicrobial capabilities of bacteria growing on feathers. In any case, the detected effects of feathers suggest that this nest material can be seen as antimicrobial material that influence bacterial environment of avian nests, diminishing probability of offspring infection. Thus, these results support the hypothesis that one of the mechanisms explaining the detected antimicrobial effects of nest-lining feathers is related to the antimicrobial properties of bacterial colonies growing on this material.

It could be argued that an additional mechanism to account for our results involves the role of feathers as sexual display between adult birds (see Introduction), which may induce, for instance, differential investment in nest sanitation behaviours and originate differences in the nest microbiome. However, although our experimental approach does not allow estimating relative importance of sexual selection and antimicrobial properties of feathers explaining nest bacterial environment, our results point out independent effects of feathers. This is because antimicrobial activities of colonies from feathers resulted higher than those from the eggshell in both experimental and natural nests.

Summarising, in support of the hypothesis that feathers in nests of birds should be important sources of antibiotic producing bacteria, we detected relatively broad antimicrobial activities for colonies isolated from experimental nest-lining feathers. Consequently, antimicrobial capabilities of bacteria growing on nest-lining feathers would be one of the mechanisms underlying the previously detected antimicrobial effects of this lining-material in avian nests. Furthermore, we show that breeding activity differentially favoured growth and antimicrobial capabilities of isolated colonies, mainly regarding keratinolytic bacteria. These last results open the possibility that particular breeding activities are involved in enhancing growth of especially active antibiotic producing bacteria in bird nests, and therefore deserve further investigation.

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Statement of authorship

JJS, MRR, and GT designed the study with considerable assistance from CRC. CRC and MRR performed all bacterial analyses. GT and CRC performed most of the field work with assistance by JJS. JJS performed all the

statistical analyses and CRC and JJS wrote the manuscript with substantial contribution from all authors.

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CAPÍTULO 5

Adaptive nest material selection by spotless starlings

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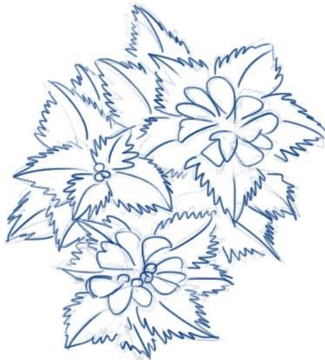

Abstract

The nest of birds is an essential structure to protect offspring during development. Nest building entails costs in terms of time, energy or exposure to predators and parasites. In particular, searching for and carrying materials to the nest is contingent on the availability of different nest materials in the surroundings of the nest. Thus, by selecting the most appropriate combination of nest materials, birds have to adjust these efforts to optimize the outcome of the balance between costs and benefits of nest building. Green plants and feathers are common nest materials used by many bird species. Different plants and feathers of different colours have been hypothesized to play key roles in sexual selection and in offspring protection against pathogens. In this study, we experimentally assessed spotless starling (*Sturnus unicolor*) preferences for pigmented *vs.* unpigmented feathers and for different green plants (aromatic *vs.* non-aromatic plants) as nest materials. Literature suggests that birds should select unpigmented feathers and plants with high antimicrobial properties. We offered these materials to nest building birds and assessed its usage in nests before and after egg laying started (i.e., during the laying stage). As expected, starlings preferentially selected unpigmented feathers both before and during egg laying, while aromatic plants were preferentially selected only during the egg laying stage. This study, together with previous evidence, suggests that birds make a sensible selection of feathers and aromatic plants as nest materials, which would confer antibacterial protection to their offspring.

Keywords: Aromatic plants, Feather pigmentation, Nest lining feathers, Nest material preference, Nest building behaviour.

Introduction

The nest of birds is an essential structure to protect offspring from a wide diversity of environmental challenges during development (Hansell 2000; Moreno 2012; Mainwaring et al. 2014). However, nest building entails costs in terms of, for instance, expended time and energy, or exposure to predators and parasites (Hansell 2000; Mainwaring & Hartley 2013). Moreover, different nest materials have different properties and, therefore, birds are expected to carefully adjust nest building behaviour to optimize the balance between costs and benefits while choosing the more appropriate combination of nest materials to maximize fitness (Soler et al. 1998; Hansell 2000).

Among the several materials employed by birds to build their nests, two are widely used and have received special attention in evolutionary ecology research: green plants and feathers (Hansell 2000; Dubiec et al. 2013). Green plants, which can be used as structural or lining material, can have several non-exclusive functions in avian nests that include sexual signalling (Fauth et al. 1991; Brouwer & Komdeur 2004; Polo et al. 2004; Veiga et al. 2006; Moreno 2012; Tomás et al. 2013). Yet, the most studied function of green plants is that related to the antimicrobial and antiparasitic properties of their volatile secondary compounds and essential oils, which protect offspring from pathogens (Clark & Mason 1985; Lafuma et al. 2001; Gwinner & Berger 2005; Shutler & Campbell 2007; Mennerat et al. 2009a; Dubiec et al. 2013; Scott-Baumann & Morgan 2015). Through different metabolic routes, others than those related to protection from parasites, many of these plants can also have therapeutic effects on nestlings improving nestling growth, health, or immunocompetence (Gwinner et al. 2000; Gwinner & Berger 2005; Mennerat et al. 2009b). This is the reason why the use of green plants in the nests of birds, to control parasite populations or to improve nestling health, is considered a form of self-medication (De Roode et al. 2013). Different plants have different properties and, therefore, birds may

differentially select some of them and adjust their nest building behaviour according to pathogen selective pressures.

Feathers are another material commonly used in bird nests, mainly for nest cup lining, but also as structural material (Hansell 2000). Like green plants, feathers can be involved in sexual signalling (Polo & Veiga 2006; Sanz & García-Navas 2011; García-López de Hierro et al. 2013; Mainwaring et al. 2016), and can improve thermal insulation (Møller 1984; Lombardo et al. 1995; Hilton et al. 2004; Pinowski et al. 2006; Dawson et al. 2011; Windsor et al. 2013), or even decrease probability of offspring microbial infections (Soler et al. 2010). This last function is explained by the antimicrobial properties of the bacterial communities that grow in feathers digesting the keratin (the main component of feathers). Antimicrobial properties of feathers, as well as their sexual functioning, likely depend on feather pigmentation. On the one hand, feathers of particular colours may be more attractive in a sexual context (Veiga & Polo 2005; Avilés et al. 2010). On the other hand, it is known that the proliferation of keratinolytic bacteria is greater in unpigmented feathers due to the absence of pigments, like melanin, that make more difficult the degradation of keratin (Goldstein et al. 2004; Gunderson et al. 2008). Thus, expected benefits mediated by antibiotic-producing bacteria would depend on feather pigmentation and on nest-lining feather colour composition (Peralta-Sánchez et al. 2014). Evidence for the antimicrobial benefits of feathers are only known for barn swallow (*Hirundo rustica*) (Peralta-Sánchez et al. 2010; 2011) and spotless starling nests (Ruiz-Castellano et al. 2016). Thus, similarly to green plants, birds may differentially select feathers of certain colour, and adjust its numbers in nests, depending on pathogen selective pressures.

Some of these hypothetical effects of feathers and green plants in nests of birds have been experimentally demonstrated but, as far as we know, adult preferences for certain plant species, and for feathers of different

colours, have never been formally tested experimentally. Previous research on European starlings (*Sturnus vulgaris*) has suggested olfactory discrimination of plant volatiles (Clark & Mason 1987; Gwinner & Berger 2008). Moreover, an observational study concluded that plant species composition of blue tit (*Cyanistes caeruleus*) nests results from individual preferences (Mennerat et al. 2009c). Nevertheless, knowledge on preferences of nest materials by nest building birds is still scarce (Hansell 2000). Exploring these preferences, as well as possible variation depending on environmental conditions, would aid understanding of the functional significance of nests and nest building behaviours.

Selection of nest materials by birds can be tackled by looking at the final assemblage of materials in nests, but it should take into account the availability of different materials in the nest surroundings (Petit et al. 2002; Mennerat et al. 2009c; Pires et al. 2012). Birds can also fly to other territories to collect nest materials (Bailey et al. 2016), which challenges a proper statistical control of nest material availability. An experimental approach is therefore the most suitable way to study any kind of preference or selection, which we accomplished here by manipulating the availability of nest materials. During nest building stage, we offered to spotless starlings unlimited pigmented and unpigmented feathers and different aromatic and non-aromatic plant species, and recorded its presence and abundance in their nests. We predicted that birds would select preferentially unpigmented feathers and aromatic plants if antimicrobial protection of the offspring is one of the functions of these nest materials.

Materials and Methods

Study species and area

The spotless starling is a medium-sized, hole-nesting passerine that mostly breeds in colonies (Cramp 1998). Green plants and feathers are commonly used as nest materials, with plants being carried by males and feathers by females (Polo & Veiga 2006). Both materials are embedded in the nests, forming part of both their structural and lining layers (Veiga & Polo 2016). Our starling population usually commences to build their nests in March, laying eggs at mid-April. Since April 10th nest-boxes were visited every three days until the first egg was laid.

