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Biodiversidad bacteriana de la secreción uropigial y la cáscara de los huevos de abubillas (*Upupa epops*): estabilidad y adquisición

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Estación Experimental de Zonas Áridas

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Memoria presentada por Mª Ángeles Martínez García 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 de la Estación Experimental de Zonas Áridas (EEZA-CSIC) y Manuel Martín-Vivaldi Martínez profesor titular de la Universidad de Granada.

En Granada a 18 de Junio de 2015

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A mis padres

"La paciencia es un árbol de raíz amarga,

pero de frutos muy dulces"

Proverbio persa

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RESUMEN

La supervivencia, reproducción y, por tanto, la eficacia biológica de un gran número de organismos depende de sus relaciones con microorganismos. Algunas bacterias viven en asociaciones mutualistas simbióticas con macroorganismos, que pueden incluso favorecer la evolución de estructuras especializadas, habitáculos, etc., donde se alojan los simbiontes y se favorece su crecimiento. La relación simbiótica entre la abubilla (*Upupa epops*) y las bacterias de su glándula uropigial es un ejemplo de estos mutualismos entre animales y microorganismos. Los simbiontes de abubillas son bacterias productoras de antibióticos que reducen la probabilidad de infección por patógenos en el ambiente del nido. Los efectos de estos simbiontes frente a bacterias degradadoras de plumas, o frente a patógenos de los embriones, pueden ser vistos como resultado de interacciones entre la comunidad de bacterias hospedada en la glándula y otras comunidades bacterianas presentes en los nidos de abubillas (huevos, plumas, piel de hembras y polluelos, etc.). La simbiosis con bacterias solo está presente en hembras incubadoras y polluelos, pero no en machos. La abubilla es la única especie de ave para la que se ha descrito que las hembras recogen la secreción con su pico y la extienden, no sólo sobre las plumas, sino también sobre las cáscaras de los huevos. Por lo tanto, mediante el acicalado, los simbiontes bacterianos de la glándula uropigial y/o sus sustancias antimicrobianas llegarán a la superficie del cuerpo de abubillas y a las cáscaras de sus huevos influyendo en las comunidades bacterianas de estos lugares. Podrían, por ejemplo, prevenir la invasión por microorganismos patógenos.

La gran mayoría de las bacterias producen sustancias antimicrobianas frente a otros microorganismos con los que se encuentran en competencia por los recursos y el espacio. En el caso de las bacterias mutualistas, estas sustancias antimicrobianas ayudarían a los hospedadores a luchar contra infecciones por patógenos. Este es probablemente el caso de las abubillas y

las bacterias que crecen en su glándula uropigial, ya que sus simbiontes son eficaces contra microorganismos que degradan plumas, y contra patógenos de los embriones. Por lo tanto, investigar la relación existente entre la comunidad bacteriana simbiótica de la secreción uropigial de abubillas y las de otros lugares dentro de los nidos, incluyendo el material del nido, ayudaría a entender los mecanismos de adquisición de las bacterias simbióticas de hembras y pollos de abubilla, incluso la posibilidad de transmisión vertical de madres a hijos. Este enfoque de meta-comunidad también serviría para ampliar nuestro conocimiento sobre los mecanismos de protección bacteriana de los hospedadores frente a microorganismos patógenos.

Esta tesis se ocupa del estudio de las comunidades bacterianas del ambiente del nido de las abubillas, incluida la de su glándula uropigial, y las relaciones existentes entre ellas. Por medio de análisis moleculares, caracterizamos las comunidades bacterianas de la glándula uropigial, pico, placa incubadora de las hembras y cáscaras de los huevos al final del periodo de incubación, y exploramos las similitudes entre ellas. En términos de número de unidades taxonómicas operacionales (OTUs), la comunidad bacteriana de la secreción uropigial fue el lugar de muestreo más rico (124 OTUs), seguido por el pico (106 OTUs), la cáscara del huevo (98 OTUs) y la placa de incubación (97 OTUs). Sin embargo, la mayoría de estos OTUs sólo apareció esporádicamente, por lo que el número medio de cepas bacterianas por individuo da una mejor representación de lo que se encuentra en esas comunidades: secreción uropigial 22 OTUs, pico 9 OTUs, placa incubadora 9 OTUs y cáscaras de los huevos 8 OTUs. Las comunidades bacterianas del pico y la placa incubadora fueron bastante similares entre sí en composición, y significativamente diferentes de las de la secreción y cáscaras de los huevos, que a su vez también difirieron entre sí. Varios de los OTUs detectados aparecieron en todas las comunidades estudiadas y algunos de los más prevalentes en las muestras de secreción uropigial también aparecieron en las

del pico, en la placa incubadora y en las cáscaras de los huevos con frecuencias relativamente altas, lo que sugiere que las bacterias de la secreción uropigial juegan un papel determinante en la comunidad bacteriana de las cáscaras del huevo. De acuerdo con esta posibilidad, se detectó un patrón de anidamiento entre las comunidades bacterianas exploradas. La comunidad bacteriana de las cáscaras de los huevos está anidada dentro de la comunidad de la placa incubadora; a su vez, la de la placa incubadora está anidada dentro de la comunidad del pico, y la comunidad del pico dentro de la comunidad de la secreción uropigial. Todos estos resultados sugieren que el comportamiento de acicalamiento de las hembras de abubilla, usando para ello la secreción uropigial que contiene bacterias simbiontes, se utiliza para transmitirlas a las cáscaras de los huevos y poder proteger al embrión de infecciones por patógenos.

Con el objetivo de explorar el posible papel de las comunidades bacterianas existentes en los nidos de abubilla como fuentes de bacterias simbióticas para las hembras y sus polluelos, se realizaron dos experimentos diferentes. Por un lado, manipulamos las comunidades bacterianas presentes en el material del nido, caracterizamos la comunidad bacteriana de la cloaca de las hembras, y exploramos sus asociaciones con las comunidades de la secreción de las hembras y de las cáscaras de los huevos. Los resultados mostraron que la comunidad bacteriana de la secreción de las hembras no dependía de la del material de nido o de la cloaca. Sin embargo, la modificación experimental de la comunidad bacteriana de los materiales del nido afectó a la comunidad bacteriana de las cáscaras de los huevos y, por lo tanto, a la probabilidad de infección del embrión.

Por otro lado, hemos realizado experimentos de intercambio de pollos (cross-fostering) entre parejas de nidos para los que habíamos previamente caracterizado las comunidades bacterianas de las hembras. Los resultados

mostraron un importante componente genético determinante de la comunidad bacteriana existente en la glándula uropigial de los polluelos, dado que el nido de origen explicó una mayor cantidad de varianza que el nido de crianza. Las comunidades bacterianas de los polluelos que se cambiaron de nido fueron más similares a las de sus hermanos y madre que a las de sus hermanastros y madre adoptiva. Estos resultados podrían explicarse por una transmisión vertical de simbiontes de madre a hijo antes del experimento, o por la existencia de particularidades de la glándula uropigial de los polluelos que fueran heredadas de las madres y, que de alguna forma, aumentaran la probabilidad de adquirir determinadas bacterias simbiontes de entorno (el nido).

Cada capítulo de la tesis incluye una discusión de los resultados en un escenario de relación mutualista entre abubillas y bacterias, y en el apartado de discusión general, los resultados de los diferentes capítulos se relacionan entre sí, para concluir que el enfoque meta-poblacional usado en este trabajo ha permitido la detección de patrones de interacción entre las comunidades de los nidos de abubillas que son esenciales para la comprensión de la asociación simbiótica entre abubillas y bacterias.

ABSTRACT

Survivorship, reproduction and, therefore, fitness of a large number of organisms depend on their relationship with microorganisms. Some bacteria live in symbiotic mutualistic associations with macro-organisms, which may even evolve specialized structures, dwellings, etc. to host and enhance growth of mutualistic symbionts. The symbiotic relationship between hoopoes (*Upupa epops*) and the bacteria of their uropygial gland is an example of these mutualisms between animals and microorganisms. Symbionts of hoopoes are antibiotic producing bacteria that reduce probability of pathogenic infection in the nest environment. The effects of these symbionts on feather degrading bacteria or embryo pathogens can be seen as the results of an interaction between the community of bacteria hosted in the gland and those present in the nests of hoopoes (eggs, feathers, skin of females and nestlings, etc.). The symbiosis with bacteria is only apparent in incubating females and nestlings, but not in males. The hoopoe is the only bird species for which it has been described that females collect secretion with the beak and spread it, not only on feathers, but also on eggshells. Thus, by mean of preening, the bacterial symbionts of the uropygial gland and/or their antimicrobials will reach the body surface of hoopoes and their eggshells, and influence the bacterial communities of these locations by, for instance, preventing the invasion of microorganisms that are host pathogens.

The vast majority of bacteria produce antimicrobials against other microorganisms with which they are in competition for resources and space. In the case of mutualistic bacteria these antimicrobials would help hosts fighting against pathogenic infections. This is likely the case for hoopoes and their bacteria growing in their uropygial gland that are effective against feather degrading microorganisms and some potential pathogens of embryos. Thus, investigating the connection between the symbiotic bacterial

community of the uropygial secretion of hoopoes and those of other locations within the nests, including nest materials, would help to understand mechanisms of symbiotic bacterial acquisition by hoopoe females and nestlings, including the possibility of vertical transmission from mothers to offspring. This meta-community approach would also serve to extend our knowledge on mechanisms of bacterial protection of hosts from pathogenic microorganisms.

This thesis deals with the study of bacterial communities of the nests environment of hoopoes, including those of their uropygial gland, and the relationships among them. By mean of molecular analyses, we characterized bacterial communities of the uropygial gland, beak, brood patch of incubating females and eggshells at the end of the incubation period, and explored similarities among them. In terms of number of Operational Taxonomic Units (OTUs), the bacterial community of the uropygial secretion was the richest (124 OTUs) followed by that of the beak (106 OTUs), eggshells (98 OTUs) and brood patch (97 OTUs). However, most of these OTUs appeared only sporadically, and the average number of bacterial strains per individual, gives a better representation of what these communities are: uropygial secretion (22 OTUs), beak (9 OTUs), brood patch (9 OTUs) and eggshells (8 OTUs). Bacterial communities of the beak and brood patch were quite similar to each other in composition, and significantly different from those of the secretion and eggshells, which also differed to each other. Several of the detected OTUs did appear in all studied communities, and some of the most prevalent in secretion samples also appeared in samples of the beak, brood patch and eggshells at relatively high frequencies. This suggests that the bacteria of the uropygial secretion play a role determining the bacterial community of the eggshells. In accordance with this possibility, we detected a nested pattern among explored bacterial communities. That of the eggshells was nested within the community of the brood patch; the brood patch community was

nested within the community of the beak, and the community of the beak within the community of the uropygial secretion. All these results suggest that preening behaviour of female hoopoes with uropygial secretion containing bacterial symbionts is used to transmit them to eggshells to prevent embryo pathogenic infections.

With the aim of exploring the role of bacterial communities within the nests of hoopoes as sources of symbiotic bacteria for females and for developing nestlings, we performed two different experiments. On the one hand, we manipulated bacterial communities of nests of hoopoes, characterized the bacterial community of the cloaca of females, and explored their associations with those of the secretion of females and of the eggshells. Results showed that the bacterial community of the secretion of females did not depend of that of the nest material or of the cloaca. However, the experimental modification of the bacterial community of nest materials did affect the bacterial community of the eggshells and, then, the probability of embryo infection.

On the other hand, we performed cross-fostering experiments moving nestling hoopoes between pairs of nests for which we had previously characterized the bacterial communities of brooding females. The results pointed out a significant genetic component determining the bacterial community of the uropygial gland of nestlings given that the nest of origin explained larger amount of variance than nest of rearing. Bacterial communities of cross-fostered nestlings were more similar to those of their siblings and mothers than to the bacterial communities of stepsiblings and stepmother. These results may be explained by vertical transmission of symbionts from mother to offspring before the experiment, or by particularities of the uropygial gland of offspring that were inherited from

mothers that enhance probability of acquiring particular bacterial symbionts from the nest environment.

Each of the thesis chapters includes a discussion of the results in a scenario of mutualistic relationship between hoopoes and bacteria and, in the general discussion section the results from different chapters are related to each other, concluding that the meta-population approach adopted here has allowed the detection of patterns of interactions among communities of the nests of hoopoes that are essential for the understanding of the symbiotic association between hoopoes and bacteria.

INTRODUCCIÓN

Los procesos coevolutivos entre especies o grupos de individuos (cambios evolutivos recíprocos como consecuencia de sus interacciones) son unos de los principales responsables de la biodiversidad y de su organización, tal y como la conocemos actualmente (Thompson 1998, 1999a). La supervivencia y reproducción de muchos de los seres vivos depende en gran medida de su relación con otros organismos, pudiendo estas relaciones llegar a ser muy especializadas (Thompson 1999b). Algunos ejemplos clásicos de procesos coevolutivos son los de las higueras y sus avispas polinizadoras, el de las yucas y las polillas, o el de parásitos de cría y sus hospedadores (Thompson 1994). Incluso, un mismo organismo puede mantener relaciones coevolutivas estrechas con más de una especie, como ocurre con las plantas con flores y los insectos polinizadores, las plantas leguminosas y los rizobios del suelo, o los animales y la microbiota intestinal (Thompson 1998). Los cambios producidos por procesos coevolutivos en las poblaciones de organismos, en su dinámica y/o en su estructura, pueden ocurrir muy lentamente si las relaciones entre las partes son más o menos difusas, o pueden ser rápidos cuando las relaciones coevolutivas son más estrechas (Thompson 1998).