The study was performed during the 2013 breeding season in Hoya de Guadix, southeast Spain (37°18' N, 3°11' W), a high-altitude plateau 1000 m a.s.l., with a semi-arid climate. Apart from a few sparse almond trees (*Prunus dulcis*), vegetation in the area is typical of an agro pastoral steppe, with abandoned fields interspersed within barley (*Hordeum vulgare*) and lettuce (*Lactuca sativa*) cultivated fields. There were 80 cork-made nests boxes (internal height * width * depth: 350 * 180 * 210 mm, bottom-to-hole height: 240 mm) available for spotless starlings attached to tree trunks or walls at 3-4 m above ground. Nest-boxes were often less than 1m apart from each other (as in natural holes).

Experimental procedures

Feathers and plants were offered *ad libitum* to nest building starlings. Feathers were provided in 24 * 24 cm broad plastic meshes placed in the ground, and distributed around the study area, so that all breeding pairs could have access to feathers. Meshes were located in adjacent pairs, one containing 50 unpigmented and the other containing 50 pigmented feathers. Body feathers of approximately 10 cm long were collected from domestic turkeys

(*Meleagris gallopavo*) to distinguish them from chicken feathers that were the most abundant in our study area. In addition, turkey feathers were marked on the quill with a permanent marker. Meshes were checked daily and feathers were replenished whenever there were 15 feathers or less in the mesh.

Plants were provided in a plastic container (length * width * height: 9.5 * 7 * 6 cm) filled with insulating foam and water to maintain them fresh. In each container, four apical plant fragments of approximately 10 cm length of four aromatic species (*Marrubium vulgare*, *Artemisia barrelieri*, *Lamium amplexicaule* and *Anacyclus clavatus*) and one non-aromatic plant (barley, *Hordeum vulgare*) were provided. Plants were marked in the stem with seal ink. Containers were attached on top of 2/3 of nest-boxes and were replaced every three days to provide fresh plants, when we counted the number of plant fragments collected by starlings. Starlings made use of plants irrespective of whether those were above their own or above an adjacent nest-box (authors' personal observation).

Plants and feathers were provided to starlings during three weeks, starting one week before the expected laying date of the first egg (starling pairs are quite synchronous about the onset of egg laying in our population). Nest-boxes were checked every three days, from the day we offered plants and feathers until eggs were found in the nest. In each visit, we counted the number of feathers present in nests, distinguishing between pigmented and unpigmented, and between experimental and non-experimental feathers. In each visit, we also weighted plants (± 0.1 g) and counted fragments of experimental plants found in nests. None of the nests contained eggs during the first visit, when offering experimental nest material for the first time. Some nests (N = 21) were detected with eggs three days later, during the second visit. While for 27 nests eggs were detected in subsequent visits. These two groups of nests were then used for comparing nest material composition

in nests with and without ad libitum materials available three days before egg laying.

In addition to monitoring nest material composition, we used the number of times that each experimental mesh containing either pigmented and unpigmented feathers needed to be replenished, as well as the number of steams of each plant species that were collected from the containers, as proxies of the selection made by starlings of feathers and plants.

To estimate plant availability around nests, we recorded the percentage of a circular area of 20 m-radius around each nest-box that was covered by green plants (hereafter percentage of vegetation coverage). These values were used to control plant availability when analysing variation in mass of green plants found in starling nests.

Antimicrobial activity of green plants offered to starlings

We assesses the antimicrobial capabilities of the five green plants offered to spotless starlings. Plants were sterilized in the laboratory using a UV sterilizer chamber (Burdinola BV-100) and 1 cm fragments of each species were placed in Brain Heart Agar (BHA, Scharlau Chemie S.A., Barcelona) plates, inoculated with 100 µL of a 24 h culture of the indicator bacteria strain (see below), and then incubated 24 h at 37 ºC. The antimicrobial activity of each plant was revealed by the presence of growth-inhibition halos around the plant fragment. We distinguished between two kinds of halos: a clear halo, where growth of the indicator bacteria was completely inhibited, and a colored halo, presumably due to pigments from plants that do not completely inhibit growth of the indicator bacteria.

Antimicrobial activity of each plant was summarized by an index resulting from the addition of standardized values (i.e., mean = 0 and SD = 1) from three different variables describing (i) halo transparency: 0 (no halo), 1

(not clear halo), 2 (clear halo); (ii) size of halo: 0 (no halo), 1 (1-2 mm), 2 (3-4 mm), 3 (5-6 mm) and 4 (>6 mm), and (iii) number of indicator bacteria to which tested plants demonstrated antimicrobial activity (from 0 to 11).

Antimicrobial activity assays were performed against 11 bacterial typified strains from the Spanish Type Culture Collection (CECT) and from our laboratory collection: *Bacillus licheniformis* D13, *Enterococcus faecalis* MRR10, *Listeria inocua* CECT340, *Listeria monocytogenes* CECT4032, *Staphylococcus aureus* CECT240, *Enterococcus faecalis* F-58, *Enterococcus faecalis* JH2, *Enterococcus faecalis* S47, *Enterococcus faecium* 115, *Lactobacillus paracasei* 11-2 and *Lactococcus lactis*. However we did not find antimicrobial activity of any tested plants against the last six indicator bacteria.

Sample size and statistical analyses

We used information on feathers and plants in 48 nests at the two different times of interest: once eggs were found in the nest for the first time (hereafter laying stage), and in the previous visit three days before (hereafter pre-laying stage). Total and experimental number of pigmented and unpigmented feathers, as well as mass of plants found in starling nests, were log10 transformed to approach Gaussian distributions.

Because of differences in laying dates, experimental feathers and plants were only available for 27 out of the 48 nests sampled during the pre-laying stage, but for all of them during the laying stage. Thus, to explore differences in total number of feathers and in colour composition of feathers in nests due to the presence of ad libitum experimental feathers, we performed two different models. First, we explored the effects of availability of feathers (nests with vs without experimental feathers available) (fixed factor) on the total number of feathers detected in nests in the pre-laying stage (dependent variable) in a General Linear Model (GLM). Second, to explore possible

differential effects for feathers of different pigmentation, we used Repeated Measures ANOVAs with feathers of different colour detected in the same nest as repeated measures, and availability of experimental feathers as between factor. Furthermore, to explore possible differences in total number of feathers and in colour feather composition due to reproductive stage (i.e., pre-laying vs laying), we used nests that had experimental feathers available both before and during egg-laying. These effects were explored in Repeated Measures ANOVAs with two within factors, feather colour and reproductive stage. In a separate model we estimated the effect of laying date on feather composition across the breeding season (interaction between laying date and the repeated measures). In addition, to analyse feather colour preference from meshes by starlings, we used the number of times that meshes of different colour were replenished as dependent variable and feather colour as fixed factor in a GLM.

The effect of plant availability on the amount of green plants found in starling nests was explored in GLM models with availability of experimental plants as the categorical factor, and the percentage of vegetation coverage as a covariable. The effect of nest stage on the amount of green plants in starling nests was explored using nests with available experimental green material during the pre-laying and laying visits. We used Repeated Measures ANOVAs with nest stage as repeated measure variable. The effect of laying date on the repeated measure variable (i.e., interaction between laying date and nest stage) was estimated in a separate model. In addition, to further explore the preference of starlings for any of the experimental plants offered, we used the presence/absence information of experimental plants of each species in starling nests (during the pre-laying and laying stages) as dependent variable, plant species and nesting stage as categorical independent factors in a Generalized Linear Model (GLZ) with a binomial distribution and logit-link function while controlling for overdispersion. Plant preferences were also

assessed by analysing number of plants of each species that were collected by starlings from the containers in a GLM. We used the log10 number of steams collected by starlings as dependent variable, and species and container identity as factors in a GLM.

All analyses were performed with Statistica 8.0 (Statsoft Inc. 2011). Values reported are means ± 95% CI.

Results

Feather colour preferences

During the pre-laying stage, total number of feathers in starling nests did not depend on feather availability ($F_{1,46}$ = 0.11, P = 0.738). However, unpigmented feathers were significantly more abundant than pigmented feathers ($F_{1,46}$ = 6.73, P = 0.013) while feather colour composition did not depend on availability of experimental feathers ($F_{1,46}$ = 0.04, P = 0.549; Fig. 1A).