La evolución de la virulencia en parásitos y la de la resistencia en sus hospedadores, el ajuste de los rasgos fenotípicos entre especies depredadoras y sus presas, o la aparición de relaciones mutualistas entre taxones filogenéticamente distantes, son en la mayoría de los casos consecuencia de los procesos coevolutivos (Thompson 1998). Muchas de las asociaciones coevolutivas incluyen a bacterias como una de las partes. Las bacterias son microorganismos ubiquistas capaces de vivir en simbiosis con macroorganismos (Moran 2006), formando asociaciones parasitarias, comensalistas, o incluso mutualistas en las que tanto el hospedador como el simbionte se ven beneficiados (Steinert et al. 2000). A pesar de que la

infección microbiana es una de las mayores causas de mortalidad natural de animales (Price 1980), existe una gran cantidad de ejemplos de interacciones mutualistas entre animales y bacterias (Moran 2006). Algunos de los beneficios más conocidos que ofrecen los simbiontes a sus hospedadores se relacionan con la asimilación de nutrientes en el aparato digestivo (Nalepa 1994, Hill 1997, Ley et al. 2008). Las bacterias juegan también un papel fundamental en la producción de vitaminas (Hill 1997), en el mantenimiento del sistema inmune (Umesaki et al. 1999, Macpherson and Harris 2004), o en la protección de su hospedador frente a infecciones por patógenos (Fons et al. 2000, Dillon et al. 2005). Estas interacciones pueden llegar a ser muy estrechas, originando cambios evolutivos en los animales que favorezcan a determinadas cepas de bacterias, y en las bacterias cambios que favorezcan a los animales. Un caso particular es la evolución en los hospedadores de compartimentos especializados en el cultivo de los simbiontes, muchas veces con aporte glandular que favorece el crecimiento de determinadas cepas de bacterias (Barbieri et al. 2001, Currie et al. 2006).

Entender cómo los microorganismos simbiontes se establecen y mantienen en sus hospedadores es una cuestión de gran importancia en biología y está siendo explorada en la actualidad desde perspectivas tan diferentes como la biología molecular, la ecología del comportamiento y la ecología de comunidades (Bright and Bulgheresi 2010, Archie and Theis 2011, Ezenwa et al. 2012, Scheuring and Yu 2012, Heath-Heckman et al. 2013, McFall-Ngai et al. 2013). Desde el punto de vista de la ecología de comunidades, la adquisición de simbiontes por los hospedadores se podría ver como un tipo de interacción o de relación entre comunidades de bacterias. Las comunidades de bacterias, incluyendo aquellas en simbiosis con los animales, no están aisladas unas de otras sino que existe un cierto grado de relación entre ellas debido a la expansión de las mismas o a fenómenos de dispersión de algunas de sus cepas (Long and Azam 2001, Prasad et al. 2011, Long et al.

2013). Esas relaciones podrían influir en algunas de las características funcionales de las comunidades simbiontes de animales, como puede ser la adquisición de cepas adecuadas (Scheuring and Yu 2012) o la producción de sustancias antibióticas (Cordero et al. 2012), y en la estabilidad de la comunidad (Prasad et al. 2011, Long et al. 2013). Por tanto, en un escenario de meta-comunidades, conocer el grado de relación o conexión entre comunidades bacterianas simbiontes de animales y otras existentes en el medio que los rodea es esencial para entender los mecanismos por los que ciertas bacterias protegen a sus hospedadores. La teoría de redes es el marco de trabajo para explorar interacciones entre comunidades bacterianas. Sin embargo, esta aproximación al estudio de comunidades simbióticas, hasta ahora solo se ha aplicado al estudio de sistemas de micorrizas y plantas (Chagnon et al. 2012, Montesinos-Navarro et al. 2012, Jacquemyn et al. 2015).

Una aproximación que permite detectar interacciones entre comunidades, que afectan o explican los patrones de distribución de múltiples especies a través de múltiples localidades, es el análisis de anidamiento (Ulrich et al. 2009, Ulrich and Almeida-Neto 2012, Traveset et al. 2014). El concepto de anidamiento se originó en contextos de trabajo que trataban de explicar la biodiversidad de islas como resultado de la colonización de fuentes más cercanas al continente. Los mejores dispersores colonizarían islas más alejadas, mientras que la distribución de los organismos que se dispersaran peor quedaría restringida a las islas más cercanas, dando lugar a un patrón de distribución de especies encajonado o anidado, desde el continente a las islas más alejadas (Ulrich and Almeida-Neto 2012). El análisis de anidamiento entre comunidades, por tanto, detecta patrones no aleatorios en la composición de distintas comunidades en un gradiente ambiental. En escenarios de meta-comunidades, un patrón de anidamiento indicaría una relación entre gradientes ambientales y características de las especies que

componen la comunidad (Ulrich et al. 2009). Los patrones de anidamiento son comunes en redes ecológicas de interacciones entre especies (Bascompte et al. 2003, Fortuna et al. 2010) y han sido raramente explorados en comunidades bacterianas (Poisot et al. 2011, Aguirre-von-Wobeser et al. 2014). Por tanto, estimar el grado de anidamiento de metacomunidades de simbiontes ayudará a comprender la dinámica y estabilidad de las comunidades microbianas de los animales. En esta tesis nos planteamos estudiar la comunidad bacteriana de la glándula uropigial de la abubilla europea (*Upupa epops*) en un escenario de meta-comunidades. Es decir, no solo centrándonos en la comunidad de simbiontes sino también en aquellas cercanas con las que la comunidad simbionte podría estar relacionada siguiendo patrones de anidamiento.

La abubilla europea (Soler et al. 2008) y la abubilla arbórea (*Phoeniculus purpureus*, Law-Brown and Meyers 2003) son las únicas especies de aves para las que se conoce la existencia de una comunidad de bacterias en su glándula uropigial. En el nido se pueden explorar distintos tipos de comunidades bacterianas (en hembras, en pollos, en huevos y en material del nido), y el estudio de las relaciones o conexiones existentes entre cada una de ellas nos ayudaría a comprender importantes particularidades del sistema mutualista entre bacterias y abubillas. En concreto, en esta tesis, después de caracterizar los distintos tipos de comunidades en nidos de abubillas (Capítulos I y III), comprobamos la existencia de una conexión entre la comunidad de la secreción y la de los huevos (Capítulo II) que ayuda a comprender los efectos detectados en trabajos anteriores de la secreción uropigial sobre la carga bacteriana y la probabilidad de infección de embriones (Martín-Vivaldi et al. 2014). Además, esta aproximación también proporciona importantes pistas que ayudan a comprender cómo llegan las bacterias a la glándula de las hembras (Capítulo III), y de los pollos (transmisión vertical y/o horizontal, componente genético y ambiental de la comunidad) (Capítulo IV). Para comprender la importancia del estudio de

esas cuestiones es necesario primero conocer distintos aspectos de las comunidades bacterianas y de las relaciones simbióticas entre animales y bacterias que se explican brevemente en los siguientes apartados.

RELACIONES ENTRE BACTERIAS: INTERFERENCIA BACTERIANA

Como consecuencia de la competencia por el hábitat y los recursos, se ha seleccionado en las bacterias la producción de una gran variedad de compuestos bioactivos, utilizados como sistema de defensa contra otros microorganismos. Estos compuestos determinan el fenómeno conocido como interferencia bacteriana, por el que la colonización de un ambiente por una cepa bacteriana afecta a la posibilidad de que otras cepas se establezcan (Brook 1999). Una de las principales armas con que cuentan las bacterias para la competencia a este nivel son las bacteriocinas (Riley and Wertz 2002a). Estas sustancias son pequeños péptidos capaces de modificar el ambiente, generando una barrera química que impedirá el establecimiento de otros microorganismos, a menos que estos produzcan sustancias químicas capaces de contrarrestar las producidas por los primeros.

Las bacteriocinas están ampliamente extendidas tanto en las bacterias Gram negativas (Riley and Wertz 2002b) como en las Gram positivas (Jack et al. 1995) y pueden tener un amplio espectro de acción frente a otras cepas bacterianas (Riley et al. 2003). Por ello, tanto las bacteriocinas como sus cepas productoras pueden constituir una herramienta de lucha frente a especies de bacterias patógenas para los animales con los que establezcan simbiosis (Haine 2008). De esta forma los hospedadores pueden verse favorecidos por la colonización de bacterias que, gracias al fenómeno de interferencia, impedirían la infección por otras patógenas (Soler et al. 2010).

RELACIONES ENTRE HOSPEDADOR Y BACTERIAS: ADQUISICIÓN Y TRANSMISIÓN BACTERIANA

Para entender cualquier relación simbiótica, es imprescindible conocer cómo los simbiontes son capaces de colonizar nuevos hospedadores y cómo se transmiten entre generaciones. Diferentes modos de transmisión conllevan diferentes tasas de coevolución entre las contrapartes (Dillon and Dillon 2004) y, por tanto, niveles de especificidad de las relaciones mutualistas (Douglas 1998). En general, si los microorganismos dependen totalmente de su hospedador, la transmisión de estos simbiontes suele ocurrir de padres a hijos y es conocida como transmisión vertical (Moran 2006). En la transmisión horizontal los simbiontes se reclutan directamente del ambiente o de contactos directos entre individuos no relacionados (Haine 2008).

En relaciones mutualistas en las que la transmisión de bacterias se efectúe de padres a hijos, la selección de cepas en función de los efectos positivos sobre sus hospedadores ocurrirá más rápidamente, ya que la eficacia biológica de cada cepa (o grupos genéticos de cepas) estaría asociada a la del hospedador y su descendencia, existiendo una relación estrecha entre genotipo del simbionte y el del hospedador (Currie et al. 2006, Hosokawa et al. 2006). Sin embargo, la transmisión vertical no es requisito indispensable para que se establezcan relaciones mutualistas estrechas entre micro y macroorganismos, sino que éstas son posibles incluso en el caso de que las bacterias simbiontes se adquieran periódicamente del ambiente de forma independiente en cada generación. Este es el caso de la mayoría de las bacterias digestivas de los animales, o de las bacterias fijadoras de nitrógeno (por ejemplo, *Rhizobium*) en plantas leguminosas (Heath and Tiffin 2007). La posibilidad de seleccionar simbiontes del ambiente puede ser incluso ventajosa para los hospedadores ya que aumentaría las probabilidades de encontrar el genotipo óptimo del simbionte, que puede variar dependiendo de las condiciones ambientales y de

las características del hospedador (Heath and Tiffin 2007, Scheuring and Yu 2012).

En un contexto coevolutivo más general, estas relaciones mutualistas dependientes del ambiente y de las características del hospedador jugarían un importante papel manteniendo la variabilidad genética en el simbionte con todas sus implicaciones evolutivas (Heath and Tiffin 2007). Por tanto, en el análisis del funcionamiento de un sistema mutualista es fundamental conocer el modo de transmisión de las comunidades de simbiontes que se establecen en el hospedador.

SIMBIOSIS MUTUALISTAS: BACTERIAS USADAS CONTRA BACTERIAS

Una gran variedad de animales y plantas albergan microorganismos simbiontes con los que establecen relaciones mutualistas (Douglas 1998). A pesar de ello, en pocas ocasiones han sido descubiertas simbiosis con bacterias productoras de sustancias antibióticas que proporcionen a sus hospedadores protección frente a enfermedades. Este tipo de interacciones se han descrito en algunas plantas (Saikkonen et al. 1998, Heath and Tiffin 2007) y animales. En animales se han descrito sobre todo en invertebrados como son los isópodos marinos (Lindquist et al. 2005), calamares (Barbieri et al. 2001, Heath-Heckman et al. 2013), langostas (Gil-Turnes et al. 1989, Gil-Turnes and Fenical 1992), hormigas (Currie et al. 1999), avispas (Kaltenpoth et al. 2005), áfidos (Oliver et al. 2003) o escarabajos (Cardoza et al. 2006, Scott et al. 2008). La función de los simbiontes en estos sistemas puede variar por el tipo de enemigos frente a los que defienden a sus hospedadores (hongos, bacterias o incluso parasitoides), o por el recurso del hospedador que es protegido (huevos, habitáculos o incluso cultivos de hongos).

En vertebrados, la existencia de este tipo de bacterias defensivas ha sido descrita solamente en tres ocasiones, una, en la piel de la salamandra *Hemidactylium scutatum* (Banning et al. 2008) y las otras dos en la secreción uropigial de dos especies de aves pertenecientes a dos familias diferentes del orden upupiformes: la abubilla arbórea de pico rojo africana (Law-Brown and Meyers 2003) y la abubilla europea (Soler et al. 2008). La glándula uropigial segrega una sustancia que las aves utilizan durante el acicalamiento para impregnar el plumaje para su cuidado (Jacob and Ziswiler 1982). En el caso de estas especies, la presencia de bacterias simbiontes productoras de sustancias antibióticas ayudaría a eliminar bacterias degradadoras de plumas (Burger et al. 2004, Martín-Platero et al. 2006, Ruiz-Rodríguez et al. 2009a).

GLÁNDULA Y SECRECIÓN UROPIGIAL

La glándula uropigial es la única glándula exocrina de las aves y es un complejo glandular holocrino (la célula se destruye para liberar su contenido) situado sobre la base de la cola de la mayoría de las aves. Está formada por dos lóbulos separados por un tabique (Jacob and Ziswiler 1982), y contiene además una papila nítidamente separada de los lóbulos por un istmo (Fig. INT-1).

Los lóbulos contienen el tejido secretor activo. La secreción, antes de salir al exterior, normalmente llega a una papila en la que desemboca un sistema de conductos. Esos conductos pueden llegar al extremo de la papila, abriéndose en la superficie corporal. La papila, con frecuencia, se encuentra rodeada por un penacho de plumas. Aunque la forma externa y el tamaño relativo de la glándula varían dependiendo de la especie. En casi todas las especies existen dos lóbulos y dos o más conductos que desembocan en la papila (Jacob and Ziswiler 1982).