Nest stage (laying vs pre-laying) affected total number of feathers in nests. Feathers were more abundant in starling nests at the time of laying (mean (95% CI) = 9.37 (6.27 – 12.47)) than before egg-laying began (mean (95% CI) = 4.89 (2.50 – 7.28)) ($F_{1,26}$ = 24.49, P < 0.001), with laying date explaining a non-significant proportion of variance ($F_{1,25}$ = 1.17, P = 0.290). In addition, feather colour composition was affected by nest stage ($F_{1,26}$ = 4.73, P = 0.039), and unpigmented feathers (mean (95% CI) = 9.93 ± (6.69 – 13.16)) were significantly more abundant than pigmented feathers (mean (95% CI) = 4.33 (1.19 – 7.18)) ($F_{1,26}$ = 18.00, P = 0.0002, Fig. 1B). Laying date affected differences in feather colour composition since unpigmented feathers were relatively more abundant than pigmented feathers as the season progressed; $F_{1,25}$ = 4.47, P = 0.045) but not differences in feather composition due to nest stage were detected ($F_{1,25}$ = 0.12, P = 0.737). Similar results were

achieved when only experimental feathers found in nests were considered. Unpigmented experimental feathers were significantly more abundant (mean (95% CI) = 4.81 (2.94 – 6.69)) than experimental pigmented feathers (mean (95% CI) = 0.02 (-0.01 – 0.38)) ($F_{1,25}$ = 48.12, P < 0.001), being these differences more pronounced during the egg-laying stage (Pre-laying: unpigmented feathers, mean (95% CI) = 1.37 (0.14 – 2.6); pigmented feathers, mean (95% CI) = 0.04 (-0.04 – 0.11); Laying: unpigmented feathers, mean (95% CI) = 3.44 (2.12 – 4.77); pigmented feathers, mean (95% CI) = 0.15 (-0.03 – 0.33)) ($F_{1,25}$ = 17.99, P = 0.0002).

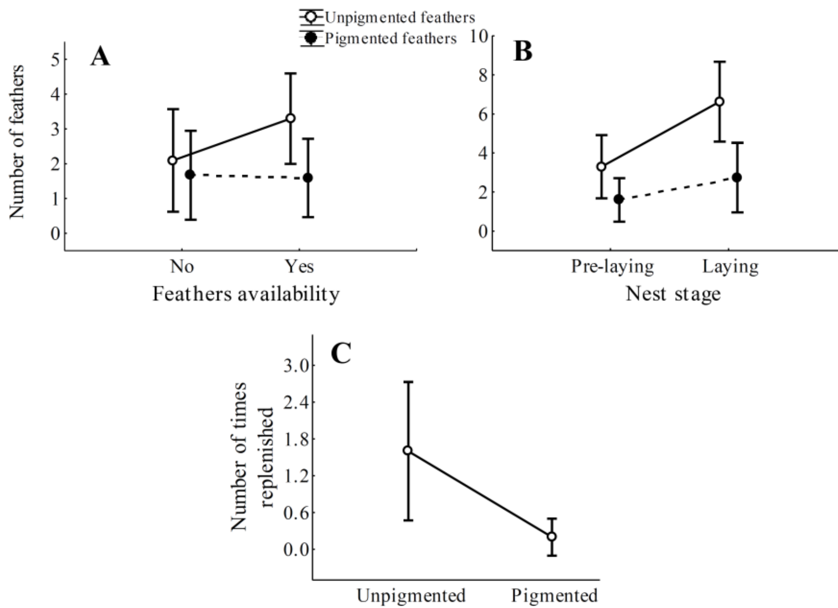

Figure 1: (A) Feather colour composition (± CI 95%) in spotless starling nests during the pre-laying stage, with or without experimental feathers available. (B) Number of pigmented and unpigmented feathers (± CI 95%) in spotless starling nests during the pre-laying and the laying stages. (C) Mean number of times (± CI 95%) that meshes containing the experimental pigmented and unpigmented feathers offered to spotless starlings needed to be replenished.

Finally, meshes with unpigmented feathers needed to be replenished more frequently than those of pigmented feathers (unpigmented feathers: range: 0-4, mean ± SE = 1.6 ± 0.5; pigmented feathers: range: 0-1, mean ± SE = 0.2 ± 0.1; $F_{1,18}$ = 7.35, P = 0.014; Fig. 1C). Thus, independently of the used variable, all results invariably suggest that starlings preferentially select unpigmented feathers, mainly during the laying stage.

Green plant preferences

In the pre-laying stage, mass of plants in nests with and without plants offered did not differ significantly (plants availability: mean (95% CI) = 0.90 (0.35 – 1.46); without availability: mean (95% CI) = 0.44 (0.15 – 1.74); $F_{1,48}$ = 0.81, P = 0.371). Moreover, nest stage did not affect mass of plants in starling nests (Pre-laying: mean (95% CI) = 0.90 (0.35 – 1.46); Laying: (95% CI) = 0.67 (0.67 – 1.16)) ($F_{1,26}$ = 0.32, P = 0.579), but laying date explained a significant proportion of variance ($F_{1,25}$ = 6.60, P = 0.017). Mass of plants found in nests during the laying stage increased as the season progressed, while the opposite trend was detected for the pre-laying stage (Fig. 2A). Percentage of vegetation coverage around nests did not significantly affect mass of plants found in nests (mean (95% CI) = 78.93 (71.73 – 86.13); $F_{1,22}$ = 2.77, P = 0.110).

We analysed the preferences for experimental plants found in nests in the two nest stages separately. In the pre-laying stage, prevalence of different experimental plant species in starling nests did not differ (χ^2 = 3.13, P = 0.536; Fig. 2B). However, starlings showed some preferences during the laying stage (χ^2 = 14.79, P = 0.005; Fig. 2B). *L. amplexicaule* and *M. vulgare* were the experimental plants most frequently found in the nests, while *A. barrielieri* and *H. vulgare* appeared with the lowest prevalence (paired comparisons: *L. amplexicaule* – *H. vulgare* χ^2 = 6.00, P = 0.014; *M. vulgare* – *H. vulgare* χ^2 = 4.35, P = 0.037; Fig. 2B). Similarly, the number of plant stems collected by starlings from the containers differed significantly according to

whether collected plant species were aromatic or not ($F_{4,435}$ = 4.73, P = 0.001) with a clear preference for the four plant aromatic species in comparison with the non-aromatic specie (post-hoc LSD: *L. amplexicaule* – *H. vulgare* P = 0.0001; *M. vulgare* – *H. vulgare* P = 0.0002; *A. clavatus* – *H. vulgare* P = 0.004; *A. barrelieri* – *H. vulgare* P = 0.006; Fig. 2C). Thus, it is during the laying stage when evidence of plant selection towards aromatic plants comes out.

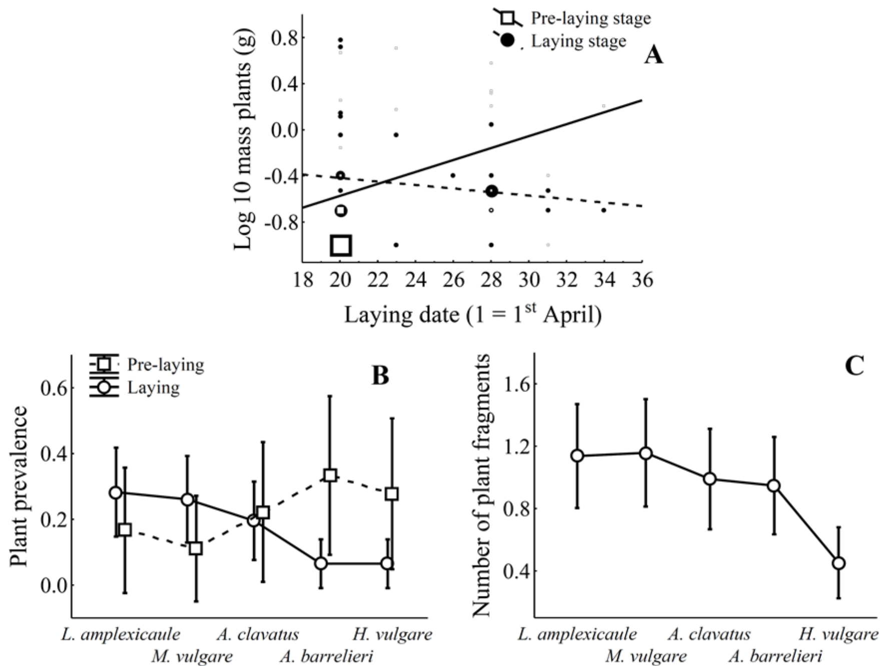

Figure 2: (A) Relationships between laying date and log10 of plants weight (g) in spotless starling nests at laying and pre-laying stages. Regression lines are shown. (B) Presence of different plant species in spotless starling nests at the pre-laying and egg laying stages; and (C) number of experimental plant fragments (± CI 95%) of the different species collected from containers by spotless starlings.

Finally, antimicrobial activity assays of green plants offered to starlings showed that *A. barrelieri* was the plant with the highest antimicrobial activity, followed by *A. clavatus*, and *L. ampleuxicaule*. The lowest antimicrobial activity was shown by *H. vulgare* and *M. vulgare* (Table 1).

Table 1: Antimicrobial activity of the different green plants offered to spotless starlings for nest building. We show the scores for halo transparency, halo size, number of bacteria for which tested plants demonstrated antimicrobial activity, and index scores for each tested plant.

Plant	Bacteria	Halo transparency	Halo size	Number of inhibited bacteria	Index score
A. barrelieri	*B. licheniformis*	2	4	5	14.990
A. barrelieri	*S. aureus*	2	4		
A. barrelieri	*L. inocua*	2	4		
A. barrelieri	*L. monocytogenes*	2	1		
A. barrelieri	*E. faecalis*	2	1		
A. clavatus	*B. licheniformis*	1	4	2	-0.054
A. clavatus	*S. aureus*	1	4		
A. clavatus	*L. inocua*	0	0		
A. clavatus	*L. monocytogenes*	0	0		
A. clavatus	*E. faecalis*	0	0		
L. ampleuxicaule	*B. licheniformis*	1	4	1	-4.198
L. ampleuxicaule	*S. aureus*	0	0		
L. ampleuxicaule	*L. inocua*	0	0		
L. ampleuxicaule	*L. monocytogenes*	0	0		
L. ampleuxicaule	*E. faecalis*	0	0		
M. vulgare	*B. licheniformis*	1	2	1	-5.369
M. vulgare	*S. aureus*	0	0		
M. vulgare	*L. inocua*	0	0		
M. vulgare	*L. monocytogenes*	0	0		
M. vulgare	*E. faecalis*	0	0		
H. vulgare	*B. licheniformis*	1	2	1	-5.369
H. vulgare	*S. aureus*	0	0		
H. vulgare	*L. inocua*	0	0		
H. vulgare	*L. monocytogenes*	0	0		
H. vulgare	*E. faecalis*	0	0		

Discussion

The experimental results showed that starlings preferred unpigmented over pigmented feathers and aromatic plants over non-aromatic plants for building their nests. Moreover, these preferences were more clearly detected during the egg laying stage than during the pre-laying stage, and it is in the laying stage when the hypothetical protective function of these nest materials for developing offspring would be more important.

Feather preferences

In nests of starlings, feathers were more abundant during the egg laying stage than during the pre-laying stage, in accordance with the common yet often overlooked assumption that nest building in birds does not end with onset of laying but it is a dynamic and multifaceted behaviour that can continue during posterior stages, likely to accommodate successive selection pressures (Hansell 2000).

Independently of the nesting stage, unpigmented feathers were more abundant than pigmented feathers in starling nests. The main studied function of feathers in nests concerns their thermoregulatory properties (Møller 1984; Windsor et al. 2013) which for instance would help to reduce clutch and brood cooling rates (Hilton et al. 2004). Although it is known that pigmentation determines thermal insulation properties of structures in nature (Hochscheid et al. 2002; Hetem et al. 2009; for a similar argument in avian eggs see Lahti & Ardia 2016), previous studies dealing with insulation properties of feathers in avian nests have not considered the potential effect of feather pigmentation (Hilton et al. 2004; Dawson et al. 2011; Windsor et al. 2013). This possibility is out of scope of the present study. However, any hypothetical effect of feather pigmentation on nest thermoregulation will mainly apply to open nests since external radiation reaching nest contents

inside holes is limited (Kilner 1999; Hunt et al. 2003; Avilés et al. 2008) and, thus, would hardly apply to starling nests.

A second functional explanation for the preference of unpigmented feathers is related to scenarios of sexual selection (Veiga & Polo 2005), with feathers in nests acting as a courtship display affecting mate choice, and/or as a post-mating sexual signal eliciting differential reproductive investment in mates (Sanz & García-Navas 2011; but see Veiga & Polo 2011). Spotless starling females carry feathers to the nest in response to green plants carried by males (Polo & Veiga 2006). Moreover, amount of feathers in starling nests is related to female experience (Polo & Veiga 2006), and the experimental addition of feathers resulted in a reduced nestling mortality (Veiga & Polo 2011). Thus it is likely that feathers induced differential paternal investment in reproduction (Veiga & Polo 2011). Nevertheless, as far as we know, no study has explored any potential effect of pigmentation of nest lining feathers on sexual selection, a possibility worth to be further explored experimentally by manipulating feather composition and recording parental investment in reproduction.

Even if feather colour preference is driven by sexual selection, it may also function in additional scenarios. In this sense, trying to figure out possible scenarios explaining the detected positive effects of experimental feathers reducing nestling mortality, Veiga and Polo (2011) discussed the role of feathers as a possible antiparasitic material that would reduce nestling mortality. Thus, it is even possible that the reason underlying the preference for unpigmented feathers is their higher antimicrobial properties that have been demonstrated in laboratory (Peralta-Sánchez et al. 2014) and in the field in nests of barn swallows (Peralta-Sánchez et al. 2010). A non-adaptive explanation of colour composition of nest-lining feathers is that it could mirror the availability or detectability of feathers of different colour in the nest surroundings. However, this possibility would not explain feather colour

composition of nests in our study, given that pigmented and unpigmented feathers were available *ad libitum* and easily locatable.

Even knowing the starling preference for unpigmented feathers, and the ad libitum availability of our experimental approach, very few nests (7 out of 48 nests) harbored only unpigmented feathers, which suggests that starlings tried to incorporate feathers to the nest following a certain combination of colours that they prefer. Interestingly, the detected combination of feathers of different colour in starling nests changed, with a relative increase of unpigmented feathers as the season progressed. We know from previous studies that bacterial colonies from pigmented feathers also have antibacterial activity (Peralta-Sánchez et al. 2014) and that the abundance of pigmented feathers has a positive effect on phenotypic quality of spotless starling nestlings (Ruiz-Castellano et al. unpublished). Moreover, we also know that antimicrobial activity of bacterial colonies isolated from unpigmented feathers in nests of barn swallows was higher in nests where experimental pigmented feathers were added (Peralta-Sánchez et al. 2014). Thus, it is possible that because environmental conditions at the end of the breeding season favour bacterial growth in avian nests (Soler et al. 2015; Møller et al. 2015), starlings tried to compensate such increase in probability of bacterial infection by carrying to the nest more feathers with the highest antimicrobial capabilities as the season progressed (Peralta-Sánchez et al. 2014). Since the only available evidence of relatively higher antimicrobial capabilities of bacteria isolated from unpigmented feathers comes from barn swallow nests with experimentally added unpigmented feathers, it is possible that certain combination of pigmented and unpigmented feathers in nests of birds maximizes feather-derived antimicrobial activity. Further investigation on antimicrobial properties of bacteria isolated from unpigmented and pigmented feathers in nests of birds with different combinations of feather colour is necessary to explore this possibility.

Green plant preferences

Starlings also showed a preference for specific green plants to build their nests. Availability of experimental plants did not affect plant abundance in starling nests, but nests that start to breed later harboured more plants than those of early breeders. This positive association between amount of green plants in the nests and laying date has also been found in European starlings (Clark & Mason 1985; Gwinner & Berger 2005; Dubiec et al. 2013). It could be argued that this is just the outcome of a higher accumulation of plants in nests that commenced egg laying later. However, this explanation is unlikely; first because birds remove part of the old dried plants after some days in the nests (Petit et al. 2002) and, second, because for a given starling nest, the amount of plants found during the laying period was smaller than during the pre-laying stage. In addition, it is known that female starlings remove from the nest the green plants carried by males (Veiga & Polo 2012), which reflect male quality (Veiga et al. 2006). This female behaviour has been explained as a mean to difficult the assessment of male and nest quality by neighboring rival females (Veiga & Polo 2012), which usually prospect other nests to gather public information (Parejo et al. 2008). Removing green plants would therefore prevent nest usurpation and reduce attractiveness for conspecific brood parasites (Sandell & Diemer 1999; Veiga & Polo 2012). Thus, it is possible that the relatively smaller amount of plants detected in starling nests during the egg-laying stage was the consequence of this female behaviour.

In agreement with a possible antimicrobial function of plants, we also found that during the pre-laying stage starlings did not show a marked preference for any of the offered plant species to be carried to their nests, but during the laying stage males preferentially selected aromatic plants. It is possible that, before egg laying, males attract females to their nests by carrying fresh green material with less emphasis on antimicrobial and antiparasitic properties of plants (Hansell 2000). However, once reproduction

has started, selecting aromatic plants would have the additional advantage of protecting the nests against parasites (Clark & Mason 1985) and microorganisms (Mennerat et al. 2009a). Most of the aromatic plants that we provided to starlings have strong antimicrobial activity (see table 1). The most preferred plant species appeared to be *L. amplexicaule*, and not *A. barrelieri* that showed the highest antimicrobial activity. We estimated antimicrobial activity of these plants by testing inhibitory potential of growth of 11 bacterial strains that include possible avian pathogens. Thus, although antimicrobial capabilities against different bacterial strains usually covary (Tabla 1; Al-Bakri & Afifi 2007; Khalil et al. 2009), we cannot rule out the possibility that the most preferred plants were active against some particular bacterial pathogens abundant in the study area. Independently of the reasons determining preferences for specific aromatic plants, the detected higher amount of green plants during the prelaying stage and the preferences for aromatic plants during the laying stage, suggest a primary role of this material in sexual signalling, with a role in nest protection against pathogens becoming more prominent during the egg laying stage (see Rubalcaba et al. 2016).