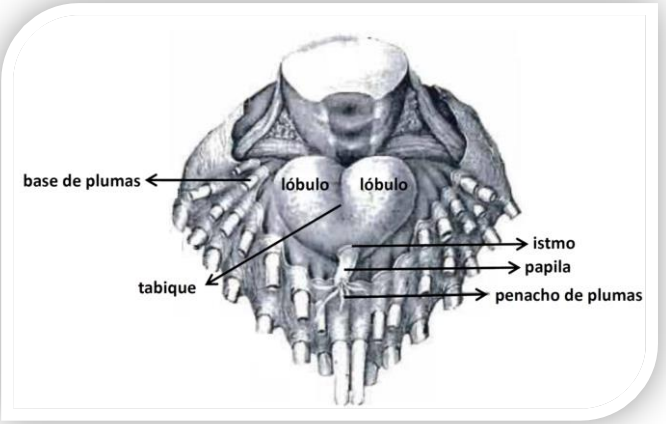

Figura. INT-1. Morfología externa de una glándula uropigial y sus principales partes (Jacob and Ziswiler 1982).

La secreción uropigial es una sustancia generalmente sebácea, hidrofóbica, espesa y de color blanquecino. Su composición es principalmente de monoésteres de alcoholes alifáticos y ácidos grasos; pero también puede contener diferentes tipos de diésteres, triésteres, glicéridos y, en menor cantidad, esteroles (por ejemplo, colesterol), e incluso ciertos hidrocarburos (por ejemplo, escualeno) como ocurre en algunos Anseriformes (Jacob and Ziswiler 1982). Se han detectado cambios en la composición química de la secreción, no solo con la época reproductora o las estaciones del año (Reneerkens et al. 2002), sino también dependiendo de la especie de ave (Jacob and Ziswiler 1982, Burger et al. 2004, Gebauer et al. 2004, Montalti et al. 2005). La secreción uropigial induce flexibilidad, impermeabilidad e higiene al plumaje. Debido a sus características antimicrobianas (Jacob et al. 1997, Bandyopadhyay and Bhattacharyya 1999, Shawkey et al. 2003), también tiene un papel en la defensa frente a microorganismos degradadores de plumas y en la prevención de infección por agentes patógenos (Pugh and Evans 1970, Bandyopadhyay and Bhattacharyya 1999, Shawkey et al. 2003). La secreción uropigial puede incluso llegar a la cáscara de los huevos y,

gracias a sus potencial antimicrobiano, contribuir a la protección del embrión frente a la contaminación (Soler et al. 2012). También puede influir en la probabilidad de depredación, ya que el olor de algunas secreciones disuade a algunos depredadores (Burger et al. 2004), o porque potenciales presas con peor calidad de secreción tendrán peores plumas (más bacterias degradadoras) y serán más fácilmente capturadas (Møller et al. 2010, 2012). También pueden ser utilizadas por sus propiedades cosméticas para colorear o maquillar sus plumas o huevos (Piersma et al. 1999, Zampiga et al. 2004, Delhey et al. 2007, Soler et al. 2014). Por todo ello, existe una gran variabilidad inter-específica en el tamaño de la glándula uropigial que, al menos en parte, se relaciona con caracteres de historia vital, probabilidad de sufrir depredaciones y, sobre todo, infecciones (Vincze et al. 2013). Todas estas capacidades, habitualmente dependen de sustancias producidas por la propia ave. Sin embargo, en abubillas y abubillas arbóreas las bacterias simbiontes ayudan a componer un arsenal de compuestos químicos bastante más amplio del que está presente en los otros grupos de aves (Burger et al. 2004, Martín-Platero et al. 2006, Martín-Vivaldi et al. 2010).

BACTERIAS PRODUCTORAS DE ANTIBIÓTICOS Y AVES: ABUBILLA EUROPEA *VS* ARBÓREA

Abubilla europea

Upupa epops

Foto: Jorge Rubio

Abubilla arbórea

Phoeniculus purpureus

Foto: Loretta Steyn

Como ya hemos mencionado, la abubilla europea y la abubilla arbórea son las dos únicas especies de aves en las que se ha detectado la presencia de bacterias simbiontes en su glándula uropigial protegiéndolas frente a patógenos y/o depredadores. En la secreción uropigial de la abubilla arbórea, perteneciente a la familia Phoeniculidae, se aisló una especie nueva de bacteria denominada *Enterococcus phoeniculicola* (Law-Brown and Meyers 2003). En la secreción de la abubilla Europea perteneciente a la familia Upupidae, se han aislado varias cepas de *Enterococcus faecalis* y especies próximas (Martín-Platero et al. 2006, Ruiz-Rodríguez et al. 2014). La secreción de la abubilla arbórea se ha comprobado que inhibe el crecimiento de varias especies de bacterias patógenas, entre ellas y con una actividad especialmente marcada *Bacillus licheniformis,* una bacteria degradadora de plumas (du Plessis et al. datos no publicados, citado en Burger et al 2004). Por otro lado, de la glándula uropigial de la abubilla europea, se han aislado varias bacterias simbiontes productoras de distintas bacteriocinas (Martín-Platero et al. 2006, Ruiz-Rodríguez et al. 2012) y, además, se ha demostrado la relación entre la presencia de bacterias y la producción de importantes sustancias antibióticas (Martín-Vivaldi et al. 2010). La mayoría de las cepas bacterianas aisladas de la secreción uropigial de la abubilla en medios tradicionales de cultivo pertenecen al género *Enterococcus* (Law-Brown and Meyers 2003, Soler et al. 2008, Martín-Vivaldi et al. 2009, Ruiz-Rodríguez et al. 2012, 2014); género que produce un bien conocido grupo de bacteriocinas (i.e. Enterocinas) con una amplia capacidad antimicrobiana (Franz et al. 2007).

La principal diferencia entre las características de las secreciones de estas especies, es que en la abubilla arbórea la comunidad bacteriana está presente en su secreción durante todo el año y se ha detectado tanto en las hembras como en machos y pollos (Law-Brown 2001). En la abubilla europea, este tipo de secreción solo aparece en las hembras y en los pollos, y solo durante su etapa de permanencia en el nido (Soler et al. 2008). Nunca ha

sido detectada en los machos ni en hembras fuera de la época de cría (Soler et al. 2008).

BACTERIAS Y LA ABUBILLA EUROPEA: CARACTERÍSTICAS ÚNICAS

Glándula y secreción uropigial

La glándula y secreción uropigial de la abubilla europea presentan cambios estacionales, puesto que sus características varían entre la época de cría y el resto del año. En temporada de reproducción, cerca del comienzo de la puesta, el tamaño de la glándula uropigial de las hembras aumenta considerablemente. Aumenta su volumen hasta 10 veces el que posee el resto del año (Martín-Vivaldi et al. 2009), y cambian considerablemente las características de su secreción; de inodora y blanca a mal oliente y marrón (Fig. INT-2). Los pollos comienzan a desarrollar su glándula a los 3-4 días de nacer, pero la producción de secreción uropigial con características similares a las de su madre ocurre días después (Martín-Vivaldi et al. 2009).

Las bacterias simbiontes de la glándula uropigial de las abubillas europeas producen sustancias antibióticas responsables de la actividad antagónica de las secreciones marrones (Martín-Platero et al. 2006, Soler et al. 2008, Ruiz-Rodríguez et al. 2009a, 2012, 2013, 2014). Esta actividad, sin embargo, no se detecta en las secreciones blancas de machos y hembras en épocas no reproductoras (Soler et al. 2008), momento en el que no se detectan bacterias (Martín-Vivaldi et al. 2009).

Figura INT-2. (1) Cambios morfológicos de la glándula uropigial de las hembras de abubilla europea en diferentes épocas del año: (1) fuera de la época de cría y (2, 3) en época de reproducción (hembras incubadoras). (3) Secreción marrón con capacidad antimicrobiana saliendo al exterior de la glándula en la fase de reproducción.

Aplicación de la secreción

La abubilla presenta un comportamiento de uso de la secreción uropigial no descrito en otras especies de aves. La particularidad es que las hembras incubadoras no utilizan su pico solo para recoger la secreción uropigial y esparcirla por su plumaje, sino que además aplican secreción directamente con el pico sobre la superficie de la cáscara de sus huevos (Martín-Vivaldi et al. 2014). Esto provoca el cambio de coloración de los huevos de azul a marrón-verdoso durante la incubación (Martín-Vivaldi et al. 2009, Soler et al. 2014) (Fig. INT-3). Dado que la secreción también es utilizada durante el acicalamiento en la placa incubadora de la hembra, los huevos reciben secreción por dos vías: directamente con el pico, e indirectamente a través de la placa durante el proceso de incubación (Martín-Vivaldi et al. 2014, Soler et al. 2014).

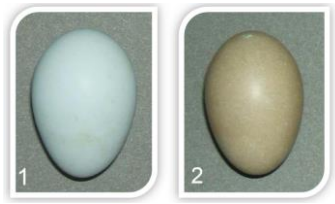

Figura. INT-3. Cambio de coloración de la cáscara de los huevos de abubilla: (1) aspecto antes y (2) después del periodo de incubación.

Superficie de los huevos

La cáscara de los huevos de las aves está formada por varias capas de naturaleza orgánica e inorgánica: la cutícula, la matriz y la membrana (Sparks 1994). Las principales funciones de la cutícula (capa más externa de la cáscara) son las de mantener la difusión gaseosa evitando la obstrucción de los poros (que conectan el interior con el exterior del huevo) por desechos del nido, reducir la probabilidad de roturas, y evitar que el agua penetre por los poros (Board and Fuller 1994). La cutícula también es la primera barrera frente a las bacterias (Board and Fuller 1994, Samiullah and Roberts 2014), y es muy variable entre especies (Kusuda et al. 2011) (Fig. INT-4).

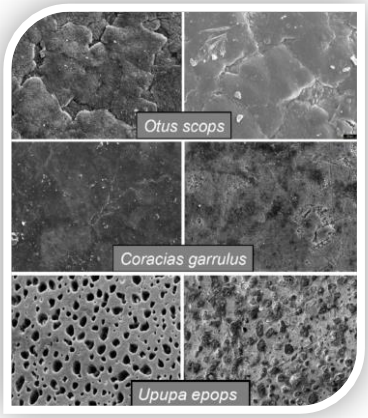

Figura INT-4. Aspecto de la superficie de las cáscaras de los huevos en tres especies de aves: *Otus scops* (autillo europeo), *Coracias garrulus* (carraca europea) y *Upupa epops* (abubilla europea). Las fotografías de la izquierda muestran la cáscara antes de la incubación y las de la derecha al final.

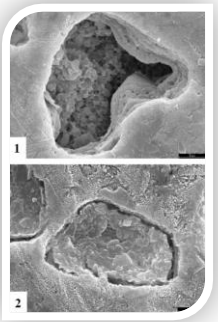

Figura INT-5. (1) Detalles de cráteres de las cáscaras de los huevos de abubilla, vacíos (huevos recién puestos) y (2) rellenos (huevos al final del periodo de incubación).

La cuticula llega incluso a no estar presente en los huevos de algunas especies como en tórtolas y palomas (Tullett 1984; Mikhailov 1997), así como en la abubilla (Martín-Vivaldi et al. 2014). La ausencia de esta protección en los huevos de la abubilla tiene que ver con la existencia en su cáscara de estructuras especializadas en la retención de la secreción uropigial que no se han descrito en ninguna otra especie de ave. Se trata de cráteres poco profundos repartidos por toda la superficie externa de la cáscara (Fig. INT-4), que no la atraviesan por completo (Fig. INT-5.1) y, aunque están vacíos en el momento de la puesta, terminan la incubación rellenos de secreción uropigial cargada de bacterias (Fig. 4, Fig. INT-5.2).

Además, se sabe de trabajos anteriores que la abundancia de estas bacterias en la cáscara se relaciona positivamente con el éxito de eclosión de los huevos (Martín-Vivaldi et al. 2014), y que la inhibición de la acción protectora de las bacteriocinas mediante su destrucción con proteasas en nidos de abubilla reduce el éxito de eclosión (Soler et al. 2008). Ambos resultados implican unos beneficios directos de las bacterias simbiontes en la eclosión que, unidos a los beneficios derivados de la efectividad de las cepas de enterococos presentes en estas secreciones en la lucha contra bacterias degradadoras de plumas (Ruiz-Rodríguez et al. 2009a), apuntan a que la relación que mantienen abubillas y bacterias es mutualista.

RELACIONES ENTRE COMUNIDADES: LA ABUBILLA Y LAS COMUNIDADES BACTERIANAS DE SUS NIDOS

El sistema formado por la abubilla y los simbiontes de su glándula uropigial es un modelo ideal para el estudio de las relaciones entre comunidades de microorganismos por varias razones. (i) El hecho de que la comunidad bacteriana de la secreción de las hembras se establezca cada año puede implicar un potencial ajuste de la comunidad bacteriana y de sus

características antimicrobianas a las del ambiente en el que se reproduce cada año (Scheuring and Yu 2012, Ruiz-Rodríguez et al. 2013, 2014). Por tanto, el estudio de relación entre comunidades bacterianas existentes en el nido (e.g., restos de reproducciones anteriores y/o bacterias digestivas en la cloaca de la hembra) podría arrojar luz sobre los mecanismos de adquisición de las bacterias simbiontes.

Además, (ii) sabemos que la secreción cargada de bacterias simbiontes se usa sobre diferentes partes del cuerpo y la superficie de los huevos, con condiciones ambientales muy diferentes. Por ello, el estudio de las relaciones entre comunidades bacterianas de las hembras en su secreción, pico, y placa incubadora, y de la cáscara del huevo, ayudarán a poner de manifiesto una posible influencia de la comunidad de la secreción en la comunidad de los huevos de esta especie. Como en otras especies, los huevos de la abubilla están en contacto con materiales del nido y, por tanto, también podríamos explorar la asociación existente entre comunidades del nido y las de las cáscaras de los huevos. (iii) También sabemos que la secreción de los pollos es muy similar a la de las hembras y, estudiar la relación entre esas comunidades en pollos que se cambien de nido poco antes del desarrollo de la secreción, nos permitirá determinar la importancia de una posible transmisión vertical de madres a pollos. Por ultimo (iv) es importante destacar que todo este complejo de interacciones entre múltiples comunidades tiene lugar en un escenario muy concreto y accesible para los investigadores: el nido.