Conclusion

Nest construction is a costly process in terms of energy and time (Mainwaring & Hartley 2013), and birds have to find the best combination of nest materials to optimize the outcome between benefits and costs of searching and carrying every nest material. Irrespective of the diverse functionality of different nests materials, and of the possible optimization of nest material composition, we have shown that starlings make a selection of nest building materials in a possible adaptive way. We have shown this preference regarding two common nest materials profusely employed by many bird species from different orders and in different habitats to build their nests, i.e., green plants and feathers (Hansell 2000; Dubiec et al. 2013). Furthermore, these two materials play pivotal roles in the study of nest building behaviours and may

aid a better comprehension of self-medication and tool use by animals (Hansell & Ruxton 2008; Healy et al. 2008; De Roode et al. 2013). Thus, we fulfill a gap to better understand the adaptive functionality of nest building processes and their involved behaviours.

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Principales resultados y discusión integradora

En esta Tesis hemos investigado los efectos que las plantas verdes y las plumas empleadas como materiales en la construcción del nido de las aves tienen sobre la carga bacteriana de los huevos y los pollos y sobre las distintas variables relacionadas con la supervivencia de los pollos en los nidos, como son el peso corporal, la repuesta inmunitaria, o incluso, la longitud y dinámica de los telómeros (indicadoras del nivel de estrés que han sufrido en el nido durante el crecimiento). Además hemos estudiado la influencia de estos materiales del nido sobre la actividad antimicrobiana de las colonias aisladas de las cáscaras de los huevos y las plumas. Por último, también hemos estudiado la selección que hacen los estorninos a la hora de introducir las plumas y las plantas en sus nidos.

Algo que debemos tener en cuenta a la hora de interpretar los resultados, es que todos los resultados sobre comunidades bacterianas se han obtenido por medio de técnicas clásicas de cultivo de bacterias en diferentes medios. Aunque sabemos que las bacterias cultivables son un número mucho menor del total existente en la naturaleza (Amann et al. 1995; Hugenholtz 2002), muchos estudios han demostrado la eficacia de esta técnica para detectar diferentes asociaciones y efectos, como pueden ser aquellos relacionados con la incubación o con el crecimiento de los pollos, etc. (Cook et al. 2005a; Peralta-Sánchez et al. 2010; González-Braojos et al. 2012b). En esta Tesis no nos planteamos describir las comunidades bacterianas, sino explorar el papel antibacteriano de las plumas y las plantas en los nidos de estornino, para lo que la utilización de técnicas clásicas de cultivo debe ser suficiente. Sin embargo, la utilización de métodos moleculares para determinar el posible papel de estos materiales en el nido podría dar una visión más amplia de los efectos sobre las comunidades bacterianas de los nidos y, por tanto, sería el siguiente paso para avanzar en el conocimiento de

las funciones de los materiales utilizados por las aves a la hora de construir sus nidos.

Son muchas las funciones que se han postulado para explicar la utilización de determinados materiales en la construcción del nido de las aves (Hansell 2000; Mainwaring et al. 2014). En el caso de las plumas y las plantas verdes, las funciones que principalmente se les han asignado son: una función de señalización sexual para ambos materiales (Dubiec et al. 2013; Mainwaring et al. 2014), una función de termorregulación en el caso de las plumas (Hilton et al. 2004; Windsor et al. 2013), y una función antiparasitaria en el caso de las plantas (Dubiec et al. 2013; Scott-Baumann & Morgan 2015) y más recientemente también en el caso de las plumas (Soler et al. 2010; Peralta-Sánchez et al. 2010). Estas funciones no tienen por qué ser mutuamente excluyentes, de modo que un mismo material puede desempeñar varias funciones. En esta Tesis hemos detectado numerosas evidencias del papel antibacteriano de las plumas y las plantas en los nidos de estornino negro, especie en la que la principal función estudiada hasta la fecha ha sido la función de señalización sexual (Polo & Veiga 2006; Veiga et al. 2006; Veiga & Polo 2011).

Efectos antibacterianos de las plantas

Los efectos antimicrobianos de las plantas han sido demostrados en pollos de otras especies como el herrerillo (Mennerat et al. 2009a) o el estornino pinto (Gwinner & Berger 2005) en condiciones naturales, pero nunca en la carga bacteriana de la cáscara de los huevos de ninguna especie de ave (D'Alba & Shawkey 2015; pero ver Møller et al. 2013). En nuestra población de estornino negro, manipulamos la cantidad de plantas presentes en el nido, tanto en la etapa de huevos como en la de pollos. En nidos naturales no encontramos un efecto de las plantas experimentales sobre la carga bacteriana de los huevos (**capítulo 1**) o de la piel de los pollos (**capítulo 2**). En el caso de

la carga bacteriana de los huevos es posible que las numerosas defensas que presenta el huevo contra los microorganismos, incluso en la cáscara (D'Alba & Shawkey 2015) o los efectos antimicrobianos de la incubación (Cook et al. 2005a; Shawkey et al. 2009) dificultara poner de manifiesto los efectos esperados. En apoyo de esta segunda posibilidad, sí encontramos un efecto de las plantas en el experimento realizado en los nidos artificiales sin incubación: éstas redujeron la carga bacteriana en nidos en los que habíamos aumentado experimentalmente la carga bacteriana y eliminado el comportamiento parental (**capítulo 1**). Tampoco podemos descartar que la manipulación experimental de las plantas de los nidos no haya sido suficiente o adecuada para detectar efectos sobre la carga bacteriana.

Conviene destacar que este tipo de manipulaciones entraña la dificultad de que los adultos pueden modificar la cantidad y composición de los distintos materiales al poco tiempo de aplicarles el tratamiento experimental. Este inconveniente se ha solventado en ocasiones utilizando como modelos de estudio especies que no usan plantas verdes en sus nidos (por ejemplo Dawson 2004; Shutler & Campbell 2007). En este sentido, encontramos un efecto de las plantas que los adultos habían incorporado en los nidos a los pocos días de nacer los pollos reduciendo la prevalencia de estafilococos en la piel de los pollos (**capítulo 2**; Fig. 1A). Este grupo bacteriano incluye algunas especies patógenas para las aves (Hubálek 2004; Benskin et al. 2009). Además, también encontramos un efecto de la interacción de los tratamientos experimentales de plumas y de plantas reduciendo la prevalencia de estafilococos en nidos con pollos (**capítulo 2**; Fig. 1B). Estos resultados sugieren que las plantas pueden tener un efecto antibacteriano que no hemos podido detectar claramente, y que además puede estar relacionado con las plumas que hay en los nidos durante la fase de pollos.

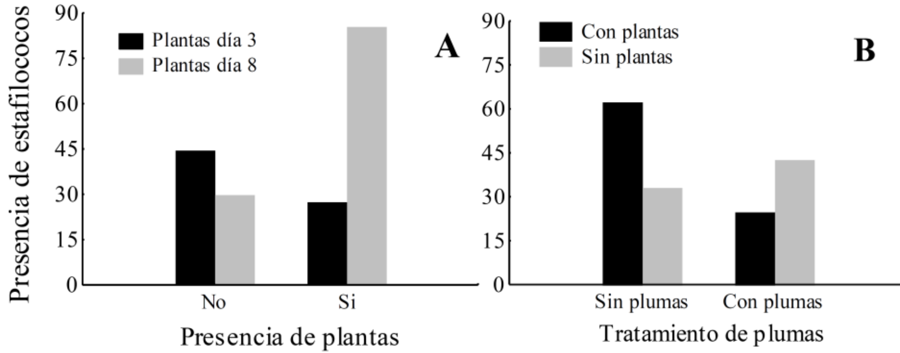

Figura 1: Asociación entre la presencia de estafilococos en la piel de los pollos de estornino y (A) la presencia de plantas en el nido a día 3 y día 8 de edad de los pollos y (B) la interacción entre los tratamientos experimentales de plumas y de plantas.

Efectos antibacterianos de las plumas

Nuestros resultados mostraron que las plumas presentes en los nidos redujeron la carga bacteriana tanto de la cáscara de los huevos (**capítulo 1**; Fig. 2A) como de la piel de los pollos (**capítulo 2**; Fig. 2B). Esta función antibacteriana de las plumas en los nidos de las aves (Soler et al. 2010) se había demostrado previamente tan sólo en nidos de golondrina durante la incubación de los huevos (Peralta-Sánchez et al. 2010; 2011), pero no en otras especies de aves, y nunca durante la fase de estancia de los pollos en el nido.