La abubilla nidifica en agujeros naturales o artificiales, pero donde se le facilitan cajas nido de dimensiones adecuadas, las prefieren a los huecos naturales y es, por tanto, relativamente sencillo manipular y hacer seguimiento de las comunidades bacterianas. Por otra parte, esta especie se reproduce bien en cautividad, lo que permite abordar experimentos que requieren un manejo

continuado de los nidos y que no serían posibles en poblaciones silvestres porque supondrían un riesgo elevado de abandono para las puestas.

OBJETIVOS E HIPÓTESIS DE TRABAJO

El estudio de las relaciones entre las comunidades bacterianas de nidos de abubillas (material del nido, huevos, secreción uropigial, cloaca, pico y placa incubadora de hembras, y secreción uropigial de pollos) se aborda a través de los siguientes objetivos concretos:

- **Objetivo I.** Determinar las similitudes entre la comunidad bacteriana de la secreción uropigial de las hembras y las establecidas en las ubicaciones donde las hembras la aplican (pico, placa incubadora y cáscara de los huevos) (CAPÍTULO I).

- **Objetivo II.** Comprobar si existen patrones de anidamiento entre las comunidades bacterianas existentes en pico, placa y huevos, poniendo de manifiesto evidencias de un proceso de colonización desde la glándula de las hembras (CAPÍTULO II).

- **Objetivo III.** Entender el modo de adquisición de los simbiontes de la glándula uropigial de las hembras y los procesos de colonización desde los posibles ambientes que puedan funcionar como fuentes de bacterias (CAPÍTULO III).

- **Objetivo IV.** Estudiar la influencia de las comunidades bacterianas del material del nido y la cloaca de la hembra sobre la establecida en la cáscara del huevo (CAPÍTULO III).

- **Objetivo V.** Comprender los mecanismos de adquisición de la comunidad bacteriana de la glándula uropigial de los pollos, explorando el componente genético y ambiental de la misma, y

discutiendo su relación con posibles mecanismos de transmisión vertical y horizontal (CAPÍTULO IV).

Para responder a estos objetivos en esta tesis se han llevado a cabo diversos experimentos durante varias temporadas de campo con los que se ha obtenido la información necesaria para poder desarrollar los 4 capítulos que la componen.

CAPÍTULO I. Las hembras de abubilla, durante el acicalado, usan su pico para recoger la secreción de su glándula uropigial y aplicarla directamente en sus huevos y en su placa incubadora. Por primera vez se ha caracterizado por métodos moleculares la comunidad bacteriana existente en la secreción uropigial de la glándula, del pico, la placa incubadora y la superficie de los huevos. Esta caracterización ha permitido distinguir las diferentes cepas presentes y comparar la riqueza y la composición de especies de las diferentes localizaciones. Hemos podido comprobar la siguiente hipótesis:

- Las abubillas aplican la secreción uropigial con el pico durante el acicalado, y hacen llegar bacterias mutualistas desde la glándula al huevo. Una predicción a la hipótesis planteada es que las comunidades bacterianas de la glándula uropigial, pico, placa incubadora y cáscaras de los huevos, presenten taxones comunes (Objetivo I). Una segunda predicción es que existan asociaciones positivas en la presencia de taxones claves en distintas localizaciones (Objetivo I).

CAPÍTULO II. Si las comunidades de los huevos dependen de las presentes en la secreción uropigial, su transmisión desde la glándula puede ser considerada como un proceso de colonización identificable por el grado de anidamiento entre las comunidades bacterianas de los sucesivos ambientes

recorridos en el camino hacia el huevo en un marco de meta-comunidades. El enfoque de meta-comunidades usado en este capítulo no había sido utilizado con anterioridad para caracterizar las comunidades mutualistas que protegen a sus hospedadores y nos ha ayudado a explorar la hipótesis planteada:

- Si las bacterias simbióticas de la glándula uropigial están determinando las de las cáscaras de los huevos (hipótesis), la comunidad del huevo debería de estar anidada en la del pico y/o placa incubadora, y estas también encajonadas en la comunidad de la secreción (Objetivo II).

CAPÍTULO III. Se desconoce el origen de la comunidad bacteriana existente en la glándula uropigial de las abubillas durante la época de cría, por lo que conocer el modo de adquisición de la comunidad simbionte sería crucial para entender la historia evolutiva y el funcionamiento de esta simbiosis mutualista. Identificar asociaciones entre las comunidades bacterianas de la glándula uropigial de abubillas y las comunidades de posibles fuentes de simbiontes ayudaría a esclarecer estas relaciones. Los nidos situados en cavidades, frecuentemente reutilizados entre estaciones de cría por distintas especies incluida la abubilla, pueden ser fuentes importantes de bacterias. En este capítulo, mediante un diseño experimental en cautividad, modificamos la carga bacteriana de los materiales de los nidos para explorar efectos sobre las comunidades de las diferentes localizaciones del ave y así comprobar las siguientes hipótesis:

- La comunidad de la glándula puede proceder de reservorios en el cuerpo de la propia abubilla (digestivo) donde permanecería el periodo del año en que la comunidad simbionte no se detecta en la secreción (Objetivo III).

- La comunidad de la glándula puede ser adquirida del ambiente en el que las abubillas se reproducen cada primavera (material del nido) (Objetivo III).

- Las comunidades presentes en el material del nido y la cloaca pueden aportar simbiontes a la comunidad de la cáscara del huevo (Objetivo IV).

Solo las hipótesis relacionadas con el material del nido se comprueban de forma experimental.

CAPÍTULO IV. Los hospedadores pueden adquirir los simbiontes por transmisión vertical si son transferidos desde sus padres a su descendencia, u horizontal si son seleccionados del ambiente, pero algunos simbiontes microbianos pueden ser transmitidos por ambos mecanismos. Las bacterias simbiontes de la glándula uropigial de abubillas solo aparecen en hembras mientras están en el nido y en pollos. Los simbiontes, por tanto, son adquiridos por los nuevos individuos durante su desarrollo en el nido y sólo por hembras para cada evento reproductivo. Mediante un experimento de intercambio de pollos entre nidos (cross-fostering) comparamos la comunidad bacteriana de la secreción uropigial de los pollos movidos a otro nido con la de sus hermanos en el nido de origen. También la comparamos con la de sus hermanastros con los que han compartido el mismo ambiente después del intercambio. Con los resultados de esas comparaciones comprobamos las siguientes hipótesis:

- Las bacterias de la glándula uropigial de los pollos de abubillas se transmiten horizontalmente desde el medio que les rodea. Si este fuera el caso deberíamos de encontrar que las comunidades de hermanastros criados en el mismo nido se parecen más entre sí que las

de hermanos criados en distintos nidos (i.e. componente ambiental de la comunidad bacteriana) (Objetivo V).

- Las bacterias de la glándula uropigial de los pollos de abubilla se transmiten verticalmente de madres a hijos. Una predicción de la existencia de transmisión vertical es que encontremos que las comunidades de hermanos criados en distintos nidos son más similares entre sí que las de hermanastros criados en el mismo nido. Una segunda predicción es que la comunidad bacteriana de los pollos cambiados de su nido de nacimiento se parezca más a la de sus madres que a la de sus madrastras que los criaron (Objetivo V).

- Una hipótesis alternativa a la de transmisión vertical es que existan caracteres en la glándula uropigial de las hembras que favorezcan el establecimiento de determinadas cepas bacterianas presentes en el ambiente, y que estas características se heredaran de padres a hijos. Las predicciones serian similares a la hipótesis de transmisión vertical y harían falta más experimentos para poderlas diferenciar (Objetivo V).

MATERIAL Y MÉTODOS GENERALES

ESPECIE DE ESTUDIO: LA ABUBILLA *(Upupa epops)*

Foto: Rien E. van Wijk

La abubilla es un ave de la familia *Upupidae*, incluida en el orden Upupiformes (Feduccia 1975, Sibley and Ahlquist 1990) junto a abubillas arbóreas (*Phoeniculidae*), que se distribuye por Europa, Asia y África, donde ocupa zonas abiertas, de clima cálido y seco, con acceso a suelo desnudo o con vegetación rala (Barbaro et al. 2008, Schaub et al. 2010). Se trata de un ave de mediano tamaño (26-28 cm) con un diseño inconfundible en el que

destaca un patrón blanco y negro en alas y cola, un largo pico de 5-6 cm curvado ligeramente hacia abajo, y una cresta desplegable con plumas de color marrón-anaranjado y manchas negras en el extremo de las mismas (Cramp 1985). Se alimenta de insectos y pequeños vertebrados, principalmente subterráneos y sublapidícolas, que captura gracias a su largo pico largo y curvado.

La abubilla es un ave troglodita, es decir que nidifica en agujeros, pudiendo utilizar para ello distintos tipos de cavidades como huecos de árboles, graneros, tejados, montones de madera, grietas de las rocas, muros, etc. (Cramp 1998). Con bastante frecuencia, también utiliza cajas nido si son de las dimensiones adecuadas (Arlettaz et al. 2000, 2010, Martín-Vivaldi et al. 2006). Suele reutilizar los nidos entre años y entre puestas del mismo año (Martín-Vivaldi et al. 1999, Hoffmann et al. 2015) ya que es habitual que los individuos reproductores saquen adelante más de una nidada durante la misma temporada de cría. El tamaño de la puesta varía entre 5 y 8 huevos, su incubación dura alrededor de 17 días, y la realiza sólo la hembra (Martín-Vivaldi et al. 1999). A partir de esa fecha, comienza la eclosión asincrónica de los huevos, normalmente uno por día (Cramp 1998). Los polluelos nacen ciegos e indefensos por completo, su piel está cubierta de plumón y permanecen entre 25 y 30 días en el nido (Cramp 1998, Martín-Vivaldi et al. 1999).

ÁREA DE ESTUDIO Y TAREAS DE CAMPO

El trabajo de campo se llevó a cabo durante las primaveras de 2010-2011-2012 en dos poblaciones diferentes de abubillas, una silvestre y otra mantenida en cautividad. La población silvestre se localiza en la comarca de la Hoya de Guadix (37°18′N, 38°11′W), al sur de España, en Granada, donde las abubillas nidifican en cajas nido de corcho instaladas por nosotros. La otra

población, mantenida en cautividad, se distribuye en dos núcleos distintos, uno con 17 parejas situado también en la Hoya de Guadix y otro con instalaciones para 20 parejas localizado en Almería (36º50´N, 2º28´W), en la Finca Experimental "La Hoya" perteneciente a la Estación Experimental de Zonas Áridas del CSIC (Consejo Superior de Investigaciones Científicas). En ambas localidades las parejas se mantuvieron en jaulas de dimensiones 3 m x 2 m x 2 m, distribuidas de manera dispersa y aislada unas de otras, para garantizar la reproducción exitosa de las parejas sin interferencias entre ellas. En la población de cautividad las jaulas fueron visitadas a diario, y alimentadas *ad libitum* con presas vivas (grillos adultos, larvas de mosca) y con carne (corazón de ternera) rebozada en pasta de cría de huevo para aves.

En la población silvestre las cajas nido se revisaron dos veces por semana desde mediados de febrero hasta finales de julio para detectar eventos reproductivos y, asumiendo la puesta de un huevo diario, estimar la fecha del comienzo de la puesta, el tamaño de puesta y calcular la fecha aproximada de eclosión. Una vez completada la puesta, las cajas nido se visitaron dos días antes de la fecha estimada de eclosión (día 17 desde la fecha de puesta del primer huevo) y cada dos días hasta que se completara la eclosión. Durante los primeros días de la etapa de pollos, o últimos días de la etapa de huevos, se capturaban las hembras dentro de las cajas nido, se muestreaba su secreción uropigial, y, en el caso que no estuvieran anilladas, se marcaban con una única combinación de tres anillas de colores y una metálica numerada.

La tasa de visitas a los nidos después de la eclosión dependió de la temporada de estudio y de los objetivos planteados en cada una de ellas.. Aproximadamente el día 19 después de la eclosión del primer pollo, se anillaban todos los pollos del nido con anillas numeradas y se tomaban distintas medidas biométricas (peso, longitud del tarso, longitud del ala, longitud de cola), información sobre características del plumaje (número de

manchas negras en la cresta), parásitos (malófagos, dípteros (hipobóscidos y del género Carnus) y ácaros de la piel)). También tomábamos muestras de sangre para aislamiento de hematíes y plasma para su posterior análisis en el laboratorio y estimas de nivel de respuesta inmunitaria innata mediada por anticuerpos. Esta información no se ha utilizado en la presente tesis, pero se obtienen de forma rutinaria para distintos estudios.

DISEÑOS EXPERIMENTALES

Para varios de los objetivos abordados, relacionados con los capítulos III y IV, se realizaron manipulaciones experimentales. En el capítulo III se pretendía poner de manifiesto posibles fuentes de bacterias simbiontes de la secreción de las hembras de abubilla durante la incubación. Nos plantemos si la cloaca y/o el material del nido podrían ser un reservorio de bacterias, y analizamos el grado de asociación entre la comunidad de la secreción y las comunidades bacterianas del nido y de la cloaca. Además, también exploramos la relación entre las comunidades anteriores y la de las cáscaras de los huevos. Para comprobar experimentalmente el efecto de las comunidades bacterianas del material del nido, antes de que se instalaran las parejas de abubillas en las jaulas de cautividad, instalamos cajas nuevas en las jaulas experimentales y, a la mitad de ellas se rellenaron con material procedente de cajas nido de nuestra población controlada de libertad en las que el año anterior habían anidado abubillas (i.e, con un posible reservorio de bacterias simbiontes). La otra mitad de cajas nido experimentales se rellenaron con restos de hueso de aceituna prensados y machacados (material comercializado para calefacción, llamado orujillo). Este material tiene características antimicrobianas (Fleming et al. 1973) y, por lo tanto, esperábamos que tuvieran una carga bacteriana baja y, en cualquier caso, distinta a la de una cavidad anteriormente usada por abubillas. Antes de que comenzaran a reproducirse, se obtuvieron muestras del material del nido en el que se instalaron las parejas de abubillas y, una vez

que comenzó la puesta en cada nido, se muestrearon las comunidades bacterianas de la hembra (cloaca y secreción uropigial) y de los huevos (siguiendo el protocolo descrito en el apartado anterior). Exploramos el efecto del experimento en las comunidades bacterianas estudiadas, y también las relaciones entre ellas, intentando poner de manifiesto asociaciones con la comunidad de la secreción y la de las cáscaras de los huevos.

En el capítulo IV, con el fin de estudiar los mecanismos de adquisición de la comunidad bacteriana de la glándula uropigial de los pollos de abubillas, llevamos a cabo un experimento de intercambio de pollos entre nidos (cross-fostering). Los experimentos de intercambio parcial de pollos entre nidos, son una técnica empleada para estimar la influencia de los genes y del ambiente en la determinación del fenotipo de los individuos. Al comparar caracteres fenotípicos de individuos emparentados genéticamente (hermanos) que se crían en condiciones ambientales diferentes (dos nidos distintos cuidados por diferentes adultos) permiten separar sus componentes genético y ambiental (Mërila 1996). Los pollos se intercambiaban cuando el pollo de edad mayor en el nido tenía 8 días de edad. Se intercambiaban dos de los pollos de mayor peso de cada nido, alternando la secuencia de pesos de los pollos que intervenían en el experimento. De este modo, las diferencias en edades medias de los pollos de los nidos intercambiados no fueron mayores a tres días. Se intercambiaron igual número de pollos entre nidos con igual fecha de eclosión y similar tamaño de pollada. El transporte de los polluelos de un nido a otro se realizó con una incubadora portátil enchufada al mechero del coche. Para poder identificar los pollos nativos y los foráneos en cada caja nido experimental se marcaron individuamente pintando sus patas con rotuladores indelebles de diferentes colores. Posteriormente, poco antes de que los pollos abandonaran el nido, los muestreamos de nuevo (día 18 del pollo mayor).

MUESTREO DE COMUNIDADES BACTERIANAS

Los objetivos abordados en la tesis requerían la caracterización de las comunidades bacterianas existentes en la secreción uropigial de hembras y de pollos, en el material del nido, en la cáscara de los huevos y en varias localizaciones del cuerpo de la hembra: el pico, la placa incubadora, y la cloaca. Los muestreos de hembras se realizaron 14 días después de la puesta de su primer huevo, capturándolas con la mano dentro de la caja nido. Los pollos eran muestreados cuando el pollo de mayor edad de la pollada tenía 18 días. Inmediatamente después de realizar el muestreo, se introducían de nuevo en la caja nido para reducir al máximo las molestias. En cada captura usábamos guantes de látex estériles limpiados con etanol al 96% con el fin de evitar la contaminación bacteriana externa.

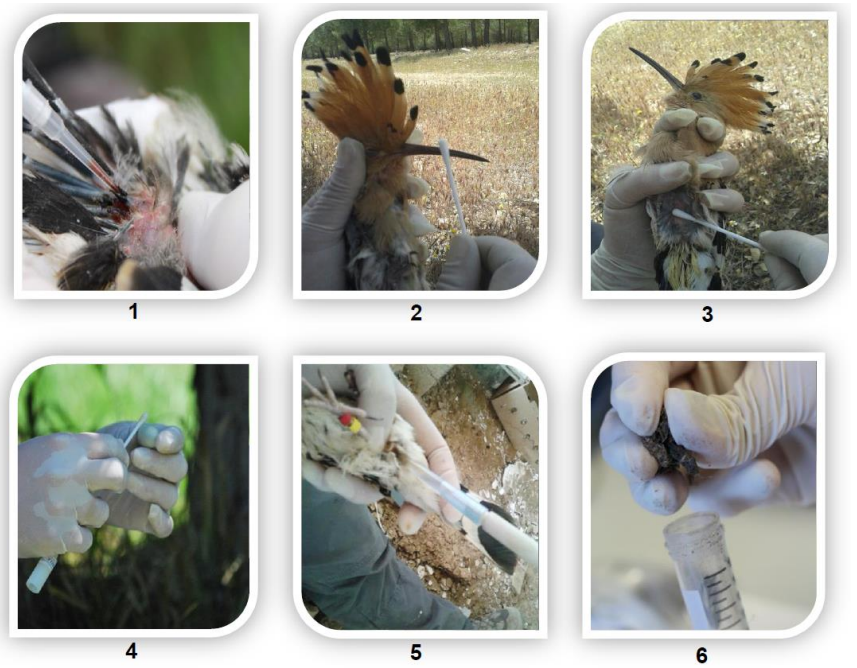

Figura MM-1. Toma de muestras de las diferentes localizaciones de estudio del ave: (1) glándula uropigial, (2) pico, (3) placa incubadora, (4) cáscaras de los huevos, (5) cloaca, (6) material del nido.

- **Secreción uropigial:** para reducir el riesgo de contaminación por bacterias externas, antes de muestrear la glándula, limpiábamos el penacho de plumas y la piel circundante a la glándula uropigial con un trozo de algodón humedecido en etanol. Una vez evaporado el alcohol, se introducía la punta estéril de una micropipeta automática (1-10 µl micropipeta [Finpipette]) en la papila de la glándula, y se llenaba las veces necesarias hasta vaciar de secreción uropigial la ampolla (Fig MM-1.1). La secreción se iba almacenando, en un tubo de microcentrífuga estéril que se mantenía en una nevera portátil a 4ºC hasta llegar al laboratorio.

- **Pico, placa incubadora y superficie de los huevos:** estas muestras se tomaron restregando la superficie de cada zona de muestreo con un hisopo (uno por muestra) humedecido ligeramente con tampón de fosfato estéril (Na_2HPO_4, NaH_2PO_4 0,1 M y 0,1 M, pH 7.2). Para las muestras de pico, restregamos el hisopo por toda la superficie (Fig MM-1.2), para la placa incubadora se muestreó la parte del vientre de la hembra que está más en contacto con los huevos (Fig. MM-1.3), y para los huevos usábamos el mismo hisopo para tomar la muestra de toda la puesta (i.e., un hisopo por nido) (Fig MM-1.4). Inmediatamente después del muestreo, los hisopos se almacenaron individualmente en tubos de microcentrífuga estériles con 1,2 ml de solución tampón esterilizada (Martín-Platero et al. 2010).

- **Cloaca:** utilizando una punta estéril, y con la ayuda de una pipeta automática (100-1000 µl micropipette Finpipette), las muestras cloacales se obtenían introduciendo 500 ml de tampón de fosfato estéril en el interior de la cloaca y repipeteando tres veces. Las muestras fueron almacenadas en tubos de microcentrífuga. Para más

información sobre esta metodología, véase Ruiz-Rodríguez et al. (2009b).

- **Material del nido:** se recogieron aproximadamente 5 gr de cantidad de material del fondo del nido del cuenco de la taza directamente con la mano (usando guantes de látex estériles), que se introducía en tubos Falcon de 15 ml relleno con tampón fosfato estéril.

Todas las muestras se mantuvieron en frío (1-3º C) en una nevera portátil hasta ser almacenadas el mismo día en el laboratorio a -20º C para los posteriores análisis moleculares.

ANÁLISIS DE LABORATORIO

La caracterización de las comunidades microbianas de las diferentes localizaciones del ave se ha realizado mediante amplificación de las secuencias de los espaciadores intergénicos (ITS) de los ARNr. Estas secuencias muestran una elevada heterogeneidad tanto en la longitud como en la secuencia de nucleótidos. Los ITS son regiones no codificantes que separan los componentes individuales de las unidades de ADN ribosómico y presentan mucho mayor polimorfismo de secuencia que las propias regiones génicas. Por lo tanto, son muy útiles como fuente de marcadores genéticos del ADN ribosómico.

La extracción de ADN se realizó con diferentes métodos dependiendo del tipo de muestra. Para muestras tomadas con hisopo (pico, placa incubadora y superficie de los huevos) se usó el método del Chelex (Martín-Platero et al. 2010) y, para aquellas muestras de secreciones uropigiales, cloacas y materiales del nido que no fueron tomadas con hisopo, se usaron diferentes kits comerciales. Las muestras del material del nido se extrajeron

con el Kit comercial PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA), mientras que para las muestras de cloaca y secreción uropigial se usó FavorPrep™ Blood Genomic DNA Extraction Kit (Favorgen Biotech Co., Ping-Tung, Taiwan). Para la extracción de ADN de las muestras de cloaca y las tomadas con hisopo centrifugamos el total de la muestra recogida y utilizamos el pellet completo. Para el material del nido usamos 0.25 gr del total de la muestra tomada y para las muestras de secreción uropigial partimos de un volumen de 5 µl. Los ITS se amplificaron usando la técnica ARISA (Automated Rybosomal Intergenic Spacer Analysis) mediante PCR (Polymerase Chain Reaction) del ADN total de la comunidad bacteriana correspondiente a la región intergénica (ITS) entre los ADNr 16S-23S. Los cebadores específicos usados para estas regiones conservadas fueron el ITSF (5´-GTCGTAACAAGGTAGCCGTA-3´) y el ITSReub (5´-GCCAAGGCATCCACC-3´) (Cardinale et al. 2004). El cebador se marcó con fluorescencia ITSReub con 6-FAM. Las amplificaciones fueron llevadas a cabo en 50 µl de volumen de reacción conteniendo H_2O ultrapura, 20 µl de 5 PRIME MasterMix (2.5x), que incluía 1.5mM $Mg(OAC)_2$, 200 µM dNTPs, 1.25 U Taq DNA polymerase, además usábamos 0.2 µM of primers y 5µl of diluted DNA 1:10. Las PCRs fueron realizadas en el termociclador Eppendorf Mastercycler Nexus Family. Los fragmentos se amplificaron bajo las siguientes condiciones: desnaturalización inicial a 94°C 2 min, seguido de 30 ciclos con una desnaturalización a 94°C 45 s, una hibridación a 52°C 45 s, and una extensión a 72°C 1 min, con una extensión final a 72°C 5 min. Los productos de PCR se diluyeron 1:10 y se calentaron en formamida para desnaturalizarlos. La longitud de los fragmentos se determinó por electroforesis capilar fluorescente automatizada en un 3130 Genetic Analyzer. Los valores de pico electroferograma se calcularon después de la interpolación con un tamaño estándar interno llamado GeneScan ™ 1200 LIZ tintado con tamaño estándar (ambos de Applied Biosystems). Estos análisis se

realizaron en el Centro de Información Científica de la Universidad de Granada.

Entre las ventajas de la técnica de ARISA destaca su reproducibilidad, alta resolución y la automatización, proporcionando un análisis muy completo de las comunidades microbianas. Por su propia naturaleza automatizada, permite además un análisis rápido de una gran cantidad de muestras (Cardinale et al. 2004). Con el programa Peak Scanner v1.0 (AppliedBiosytems) se han diferenciado las cepas bacterianas existentes en cada comunidad por el tamaño del ITS (región amplificada) en número de pares de bases (pb). Se consideran cepas diferentes las unidades taxonómicas operativas (OTUs) (Atlas, R. M. and Bartha 1997) distinguidas por el análisis de ARISA. Por cuestiones metodológicas, el tamaño estimado de la secuencia de la misma cepa bacteriana de diferentes muestras puede diferir ligeramente. Por ello se realizó un tratamiento previo de unificación de OTUs (binning) (Ramette 2009).

ANÁLISIS ESTADÍSTICOS

Las predicciones asociadas a los distintos objetivos se han estudiado utilizando los siguientes análisis estadísticos:

- **Modelos lineales generales.** Para explorar los efectos de las diferentes variables estudiadas en la población sobre las variables respuesta, usamos Modelos Generales Lineales (GLM). Las variables respuesta fueron riqueza de cepas bacterianas (nº medio de OTUs por individuo) (CAP. I, III, IV) y el índice de anidamiento entre comunidades dentro de un mismo individuo (CAP. II). Ambas variables dependientes y continuas siguieron una distribución normal, aplicando la transformación logarítmica (Kolmogorov-Smirnov) en los casos que fue necesario. Los factores explorados fueron: el tipo de

población (libertad *vs* cautividad), el año de estudio (2010-2011), el tipo de comunidad estudiado dentro del mismo individuo (glándula, pico, placa incubadora, cáscaras de los huevos, cloaca, material del nido), la zona geográfica (Almería *vs* Granada), el evento reproductivo dentro del año (número de puesta), el tipo de individuo muestreado (madre *vs* pollo), y el tratamiento experimental (experimental *vs* control). En algunos casos, se utilizaron comparaciones Post-hoc LSD para inferencias sobre valores particulares de distintos factores.

- **Análisis de frecuencias (modelos log-lineales).** Por un lado se estimaron la prevalencia de cada uno de los OTUs en cada una de las comunidades bacterianas muestreadas y se analizaron la influencia en las mismas de factores como edad, población etc. (detallados en cada capítulo). Por otro lado, para cada cepa bacteriana, estudiamos el grado de asociación entre las comunidades bacterianas muestreadas; i.e., si la probabilidad de aparición de una cepa en una comunidad de un nido (p.ej., cáscara de los huevos) es significativamente mayor (o menor) cuando también es detectada en otra comunidad (p.ej., secreción). Estimamos para ello el coeficiente de correlación de Spearman de tablas de contingencia para parejas de comunidades.