Las plumas en los nidos de estornino negro se utilizan como una señal sexual entre los adultos (Polo & Veiga 2006), por lo que un diferente número de plumas o quizás su diferente pigmentación puede provocar cambios en los comportamientos parentales incluidos los de higiene y, por tanto, en condiciones del nido directamente relacionadas con el ambiente bacteriano. Por otra parte, es conocido que la actividad de incubación es capaz de reducir la carga bacteriana en las cáscaras de los huevos, ya que ésta depende de la humedad y de la temperatura a la que se encuentran (Bruce & Drysdale 1994; Berrang et al. 1999; D'Alba et al. 2010; Ruiz-De-Castañeda et al. 2011). Cuando excluimos el efecto de la incubación en el experimento con nidos

artificiales (**capítulo 1**), detectamos un claro efecto antimicrobiano de las plumas, independientemente del tipo de pluma (pigmentadas y no pigmentadas). Además los efectos variaron entre los diferentes años de estudio, lo que sugiere que las condiciones ambientales, además del comportamiento parental en el nido, pueden jugar un papel muy importante a la hora de explicar los efectos antibacterianos de las plumas. En trabajos previos tanto en condiciones de laboratorio (Peralta-Sánchez et al. 2014), como en nidos naturales de golondrina (Peralta-Sánchez et al. 2010), los efectos antibacterianos detectados fueron mayores para plumas no pigmentadas. En nuestro caso, encontramos que en nidos naturales de estornino negro, fueron las plumas pigmentadas las que tuvieron un mayor efecto reduciendo la carga bacteriana durante la etapa de huevos (**capítulo 1**; Fig. 2A). Esto sugiere de nuevo que la incubación puede jugar un papel importante a la hora de explicar la función antibacteriana de los diferentes tipos de plumas en los nidos.

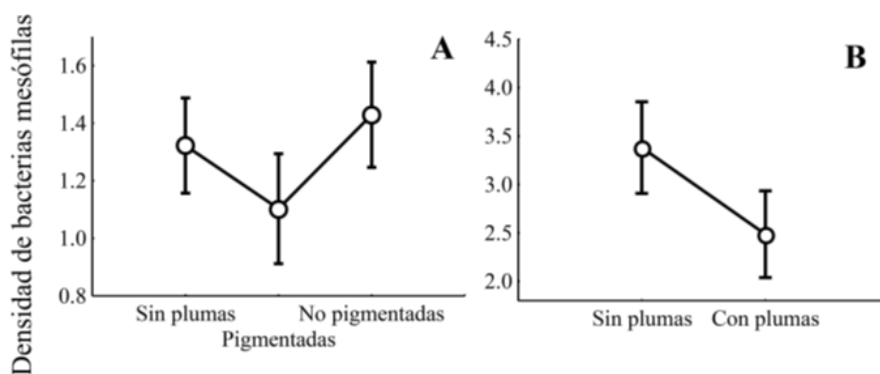

Figura 2: Relación de los tratamientos de plumas con la densidad de bacterias mesófilas (± 95% CI) en (A) la cáscara de huevos de nidos naturales y (B) en la piel de los pollos de estornino

En el caso de los efectos sobre los pollos (**capítulo 2**), también detectamos que las plumas redujeron la carga bacteriana de la piel. Pero los efectos fueron diferentes según la pigmentación de las plumas. Para el grupo de bacterias mesófilas, los efectos fueron similares para plumas pigmentadas

y no pigmentadas. Sin embargo, en el caso de las enterobacterias, su prevalencia fue mayor en nidos con más plumas no pigmentadas, mientras que la prevalencia de los estafilococos fue menor en nidos con plumas pigmentadas experimentales. De forma similar a lo que ocurría con los tratamientos experimentales de plantas, los adultos modificaban el número y composición de las plumas en sus nidos y, aunque cada poco tiempo renovábamos los tratamientos, es posible que las manipulaciones experimentales no fueran suficientes para detectar efectos claros de la pigmentación de las plumas, aunque sí el efecto antibacteriano de las plumas independientemente de su pigmentación.

Efectos de las plumas y las plantas sobre las variables de calidad de los pollos

Encontramos un efecto de las plumas y las plantas en los nidos sobre las variables relacionadas con el estado de salud de los pollos y sus probabilidades de supervivencia, como son el peso corporal, la respuesta inmune, y el éxito de vuelo (**capítulo 2**). Los posibles efectos beneficiosos que las plantas verdes pueden tener sobre el estado de salud de los pollos han sido encontrados sobre el peso corporal y sobre los niveles de hematocrito en pollos de herrerillo (Mennerat et al. 2009b) y de estornino pinto (Gwinner et al. 2000). En nuestro caso, no encontramos un efecto claro de las plantas sobre las distintas variables que hemos medido en relación al estado de los pollos. No obstante, sí hemos encontrado un efecto positivo del número de plumas sobre las diferentes variables, como el peso corporal, la respuesta inmune y el éxito de vuelo. En este caso, no podemos descartar que las plumas y las plantas sirvan como señales sexuales entre los adultos y que tengan un efecto aumentando el esfuerzo parental que mejore la condición física de los pollos (Veiga & Polo 2005; García-López de Hierro et al. 2013). Cabe destacar que también hemos encontrado una relación entre la carga bacteriana de la piel y el peso corporal y la respuesta inmune de los pollos.

Estos resultados apoyan la idea de que el efecto detectado de los materiales del nido sobre las variables de calidad de los pollos esté mediado por los efectos antibacterianos de las plumas y las plantas.

Además encontramos un efecto de los materiales del nido sobre la longitud de los telómeros de los pollos (**capítulo 3**), la cual está relacionada con el nivel de estrés sufrido por los pollos en el nido (Monaghan & Haussmann 2006; Monaghan 2014). En nidos en los que los adultos habían acarreado un mayor número de plumas los pollos tuvieron telómeros más largos al nacer. Este efecto podría estar relacionado con el papel que tienen las plumas en los nidos en la señalización sexual, ya que son las hembras las que mayormente llevan plumas a los nidos (Polo & Veiga 2006) y la longitud de los telómeros es un carácter que se hereda por vía materna (Asghar et al. 2015a). Así, hembras de mayor calidad podrían ser las que llevaran más plumas al nido y las que produjeran pollos con los telómeros más largos. Por otro lado, no encontramos una relación entre la presencia de plantas en el nido en el momento de la eclosión con la longitud de los telómeros, en concordancia con la herencia materna de la longitud de los telómeros, ya que son los machos de estornino los que llevan las plantas al nido (Polo & Veiga 2006).

Los materiales del nido también estuvieron relacionados con la longitud y acortamiento de los telómeros de los pollos al final de su estancia en el nido. Los pollos que crecieron en nidos con plantas experimentales tuvieron telómeros más cortos (Fig. 3A). Este resultado quizás pueda ser explicado por el efecto que las plantas tienen estimulando el crecimiento de los pollos (Gwinner et al. 2000; Mennerat et al. 2009b), aumentando la tasa de divisiones celulares. En cuanto a las plumas, los pollos que crecieron en nidos con plumas no pigmentadas experimentales tuvieron telómeros más largos (Fig. 3B). Esta asociación podría ser debida a las propiedades antibacterianas de las plumas no pigmentadas, ya que los pollos en estos nidos también tenían

menos bacterias en su piel. No obstante, estas asociaciones sólo las observamos en una de las dos poblaciones de estudio (Huéneja), por lo que no podemos descartar otras hipótesis alternativas. Es posible por ejemplo que haya un efecto de las plumas no pigmentadas y plantas sobre el esfuerzo parental alimentando a los pollos durante su estancia en el nido, y que este hecho afecte indirectamente a la dinámica de los telómeros (Badás et al. 2015; Kim & Velando 2015). En cualquier caso, nuestros resultados muestran que de una forma u otra los materiales de nido afectan claramente a la longitud de los telómeros de los pollos.

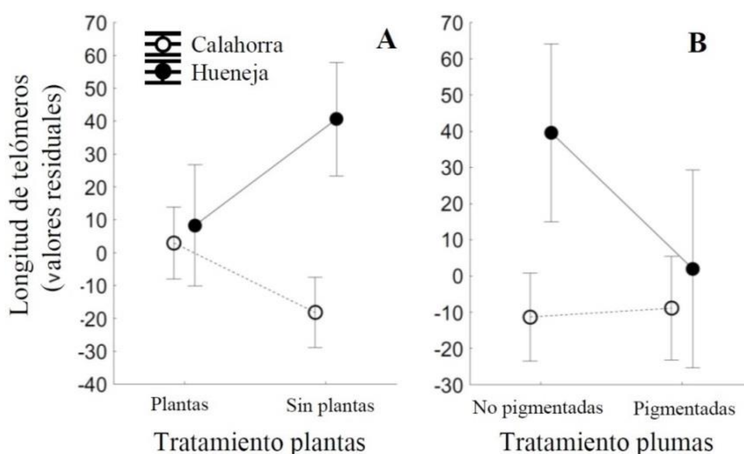

Figura 3: Efectos de los tratamientos de (A) plantas (con plantas y sin plantas aromáticas) y (B) plumas (pigmentadas y no pigmentadas) sobre la longitud de los telómeros de los pollos al final de su estancia en el nido.