Todos estos análisis se realizaron con el programa estadísticos Statistica v.8 (StatSoft 2006).

- **Análisis multivariantes: (i) composición de comunidades.** Utilizamos PERMANOVAs para estudiar las similitudes en la composición de las diferentes comunidades bacterianas analizadas. Básicamente, estos análisis permiten utilizar matrices de similitud como variable dependiente y explorar su asociación con factores de

interés (tipo de comunidad bacteriana analizada, edad, tipo de población, etc.). La representación gráfica de esas diferencias entre comunidades se realizaron con análisis de coordenadas principales (PCoA). Para realizar estos análisis usamos datos de presencia/ausencia, y las matrices de similaridad se construyeron usando el coeficiente de Jaccard (Zuur et al. 2007). Estos análisis se han realizado con el programa PAST version 2.16, con PRIMER + V.7 y/o con diferentes paquetes de R (Hammer et al. 2001, Anderson et al. 2008, R Core Team 2014).

- **Análisis multivariantes: (ii) anidamiento de comunidades**. El grado de jerarquización de comunidades de organismos nos indica la interdependencia que existe entre ellas y se ha utilizado en ecología de islas para inferir patrones de colonización desde el continente a islas más cercanas y, de ahí a islas más alejadas. Los patrones de anidamiento son fruto de la existencia de taxones más o menos abundantes, y que difieren en su capacidad de dispersión (Ulrich et al. 2009). Por tanto, la detección de un patrón de anidamiento indicaría la existencia de gradientes ambientales y de características de los taxones que componen las comunidades relacionadas con la dispersión. En el caso de nuestras comunidades bacterianas, la detección de patrones de anidamiento entre las comunidades de la secreción, pico y cascara del huevo nos indicaría que existen bacterias en la secreción con distintas capacidades de llegar y de colonizar la cáscara de los huevos. También que la comunidad de los huevos vendría en parte determinada por la comunidad de la secreción. Los análisis de anidamiento se llevaron a cabo con el programa NeD (Nestedness for Dummies) (Strona et al. 2014) y en el capítulo II se encuentra una descripción más detallada de la metodología incluyendo el tipo de índice empleado etc.

CAPÍTULOS

Foto: Ángela M.G.

CAPÍTULO I: *Preening as a vehicle for key bacteria in hoopoes*

Autors: *Martínez-García, Ángela; Soler, Juan J.; Rodríguez-Ruano, Sonia; Martínez-Bueno, Manuel; Martín-Platero, Antonio Manuel; Juárez-García, Natalia; Martín-Vivaldi, Manuel*

Journal: ***Microbial Ecology*.** doi: 10.1007/s00248-015-0636-1

Year: 2015

CAPÍTULO II: *Nestedness of hoopoes´ bacterial communities: symbionts from the uropygial gland to the eggshell*

Autors: *Soler, Juan J.; Martínez-García Ángela; Rodríguez-Ruano, Sonia M.; Martínez Bueno, Manuel; Martín-Platero, Antonio Manuel; Peralta-Sánchez, Juan Manuel; Martín-Vivaldi, Manuel.*

Journal: ***Functional Ecology*** (submitted)

Year: 2015

CAPÍTULO III: *Nest bacterial environment affects microbiome of hoopoe eggshells, but not that of the uropygial secretion*

Autors: *Martínez-García, Ángela; Martín-Vivaldi, Manuel; Rodríguez-Ruano, Sonia M.; Peralta-Sánchez, Juan Manuel; Valdivia, Eva; Soler, Juan J.*

CAPÍTULO IV: *Nest of origin shapes the microbiome of the uropygial secretion of hoopoes.*

Autors: *Martínez-García, Ángela; Soler, Juan J.; Ruiz-Rodríguez Magdalena; Martínez-Bueno, Manuel; Arco, Laura; Rodríguez-Ruano, Sonia M.; Peralta-Sánchez, Juan Manuel; Martín-Vivaldi, Manuel.*

CAPÍTULO I

Preening as a vehicle for key bacteria in hoopoes

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ABSTRACT

Oily secretions produced in the uropygial gland of incubating female Hoopoes contain antimicrobial-producing bacteria that prevent feathers from degradation and eggs from pathogenic infection. Using the beak, females collect the uropygial gland secretion and smear it directly on the eggshells and brood patch. Thus, some bacterial strains detected in the secretion should also be present on the eggshell, beak, and brood patch. To characterize these bacterial communities, we used Automatic Ribosomal Intergenic Spacer Analysis (ARISA), which distinguishes between taxonomically different bacterial strains (i.e. different Operational Taxonomic Units [OTUs]) by the size of the sequence amplified. We identified a total of 146 different OTUs with sizes between 139 bp and 999 bp. Of these OTUs, 124 were detected in the uropygial oil, 106 on the beak surface, 97 on the brood patch, and 98 on the eggshell. The highest richness of OTUs appeared in the uropygial oil samples. Moreover, the detection of some OTUs on the beak, brood patch, and eggshells of particular nests depended on these OTUs being present in the uropygial oil of the female. These results agree with the hypothesis that symbiotic bacteria are transmitted from the uropygial gland to beak, brood patch, and eggshell surfaces, opening the possibility that the bacterial community of the secretion plays a central role in determining the communities of special hoopoe eggshell structures (i.e. crypts) that, soon after hatching, are filled with uropygial oil, thereby protecting embryos from pathogens.

INTRODUCTION

Symbiotic bacteria are fundamental for animal life. For instance, they are essential to the digestive system of animals (Nalepa 1994, Hill 1997, Ley et al. 2008), play an important role in training the immune system (Umesaki et al. 1999, Macpherson and Harris 2004), and protect the respiratory and gastroinstestinal tracks of animals from pathogenic infections (Fons et al. 2000, Dillon et al. 2005). Some bacteria establish more intimate mutualistic associations with animals harboring them in specialized glands or compartments (Barbieri et al. 2001, Currie et al. 2006), and may protect hosts or their offspring from particular parasites (Moran 2006). For example, such mutualistic associations have been described in marine isopods (Lindquist et al. 2005), shrimps and lobsters (Gil-Turnes et al. 1989, Gil-Turnes and Fenical 1992), ants (Currie et al. 1999), aphids (Oliver et al. 2003), salamanders (Banning et al. 2008), and birds (Soler et al. 2008, Martín-Vivaldi et al. 2014). The only cases of mutualism between bacteria known to produce antimicrobials and birds have been described from the uropygial gland of the European hoopoe *(Upupa epops)* (Martín-Platero et al. 2006, Soler et al. 2008) and red-billed woodhoopoe (*Phoeniculus purpureus*) (Law-Brown and Meyers 2003), two closely related species (Mayr 2008). Unlike the red-billed woodhoopoes, symbiotic bacteria of European hoopoes (hereafter hoopoes) appear only in nesting females and chicks, but apparently never in males (Soler et al. 2008). Moreover, the uropygial oil of nesting female hoopoes, which is malodorous and brown in color, is used to coat their eggs (Martín-Vivaldi et al. 2009, Soler et al. 2014). Consequently, it is quite likely that bacteria from the uropygial oil reach eggshells and help protect embryos against trans-shell bacterial contamination (Martín-Vivaldi et al. 2014). In this case, bacterial communities of the secretion and eggshells should have some bacterial strains in common.

The uropygial gland is the only exocrine gland of birds. Located dorsally at the base of the tail, it produces oily secretions that birds use for preening (i.e. to clean their feathers and make them more waterproof and flexible (Jacob and Ziswiler 1982)). Using the beak, birds collect the uropygial oil and spread it over the plumage to prevent physical abrasion and bacterial contamination of feathers (Reneerkens et al. 2002, Delhey et al. 2007, 2008, Ruiz-Rodríguez et al. 2009, Lopez-Rull et al. 2010). Incubating hoopoes smear uropygial oil on the eggshells and the brood patch (Martín-Vivaldi et al. 2014, Soler et al. 2014), and the eggshells of this species are full of crypts of different sizes and depths that end at the spongy palisade layer (i.e. they do not pierce the eggshell) and that become filled with uropygial oil and symbiotic bacteria throughout the incubation period (Martín-Vivaldi et al. 2014, Soler et al. 2014). Since hoopoes handle the uropygial oil with the beak and spread it on their body and eggs, some bacterial strains in the uropygial oil should appear in bacterial communities of the beak, brood patch, and eggshells (the two latter are in contact during incubation).

Some of the symbiotic bacteria from uropygial oil of hoopoes and their antimicrobial products are known to protect feathers (Ruiz-Rodríguez et al. 2009) and embryos (Soler et al. 2008, Martín-Vivaldi et al. 2014) from pathogenic infection. In addition to the uropygial oil, there are many more possible sources of microbes for the eggshells, brood patch, and beak, but the antimicrobial properties and the bacterial symbionts of the uropygial oil should affect microbial communities of beak, brood patch, and eggshells. Characterization of bacterial communities of uropygial oil, beak, brood patch, and eggshells and determination of the relationships among them will help us understand the effect of the symbiotic bacteria of hoopoes. In particular, the determination of the frequency at which uropygial oil bacterial strains are present on the eggshells, beak, and brood patch of female hoopoes would help to identify strains that may act outside the uropygial gland. Current

knowledge of the bacterial community from hoopoe uropygial oil comes from studies with traditional culture methods for bacterial isolation, and only a few species, most belonging to the genus *Enterococcus*, have been detected (Soler et al. 2008, Ruiz-Rodríguez et al. 2014). In the present study, using ARISA (Automatic Ribosomal Intergenic Spacer Analysis), we characterize the microbial biodiversity of bacterial communities in hoopoes and the places where the samples were taken were the uropygial gland, beak, brood patch, and egg (hereafter, sampled sites). ARISA, which has been broadly used to investigate complex symbiotic relationships among microorganisms and their hosts (Sepehri et al. 2007, Schöttner et al. 2009, Welkie et al. 2010, Porporato et al. 2013), identifies different bacterial strains as Taxonomic Operational Units (OTUs).

MATERIALS AND METHODS

Study species, study area, and general methods

The hoopoe is distributed throughout Europe, Asia, and Africa, inhabiting open woods or open areas as steppes, grasslands, pastures, semi-deserts, or field crops with scattered trees, walls or buildings providing holes for nesting and soil without tall vegetation for feeding (Rehsteiner 1996, Barbaro et al. 2008, Schaub et al. 2010). Females lay one or two clutches of 6-8 eggs over the breeding season, between February and July (Martín-Vivaldi et al. 1999). Incubation lasts 17 days and starts with the first or second egg, which results in eggs hatching asynchronously at 24 h or even greater intervals (Bussman 1950, Gupta and Ahmad 1993, Cramp 1998).

The fieldwork was performed during the breeding seasons of 2010-2011 in a wild population located in the Hoya de Guadix (37°18′N, 38°11′W), southern Spain, where hoopoes breed in crops, forests and gullies within nest-

boxes placed in trees or buildings. In 2011, hoopoes were also sampled in a captive population that descended from our wild population and that have been breeding in captivity since 2008. The captive pairs were distributed in two different subpopulations, one at facilities of the University of Granada in Hoya of Guadix (Granada) and the other at the facilities of Estación Experimental de Zonas Áridas (CSIC) in Finca Experimental la Hoya in Almería (36º50´N, 2º28´W), both in southeastern Spain. All females were ringed with both numbered and color rings for individual recognition.

A total of 117 nests were sampled (wild population in 2010, N = 31; wild population in 2011, N = 33; captivity population in 2011, N = 53). For 97 nests, we recorded information from the four sampled sites (uropygial oil, beak, brood patch, and eggshells). For the remaining 20 nests, one or more of the samples was missing. We successfully collected information on 87 females; 25 of which were sampled twice; in three cases the samples were from the first brood of two different years, and in the remaining 22 cases they were from two clutches of the same season (on five of these 22 occasions, females laid in two different nest-boxes). Five additional females were sampled three times in the same nest box and year. The 52 remaining females were sampled only once during their first breeding attempt.

Nest-boxes in the wild were visited twice per week from mid-February to the end of July to record laying date, clutch size and hatching date. Pairs of hoopoes breeding in captivity were housed in independent cages at least 3m x 2m x 2m installed in the open, scattered and isolated to avoid interactions between pairs and ensure successful breeding. Cages were visited daily, and the hoopoes had access to soil and were provided *ad libitum* access to live food (crickets, vitamin-enriched fly larvae) and meat (beef heart).

Incubating females were caught 14 days after laying the first egg within the nest-box by hand, briefly sampled and released again within the nest to reduce disturbance. For each capture, we wore new latex gloves cleaned with 96% ethanol for the whole process in order to avoid external bacterial contamination and ensure correct sampling. Before collecting samples from uropygial oil, we gently washed the circlet of feathers and skin surrounding the uropygial gland with a cotton swab dipped in ethanol to reduce the risk of contamination with external bacteria. After evaporation of the alcohol, a sterile micropipette tip (1-10 µl micropipette [Finpipette]) was inserted into the gland papilla after opening the circlet of feathers that covered the gland entrance. The papilla was pressed softly with a finger and the uropygial oil collected was transferred to a sterile microfuge tube. Afterwards, 5µl were separated and placed in a different sterile microfuge tube for the analyses.

Bacterial samples from beak, eggshells, and brood patch were collected by rubbing the complete surface with a sterile swab slightly wet with sterile phosphate buffer (Na_2HPO_4, 0.1 M and NaH_2PO_4 0.1 M, pH 7.2). These samples were individually stored in sterile microfuge tube with 1.2 ml of buffer solution (see Peralta-Sánchez et al. 2012) All samples were kept cool (i.e. 1-3º C) until being stored in the lab at -20º C the same day of sampling for further molecular analyses.

Laboratory work

Bacterial genomic DNA was extracted in two different ways depending on the sampled sites: those from the beak, brood patch, and eggshells were extracted with a specific procedure to obtain genetic material from swabs, called Chelex-based DNA isolation (Martín-Platero et al. 2010). On the other hand, the viscous uropygial oil samples were extracted with a commercial KIT (The

FavorPrep™ Blood Genomic DNA Extraction Kit (Favorgen Biotech Co., Ping-Tung, Taiwan).

Automated rRNA Intergenic Spacer Analysis (ARISA) (Fisher and Triplett 1999) was used to characterize the composition of bacterial communities inhabiting the different samples. ARISA amplifies an intergenic transcribed spacer (ITS) region between the prokaryotic 16S and 23S rDNA. This region is highly variable both in size and sequence between species, offering higher taxonomic resolution than do other techniques (Danovaro et al. 2006). The ITS was amplified using the primer pair ITSF (5´-GTCGTAACAAGGTAGCCGTA-3´) and ITSReub (5´-GCCAAGGCATCCACC-3´) (Cardinale et al. 2004). The primer ITSReub was labeled fluorescently with 6-FAM. The primer ITSReub was labeled fluorescently with 6-FAM. Amplifications were performed in 50 µl reaction volumes containing ultrapure H_2O, 20 µl of 5 PRIME MasterMix (2.5x) including 1.5mM $Mg(OAC)_2$, 200 µM dNTPs, 1.25 U Taq DNA polymerase 0.2 µM of primers and 5µl of diluted DNA 1:10. PCRs were conducted in the Eppendorf Mastercycler Nexus Family. Fragments were amplified under the following conditions: initial denaturation at 94°C 2 min, followed by 30 cycles with denaturation at 94°C 45 s, annealing at 52°C 45 s, and extension at 72°C 1 min, with a final extension at 72°C 5 min. Amplified PCR products were diluted 1:10 and denatured by heating in formamide. Fragment lengths were determined by automated fluorescent capillary electrophoresis in a 3130 Genetic Analyzer. Electropherogram peak values were calculated after interpolation with an internal size standard named GeneScan™ 1200 LIZ dye Size Standard (both Applied Biosystems). These analyses were performed in the Scientific Information Center of Granada University.

Statistical analysis

Peak Scanner 1.0 (Applied Biosystems) was used to determine fragment length in terms of base pairs of each peak that enables the identification of different bacterial strains (i.e. OTUs) within each site. For methodological reasons, the estimated length of the same bacterial strain from different samples may differ slightly. Thus, binning DNA fragment lengths from different samples is necessary before comparing bacterial communities. We did so by using available scripts in R-environment (http://cran.r-project.org/) at http://www.ecology-research.com (Ramette 2009) with a window size of 4 base pairs (bp) and a distance of two consecutive binning frames (i.e. shift) of 0.1. The algorithm rearranges the data and calculates the relative fluorescence intensity (RFI) of each peak by dividing individual peak areas by the total peak area for the respective sample. All peaks with RFI values of < 0.09% were not included in further analyses since they consisted of background peaks. Only fragments above a threshold of 50 fluorescence units and ranging between 100 and 1.000 bp were taken into consideration so as to include the maximum number of peaks while excluding background fluorescence (Ramette 2009). We used the presence-absence matrix generated after the binning process for all analyses. Molecular fingerprinting techniques are highly reproducible, robust, and have proven useful for comparative analysis of microbial community structure (Loisel et al. 2006, Bent and Forney 2008).

The number of OTUs detected per sample did not differ from a normal distribution after log-transformation (Kolmogorov-Smirnov test for continuous variables, $p > 0.15$). The random effect of individual females did not explain additional significant variance of species richness of uropygial oil ($F = 1.46$, $df = 76.32$, $p = 0.12$), beak ($F = 0.83$, $df = 79,35$, $p = 0.75$), brood patch ($F = 1.34$, $df = 80,32$, $p = 0.18$), or eggshells ($F = 1.49$, $df = 77,32$, $p =$

0.051). Thus, this random factor was not included in subsequent models. Rather, because some females were sampled during different breeding attempts, we included information on breeding attempt in the models as a fixed factor.

We used general lineal models (GLMs) to explore the effects of population (captive or wild) and study year on species richness (i.e., number of OTUs per sample) at different sampled sites (uropygial oil, beak, brood patch, and eggshells). The captive population was sampled only in 2011 and, thus, the effects of year were explored with samples from the wild population, while the effects of captivity were explored with samples from 2011. Models explaining species richness therefore included sample site, breeding attempt, and either population or year, as well as the interaction between these two factors as fixed effects. Estimating main effects in models without the interaction did not affect the results and, consequently, we report results from models that included the interaction as a fixed factor. Breeding attempt did not explain a significant proportion of variation of species richness (all models explained below, $p > 0.55$) and, thus, we removed this factor from all subsequent models. *Post hoc* comparisons (i.e. LSD Test) were used to explore differences between pairs of sampled sites depending on years and populations (captivity *vs*. wild) differences.

Information from different study years and populations were pooled to explore possible differences in bacterial prevalence in samples of the uropygial oil, beak, brood patch, and eggshells. Moreover, trying to reduce the probability of detecting significant differences among sampled sites due to rare OTUs, we considered only the most abundant, i.e. those that appeared in more than 30% of the samples in at least one site (uropygial oil, beak, brood patch or eggshells). Comparisons were performed by means of Log-linear analyses, and FDR (False Discovery Rate) method was used to adjust p-values

for multiple comparisons. To explore the within-individual association in OTU prevalence at different sampled sites, we built 2x2 contingence frequency tables with a target OTU absent or present at two different sites. Again, we considered only the most frequent OTUs (i.e. those that appeared at least in 20 different females). All the analyses were performed with STATISTICA 8 software (StatSoft 2006) except FDR adjustment, which was conducted by p-adjust function of stats package in R 3.1.2 (R Core Team 2014) (http://www.r-project.org/).

We analyzed differences in OTU composition among sampled sites taking into consideration the most abundant OTUs by one-way NPMANOVA based on the Jaccard distance with 9999 permutations using PAST Paleontological Statistics Software (Hammer et al. 2001). We used classical multidimensional scaling analysis (Multidimensional Scaling (MDS), Principal Coordinates Analysis (PCoA)) to represent graphically the relationships between bacterial communities of the uropygial oil, beak, brood patch, and eggshells. This technique represents the communities on a plot with canonical axes, where the relationship between communities shows their underlying similarity (Legendre and Legendre 1998). We used Jaccard's coefficient to estimate the similarity between bacterial communities of different sampled sites. Statistical analyses were conducted by "vegdist" function of "vegan" package, "cmdscale" function of "stats" package and function "ordiplot3d" of "vegan3d" package in R 3.1.2 (R Core Team 2014) (http://www.r-project.org/).

RESULTS

Richness of bacterial communities

We identified a total of 146 different OTUs (sizes between 139 bp and 999 bp) in the bacterial communities of hoopoe sampled sites. Of these, 124 OTUs

were detected in the uropygial oil, 106 on the beak surface, 97 on the brood patch, and 98 on the eggshell. We recorded complete information (uropygial oil, beak, brood patch, eggshells) from 97 nests with the richness of OTUs per nest (i.e. considering all sites together) ranging from 11 to 60 (Mean (SE) = 33 (1.1), Mode = 40). Within individuals, the highest richness in terms of number of detected OTUs appeared in the uropygial oil samples independently of the study year and whether samples were from wild or captive populations (Fig. 1, Table 1).

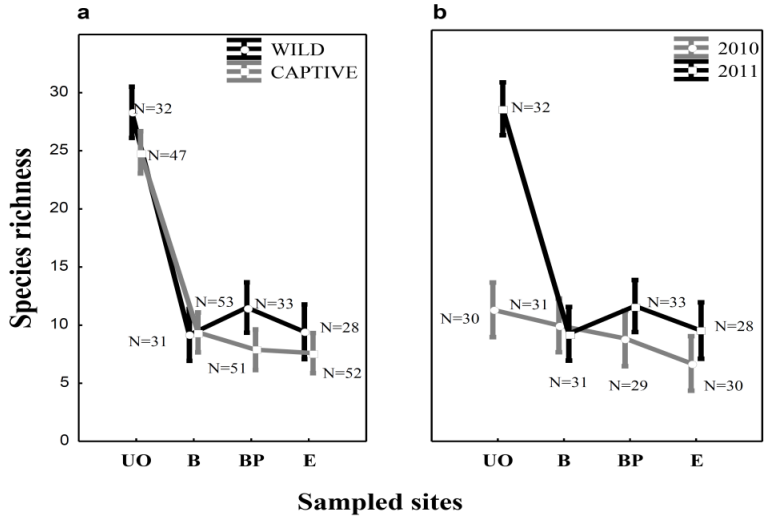

Figure. 1. Average number of OTUs (species richness) (± 95% CI) found at sampled sites from the uropygial oil (UO), beak (B), brood patch (BP), and eggshells (E) collected from wild and captive hoopoe populations during 2011 (a), and from wild populations during 2010 and 2011 (b).

Post hoc comparisons revealed that species richness of the beak differed significantly between captive and wild populations and that values for eggshells varied between years in wild populations (Table 1). Thus, study year and population (captivity or wild) had a relatively weak effect on estimated species richness and, consequently, the general effect of site in Table 1 was due to characteristics of the uropygial oil bacterial community.

Table 1. Results from General Linear Models explaining variation in species richness (i.e. number of OTUs) in relation to sampled sites [uropygial oil (UO), beak (B), brood patch (BP) and eggshells (E)], year or population [wild *vs*. captive populations (W/C)], and the interaction between site and year/population as fixed effects. *Post hoc* comparison for the effect of year or population on richness of bacterial communities of each site are also shown (normal and italic fonts show results for the wild hoopoe population sampled in 2010 and 2011 (upper sub-table), or wild and captive populations of sampled during 2011 (lower sub-table), respectively.

	Main effects				Post-hoc comparisons (LSD Test)			
	F	df	P		UO	B	BP	E
Subset of samples from the wild population (2010-2011)								
(1) Year	28.86	1.236	< **0.001**		< **0.001**	0.402	0.112	**0.019**
(2) Site	24.11	1.236	< **0.001**					
(1) x (2)	5.09	3.236	**0.002**	UO	-	< ***0.001***	< ***0.001***	< ***0.001***
				B	0.053	-	*0.632*	*0.815*
				BP	**0.029**	0.779	-	*0.482*
				E	< **0.001**	0.069	0.130	-
Subset of samples from 2011								
(1) Population								
(W/C)	7.332	1.319	**0.007**		0.227	0.804	**0.018**	0.114
(2) Site	80.89	3.319	< **0.001**					
(1) x (2)	0.775	3.319	0.509	UO	-	< ***0.001***	< ***0.001***	< ***0.001***
				B	< **0.001**	-	*0.087*	***0.049***
				BP	< **0.001**	0.583	-	*0.482*
				E	< **0.001**	0.789	0.421	-

Samples of uropygial oil, brood patch, and eggshells from the wild population were more diverse than those from captivity, but *post hoc* analyses revealed statistical significant differences only when comparing samples from the brood patch (Table1, Fig. 1a). Similarly, study year significantly affected species richness (Table 1), samples from 2011 being more diverse than those from 2010 (Fig.1b), for the uropygial oil and the eggshells (Table 1). Finally, the variation in OTUs' richness among sampled sites did not depend on population (wild vs. captivity), but on the study year. Community of the uropygial oil was more diverse than those of beak, brood patch and eggshells, especially in 2011(see *post hoc* analyses associated to the interaction terms in Table 1, Fig. 1).

Prevalence of bacterial strains in different bacterial communities

When the four sampled bacterial communities (146 OTUs) were considered, the estimated prevalence of most OTUs proved very low (mode = 0) ranging from 0.87% (OTU with 999 bp) to 85% (OTU with 183 bp). However, trying to reduce the effect of rare bacterial strains when exploring similarities between different bacterial communities, we considered 27 OTUs that appeared on at least one site in more than 30% of individuals. Length of the ITS fragment of these OTUs ranged between 139 bp and 567 bp (Fig. 2a). All the 27 OTUs selected were present in the uropygial oil samples, and three of them were exclusive to this site (sizes 139 bp, 171 bp, and 219 bp, Fig. 2). Moreover, two OTUs (sizes 307 bp and 367 bp) showed high prevalence (> 50%) in beak, brood patch, and eggshell, while being rarer (< 30%) in uropygial oil samples (Fig. 2a), suggesting that a few strains could be typical of each site.

For the OTUs considered, the prevalence in samples from the uropygial oil, beak, brood patch and eggshells significantly differed (Log-

linear analysis, χ^2 = 894.5, df = 78, p < 0.001). These differences were due mainly to higher species richness in the uropygial oil (Fig. 2a), although differences were also detected when considering the other three sampled sites (beak, brood patch, and eggs) (Log-linear analysis, χ^2 = 96.31, df = 52, p < 0.001).