Capacidades antimicrobianas de colonias bacterianas procedentes de nidos de aves

Una parte importante de la base teórica de las hipótesis que se plantean en esta Tesis recae en la asunción de la existencia de un importante potencial antimicrobiano de las bacterias que crecen en las plumas de los nidos de las aves (Soler et al. 2010). Esa asunción la intentamos comprobar en el **capítulo 4** comparando densidades bacterianas en plumas y cáscaras de huevos y

determinando las capacidades antimicrobianas de bacterias aisladas de esos sustratos. Además, debido a que las capacidades antimicrobianas de bacterias pueden depender de condiciones ambientales (Biswas et al. 1991; Mataragas et al. 2003), recogimos muestras de nidos naturales y de nidos artificiales con huevos de codorniz (i.e., sin actividad de incubación).

Detectamos un interesante efecto positivo de la actividad de incubación sobre la carga bacteriana de plumas y de huevos, y sobre la actividad antimicrobiana de colonias aisladas de plumas y huevos (**capítulo 4**). Aunque la aproximación de las diferencias entre nidos naturales y artificiales se hizo con huevos de diferentes especies (huevos de estornino en nidos naturales y de codorniz en nidos artificiales), los cuales pueden variar en sus propiedades antimicrobianas, y por tanto en el crecimiento bacteriano (Wellman-Labadie et al. 2008a; 2010; Soler et al. 2011), esta comparación ya ha sido utilizada anteriormente con éxito (Soler et al. 2015b).

Al comparar, la densidad bacteriana entre plumas y huevos de nidos artificiales y naturales, por un lado encontramos que la densidad de bacterias mesófilas y queratinolíticas en plumas era mayor en muestras de nidos naturales. Por otro lado, al estudiar la carga bacteriana de la superficie de los huevos, encontramos que, aunque la densidad de bacterias mesófilas fue similar en nidos artificiales y naturales, la densidad de bacterias queratinolíticas fue mayor en los huevos en nidos naturales (i.e., incubados) que en los huevos en nidos artificiales. Este resultado sugiere que el proceso de incubación puede favorecer el establecimiento de bacterias queratinolíticas en la superficie del huevo. De acuerdo con esta posibilidad, se ha observado un aumento de la densidad de bacterias queratinolíticas (por ejemplo géneros *Pseudomonas* o *Bacillus*) a lo largo de la incubación en distintas especies de aves (Wang et al. 2011; Potter et al. 2013; Grizard et al. 2015). Estas bacterias queratinolíticas son capaces de producir bacteriocinas eficaces frente a otras bacterias (Callow & Work 1952; Ramnani et al. 2005; Naz et al. 2015), por lo

que las aves podrían estar seleccionando un determinado tipo de bacterias con capacidades antibacterianas.

Respecto a los análisis de actividad antimicrobiana, encontramos que las colonias bacterianas aisladas de plumas mostraron mayor actividad antagónica que las aisladas de la cáscara de los huevos. Además, en ambos casos, fue mayor la capacidad antimicrobiana de las colonias aisladas de nidos naturales que la de las colonias aisladas de nidos artificiales (Fig. 4). Estos resultados refuerzan la hipótesis de que la actividad de incubación juega un papel importante en la actividad antimicrobiana del nido. Como ya hemos comentado anteriormente, la capacidad antimicrobiana de determinadas especies de bacterias se debe a la segregación de compuestos, como las bacteriocinas (Riley & Wertz 2002), la cual depende de múltiples factores, entre los que se encuentran la temperatura o el sustrato (Mataragas et al. 2003; Mataragas et al. 2004; Todorov & Dicks 2006). Por ello, la actividad que tiene lugar en los nidos puede favorecer tanto el crecimiento como la actividad de las bacterias queratinolíticas.

No encontramos efectos de la adición de plumas a los nidos, ni del tipo de pigmentación de las plumas experimentales; ni sobre la carga bacteriana, ni sobre la actividad antimicrobiana de las colonias aisladas de plumas y huevos. Trabajos anteriores encontraron que las colonias aisladas de plumas no pigmentadas recogidas de nidos con tratamiento de plumas pigmentadas tuvieron una mayor actividad antimicrobiana que las aisladas de plumas pigmentadas (Peralta-Sánchez et al. 2014), pero nosotros no hemos encontrado esos efectos. Sin embargo, sí que hemos demostrado en esta Tesis, que la diferente pigmentación de las plumas tiene diversos efectos positivos según las diferentes condiciones del nido. Por ejemplo, en nidos artificiales (sin incubación) encontramos un efecto similar de la pigmentación de las plumas, pero fueron las plumas pigmentadas las que redujeron la carga bacteriana de los huevos en nidos naturales y las no pigmentadas redujeron la

carga bacteriana de algunos grupos bacterianos en pollos. Por tanto, nuestros resultados muestran efectos positivos de las plumas en términos de reducción de cargas bacterianas que, gracias a los mayores potenciales antimicrobianos de las bacterias aisladas de plumas, se pueden interpretar como debidos a la capacidad antimicrobiana de las plumas utilizadas como material del nido por las aves. Estos resultados abren una línea de investigación interesante para determinar los mecanismos que explican la mayor capacidad antimicrobiana de bacterias aisladas de plumas en nidos con actividad incubadora.

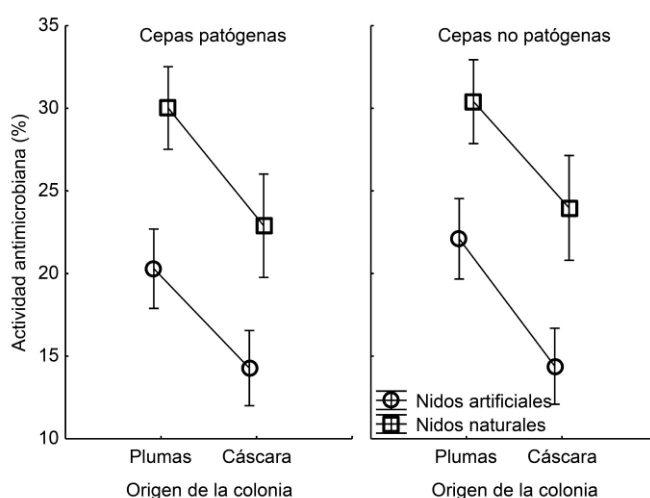

Figura 4: Actividad antimicrobiana (± SE) de las colonias aisladas de plumas y cáscaras de huevos de nidos naturales y artificiales frente a las cepas indicadoras potencialmente patógenas y no patógenas.

Selección de plumas y plantas por los estorninos para la construcción del nido

Hemos demostrado efectos positivos antimicrobianos de las plumas y las plantas presentes en los nidos y también sobre el crecimiento de los pollos. Sin embargo, la construcción del nido es un proceso costoso, debido a que buscar los materiales, llevarlos al nido y construir toda la estructura del nido, conlleva mucho tiempo y un gasto energético considerable para las aves

(Hansell 2000; Mainwaring & Hartley 2013). Por ello, la elección adecuada de una determinada cantidad y composición de los materiales que formen el nido debe ser un proceso fundamental. En el caso de plumas y plantas, se deberían de ajustar a las condiciones ambientales de, por ejemplo, disponibilidad de alimento y riesgo de infección. Este ajuste debería de estar dirigido por las diferentes funciones de los nidos, como puede ser la función sexual, según la cual el nido o su proceso de construcción pueden estar implicados en la atracción de la pareja y/o en la modificación de su inversión parental en el evento reproductivo (Soler et al. 1998; Moreno 2012; Mainwaring et al. 2014). Esta selección del material podría además estar relacionada con las propiedades antimicrobianas que puedan mejorar el ambiente bacteriano del nido donde mantener a la descendencia, como hemos observado en el caso de los estorninos. Para investigar esta selección de materiales del nido, les ofrecimos plumas pigmentadas y no pigmentadas, y plantas aromáticas y no aromáticas en las proximidades de los nidos de nuestra población de estudio.

En cuanto a las plumas, las aves mostraron una clara preferencia por las plumas no pigmentadas. Encontramos más plumas no pigmentadas en los nidos (Fig. 5A) y, además, la tasa de renovación de plumas de las mallas metálicas donde se las ofrecimos, fue mayor para las plumas no pigmentadas que para las plumas pigmentadas. Dada la mayor actividad antimicrobiana de las plumas no pigmentadas en comparación con las plumas pigmentadas detectada en otra especie de ave (Peralta-Sánchez et al. 2014), estos resultados apoyarían la idea de que los adultos elegirían las plumas no pigmentadas para reducir la carga bacteriana en los nidos. Sin embargo, nuestros resultados en el estornino negro sobre un efecto diferencial de las plumas blancas no son tan claros y no podemos descartar otras posibilidades. Es posible que esta preferencia esté también guiada por procesos de selección sexual (Polo & Veiga 2006), una hipótesis que podría ser comprobada experimentalmente.

Hasta donde nosotros conocemos, el significado funcional de la incorporación de plumas en los nidos desde un punto de vista de comunicación sexual siempre ha sido estudiado en referencia al número de plumas y no a su pigmentación, por lo que sería interesante realizar investigaciones específicas para determinar el posible efecto que la diferente coloración de las plumas de los nidos pudiera tener sobre los procesos de selección sexual.

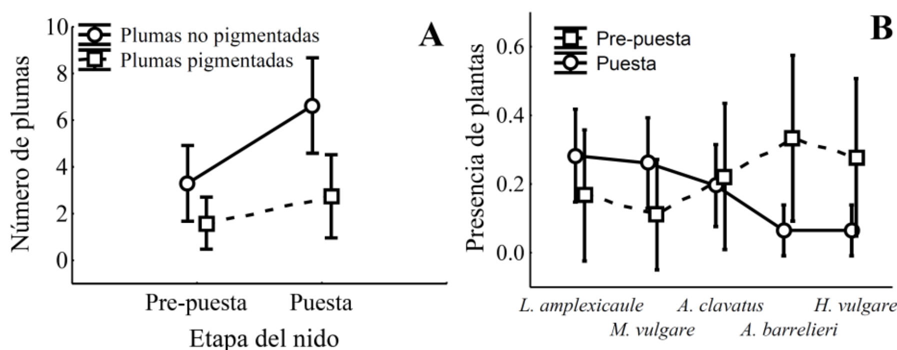

Figura 5: (A) Número de plumas pigmentadas y no pigmentadas en nidos de estornino antes y durante la puesta de los huevos, y (B) presencia de las diferentes especies de plantas en los nidos antes y durante la puesta de los huevos.

En cuanto a la selección de plantas verdes por los estorninos, encontramos que antes de la puesta de los huevos, no había diferencias en la presencia de plantas aromáticas y no aromáticas incorporadas en los nidos. Sin embargo, durante la puesta de los huevos, los adultos hacían una selección activa de las plantas, llevando preferentemente plantas aromáticas a los nidos (Fig. 5B). Estos resultados sugieren un papel tanto sexual como antiparasitario por parte de las plantas, ya que antes de la puesta de los huevos el macho puede llevar las plantas a los nidos como un despliegue sexual para atraer y motivar a la hembra (Veiga et al. 2006), mientras que una vez que la puesta ha comenzado, el llevar plantas al nido puede tener una función antiparasitaria adicional, mejorando el ambiente bacteriano donde se incuban los huevos.

Resumiendo, los resultados de esta Tesis ponen de manifiesto el papel antimicrobiano que las plumas y las plantas pueden tener en el nido de las aves, reduciendo la comunidad bacteriana de la cáscara de los huevos y la piel de los pollos y mejorando el desarrollo de la descendencia. Además hemos encontrado que estos efectos pueden estar sujetos a importantes variaciones, debidas, por ejemplo, a las condiciones ambientales o a los comportamientos parentales como la incubación. Estas propiedades antimicrobianas de las plumas y las plantas pueden ser las que determinen la selección activa de estos materiales por parte de los adultos a la hora de construir sus nidos, y de acuerdo con esta posibilidad hemos puesto de manifiesto importantes capacidades antagónicas de bacterias aisladas de plumas.

Conclusiones

1. Las plumas y las plantas usadas como materiales de nido afectaron a la carga bacteriana de los huevos. En nidos naturales de estornino negro, la reducción de la carga bacteriana fue mayor en nidos con tratamiento de plumas pigmentadas, mientras que en nidos artificiales (i.e., sin incubación) el efecto de las plumas pigmentadas y no pigmentadas sobre la carga bacteriana de los huevos fue similar. No encontramos un efecto de las plantas en nidos naturales, pero sí redujeron la carga bacteriana en nidos artificiales cuando aumentamos experimentalmente la carga bacteriana. Estos efectos los encontramos en diferentes años y zonas de estudio, sugiriendo que las condiciones ambientales, además del comportamiento parental, pueden influir en la función antimicrobiana de las plumas y las plantas en los nidos.

2. Las plumas y las plantas usadas como materiales del nido redujeron la carga bacteriana de la piel de los pollos de estornino. Estos materiales tuvieron un efecto positivo sobre diferentes variables relacionadas con el estado de salud de los pollos que, a su vez, aparecieron negativamente relacionadas con la densidad de bacterias en la piel de los pollos. Estas relaciones sugieren que los efectos beneficiosos de los materiales del nido sobre los pollos podrían estar explicados por sus propiedades antimicrobianas.

3. La adición experimental de plumas en los nidos incrementó el acortamiento de los telómeros de los pollos. Sin embargo, en nidos con plumas no pigmentadas, los pollos próximos a volar tuvieron telómeros más largos con una menor tasa de acortamiento que los pollos en nidos con plumas pigmentadas. La adición experimental de plantas aromáticas a los nidos también tuvo efectos positivos en la longitud de los

telómeros, pero solo en una de las poblaciones estudiadas. Estos resultados sugieren que el material del nido afecta a la longitud y la dinámica de los telómeros de los pollos durante su estancia en el nido. Además, detectamos una relación negativa entre la prevalencia de estafilococos en la piel de los pollos y la longitud de los telómeros y, por tanto, es posible que los efectos de los materiales del nido sobre la longitud de los telómeros estén mediados por las propiedades antimicrobianas de los materiales del nido.

4. La actividad antimicrobiana de las colonias aisladas de las plumas del nido fue mayor que la de colonias aisladas de la cáscara de los huevos, en nidos naturales y artificiales. Además, la densidad bacteriana de las plumas y la cáscara de los huevos, y la actividad antimicrobiana de las colonias aisladas de estos materiales, fueron mayores en nidos naturales que en nidos artificiales (i.e., sin incubación). Estos resultados apoyan la función antimicrobiana de las plumas y sugieren que la actividad reproductora podría aumentar el crecimiento de bacterias queratinolíticas y su actividad antimicrobiana en los nidos de las aves.

5. Los adultos de estornino seleccionaron preferencialmente plumas no pigmentadas como material de nido, antes y durante la puesta de los huevos. Los estorninos prefirieron plantas aromáticas frente a no aromáticas, pero solo durante la puesta de los huevos. Esta selección de las plumas y las plantas puede estar relacionada con sus propiedades antimicrobianas.

Conclusions

1. Feathers and plants as nest materials affected eggshell bacterial load. In natural nests of spotless starlings, bacterial load reduction was higher in nests with pigmented feathers treatment, while in artificial nests (i.e., without incubation) pigmented and unpigmented feathers had similar effects on eggshell bacterial loads. We did not find any effect of plants in natural nests, but they reduced bacterial load in artificial nests with experimentally increased bacterial load. We found these effects in different study areas and years, which suggests that both environmental conditions and parental activity influence the effect of plants and feathers as antimicrobial material.

2. Feathers and plants as nest materials reduced bacterial load on nestling skin. These materials had positive effects on several fitness-related variables that were also negatively correlated with nestling bacterial density. These relationships suggest that the beneficial effect of nest materials could be mediated by their antimicrobial properties.

3. The experimental addition of feathers to spotless starling nests increased telomere attrition of developing nestlings. However, in nests with unpigmented feathers, nestlings close to fledging had longer telomeres with lower attrition rate than nestlings in nests with pigmented feathers. The experimental addition of aromatic plants to nests also had positive effects on telomere length, but only in one of the study areas. These results suggest that nest materials affect telomere length and dynamic of nestlings during their nesting period. Since we detected a negative relationship between staphylococci prevalence on nestling skin and telomere length, it is possible that the effects of nest materials on

telomere length were mediated by the antimicrobial properties of nest materials.

4. Antimicrobial activity of bacterial colonies isolated from feather nest material feathers was higher than that of colonies isolated from eggshells, both in natural and artificial nests. In addition, bacterial densities on feathers and eggshells, as well as the antimicrobial activity of colonies isolated from these materials, were higher for natural than for artificial nests (i.e., without incubation). These findings support the antimicrobial function of feathers and suggest that breeding activity would enhance growth of keratinolytic bacteria and their antimicrobial activity in avian nests.

5. Spotless starling adults preferentially selected unpigmented feathers as nest material both before and during the egg laying stage. Starlings preferred aromatic plants as nest materials, but only during the egg laying stage. This selection of plants and feathers may be related to their antimicrobial properties.

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