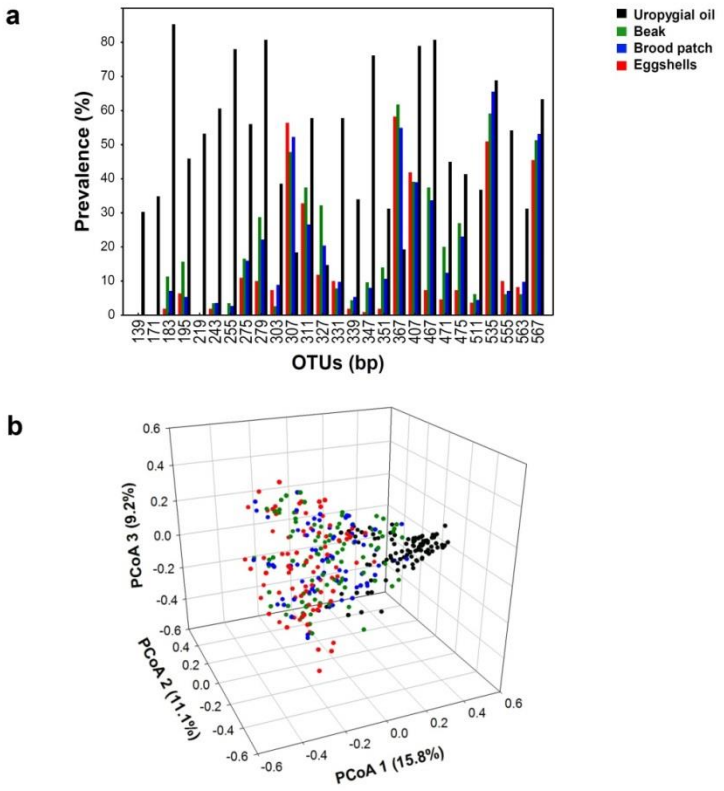

Figure. 2. Prevalence (%) of different bacterial OTUs (named by their length in base pairs (bp)) found in samples from the uropygial oil (N = 109), beak (N = 115), brood patch (N = 113), and eggshells (N = 110) of female hoopoes (a). Multidimensional space representation (PCoA) based on similarities of the most frequent bacteria communities harbored in uropygial oil, on beak, brood patch, and eggshells is also shown (total variance captured by the three axes = 36.1%) (b).

Differences in prevalence of each of the 27 most frequent OTUs revealed that only two of them (535 bp and 567 bp) did not differ significantly among sampled sites (Log-linear analysis, χ^2 > 3.04, df = 3, p > 0.34), while

the remaining 25 did (Log-linear analysis, $\chi^2 > 14.3$, df = 3, $p < 0.01$). Prevalence of two additional OTUs (311 bp and 407 bp) did not differ among samples from beak, brood patch, and eggshells ($\chi^2 > 2.68$, df = 2, $p > 0.3$, comparison for the remaining 23 OTUs, $\chi^2 > 8.2$, df = 2, $p < 0.05$) (Fig. 2a).

When exploring the association between pairs of bacterial communities connected by the preening behavior of hoopoes (i.e. uropygial oil *vs.* beak, beak *vs.* brood patch, beak *vs.* eggshells and brood patch *vs.* eggshells), we found that, in the prevalence of different OTUs, two of them appeared to be significantly related for all pairs of sampled sites. The detection of 535 bp and 567 bp in the eggshells was more likely when detected in the brood patch; detection in the latter was predicted by the detection in samples from the beak, while detecting these OTUs in beak samples were more likely when detected in samples from the uropygial oil (Fig. 3; Appendix 1). In addition, the prevalence of three more OTUs (307 bp, 367 bp, 407 bp) in samples from the beak and brood patch, brood patch, and eggshell, and from the beak and eggshells were significantly associated (Fig. 3; Appendix 1).

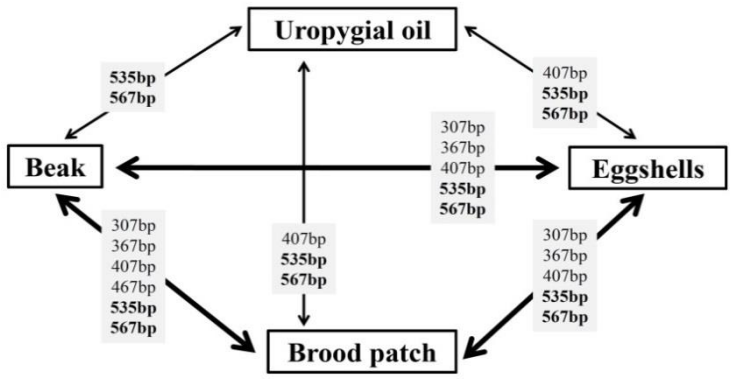

Figure. 3. Relationships between pairs of sampled sites (uropygial oil, beak, brood patch, and eggshells) within hoopoe females by the co-occurrence of particular OTUs. Broadest arrows indicate high number of OTUs with significance relation between pairs of sites. Bold fonts show OTUs with significant relations to all sampled sites.

Composition of bacterial communities

The ordination of sampled sites by PCoA was represented in three dimensions (Fig. 2b). The three axes explained 15.8%, 11.1%, and 9.2% of variance, respectively. These axes clearly separated the uropygial oil community from those of all the other sampled sites (NPMANOVA, $F > 23.39$, $p = 0.0001$; Fig. 2b). In addition, the bacterial community of the eggshell also differed from those of the beak and brood patch (NPMANOVA, $F > 23.39$, $p < 0.001$), but those of the beak and brood patch did not differ significantly (NPMANOVA, $F = 23.39$, $p = 0.266$; Fig. 2b).

DISCUSSION

In the present work, for the first time, the entire bacterial community (including non-culturable species) of hoopoe uropygial oil has been characterized by means of molecular techniques. It has previously been suggested that, because of preening, the uropygial oil including antimicrobial components (or antibiotic producing symbionts) may reach the eggshells of birds and protect the embryo from trans-shell infection (Cook et al. 2005, Soler et al. 2010, 2012, Møller et al. 2010, Martín-Vivaldi et al. 2014), but see Giraudeau et al. (2014). Thus, since incubating hoopoes harbor symbiotic bacteria in their uropygial oil inside the uropygial gland, the bacterial communities of the beak, brood patch, and eggshells may share some of their bacterial strains with the uropygial oil. In accordance with this possibility, we found that a majority of the bacteria detected in the uropygial oil were also present in the other sampled sites, and that for some bacterial strains, their detection on the beak, brood patch, and eggshells depended on their presence in the uropygial oil. There are several sources of bacteria that colonize the beak, brood patch, and eggshells of hoopoes and, thus, our results strengthen the idea that symbiotic bacteria of the uropygial gland help determine

bacterial communities of hoopoes. Below, we discuss alternative hypotheses that seek to explain such relationships between bacterial communities of hoopoes, and we speculate on possible implications on mutualistic bacteria found on the eggshells.

The community of aerobic-cultivable bacteria in hoopoe uropygial oil includes mainly few species of *Enterococcus* (Soler et al. 2008, Ruiz-Rodríguez et al. 2014). Our results suggest a more complex community of bacteria that is even more diverse than those of the beak, brood patch, and eggshells. These differences may be due to the presence of strict anaerobic bacteria that do not survive outside the uropygial gland, but also to environmental conditions such as temperature and humidity that would differentially affect bacteria on the body surfaces of animals (Ley et al. 2008, Ding and Schloss 2014). Notably, we detected a significant effect of study year on species richness but only for that of the uropygial oil, which is consistent with previous results of environment influencing the enterococci strains present in the hoopoe uropygial gland (Ruiz-Rodríguez et al. 2014) and the symbiotic bacteria found inside squid light organs (Guerrero-Ferreira et al. 2013).

Most of the 146 OTUs found were only sporadically detected, but 27 of them were present in more than 30% of the females. This pattern with a mixture of many rare species but a few highly prevalent ones is common in bacterial communities (Hulcr et al. 2012, Roggenbuck et al. 2014). Most OTUs with high prevalence (24 of 27 OTUs) were detected both inside the uropygial gland and on external sampled sites. This group includes antibiotic-producing enterococci strains (OTU307 and OTU407 for *Enterococcus faecalis*) (Martín-Platero et al. Unpublished data) that help hoopoes in their antimicrobial defense (Soler et al. 2014, Martín-Vivaldi et al. 2014, Ruiz-Rodríguez et al. 2014). These may also include other mutualistic bacteria

responsible for antibiotic production within the uropygial gland (Martín-Vivaldi et al. 2010) that would reach and be hosted in the special structures of hoopoe eggshells adapted to accumulate uropygial oil (Martín-Vivaldi et al. 2014). The eggshells of hoopoes are full of crypts (Martín-Vivaldi et al. 2014) and lack the organic cuticle that in some other species protects embryos from trans-shell infection (Sparks 1994, Wellman-Labadie et al. 2008). Crypts of eggshells became filled with uropygial oil during early incubation, and the secretion and/or symbionts that accumulate there protect embryos from pathogenic infection (Martín-Vivaldi et al. 2014). Therefore, we expected the mutualistic bacterial strains to be transmitted from the uropygial gland to the eggshells when females take uropygial oil with the beak to smear eggs directly (Martín-Vivaldi et al. 2014) or to impregnate skin and body feathers that may make contact with eggs during incubation (brood patch); i.e. an association among the microbial communities of those sites. Actually, we found that some OTUs which were more frequently detected on the beak of females were also detected in their uropygial oil as well as on the eggs when the OTUs were also detected in the brood patch or beak of females (Fig.3). These strains will be crucial in further studies such as the direction of transmission and as key mutualistic species involved in protecting hoopoes from infections outside the uropygial gland (i.e. eggshells or feathers).

Contrary to what should be expected if the uropygial secretion was the main source of bacteria for the other sampled sites, the detected associations were stronger among bill, brood patch and eggshells than those between uropygial secretion and all other sampled sites (Fig. 3). This apparently unexpected result may be explained if some strains commonly detected in the uropygial secretion were also present in nest remains and cloacal samples of hoopoes as it look to be case (Martínez-García et al. Unpublished data). Thus, we can speculate with the possibility that some of the strains in Fig. 3 could have reached bill, brood patch or eggshell of

hoopoes directly from nest materials or cloacal environment but did not successfully colonized (or were not detected in) the uropygial gland of some birds. In addition, brood patch, bill and eggshell are in close contact to each other and, consequently, the explored relationships would more easily be detected among these sites. In any case, since the bacterial community of the uropygial oil was not experimentally manipulated in this study, we cannot infer causation for the relationships detected nor can we establish the direction of the colonization. Different scenarios include the possibility of non-directional transmission among the different body parts, and differential effects of incubation on bacterial strains. Brood patch and eggshells are in contact, and brooding birds move and turn the eggs with their beak during incubation. Moreover, eggs, as well as the female's body, are in contact with the nest and, thus, bacterial communities may share some strains with nest material (Brandl et al. 2014). Moreover, it is known that incubation activity affects bacterial assemblage on the eggshells of several bird species (Shawkey et al. 2009, Brandl et al. 2014, Lee et al. 2014), for which the associations detected in only few strains could partially result from the differential effect of incubation on the communities at different sampled sites. Experimental studies manipulating bacterial presence are needed to firmly establish the causes of the composition of these communities. We hypothesize that transmission from the most diverse community of the uropygial oil of uropygial gland to beak, brood patch, and eggshells is the most likely explanation because of the antimicrobial potential of hoopoe uropygial oil (Martín-Platero et al. 2006, Martín-Vivaldi et al. 2010, Ruiz-Rodríguez et al. 2013) and also because bacteria living in the uropygial oil have to be resistant to the majority of uropygial oil antimicrobials. Therefore, a likely scenario is that the uropygial oil kills many bacteria on the beak, brood patch, and eggshell, and will therefore facilitate the colonization and growth of some of the symbiotic bacteria from the uropygial gland on hoopoe body surfaces and eggshells.

Our findings that bacterial communities living in eggshell crypts are associated with those found within the uropygial oil open the possibility that each strain has a different role, combining the antimicrobial action within glands and eggshell crypts. Further studies are necessary to fully understand the evolution of the mutualism between hoopoes and its symbionts.

Authorship statement

JJS and MM-V designed the study with considerable assistance from MM-B. AM-G and SRR performed all molecular analyses and binning with considerable assistance of AMM-P. AM-G and NJ-G performed most of the field. JJS and AMG performed all statistical analyses AM-G and MM-V wrote and first version of the manuscript with help of JJS; all other authors substantially contributed to the final version.

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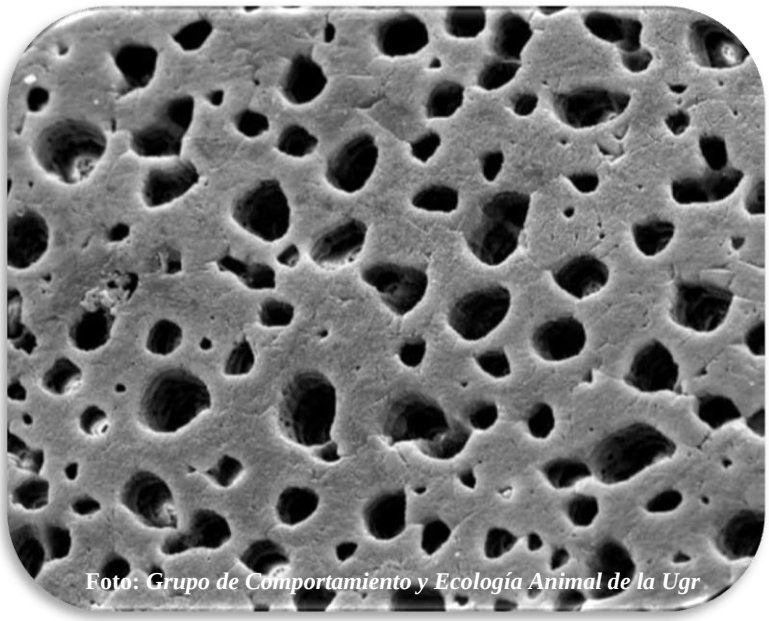
CAPÍTULO II

Nestedness of hoopoes' bacterial communities: symbionts from the uropygial gland to the eggshell



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ABSTRACT

Understanding how microbial symbionts are stablished and maintained on their hosts is a leading question that has rarely been explored from a perspective of community ecology. Acquisition or active spreading of microbial symbionts from or towards different environments by hosts can be considered a colonization process and thus be characterized by exploring nestedness of bacterial communities.

We here used this approach, and estimated nestedness of bacterial communities of European hoopoes (*Upupa epops*), a species with symbiotic bacteria in their uropygial gland that are expected to colonize eggshells where they protect embryos from pathogens.

We detected a consistent nested pattern of bacterial communities of hoopoes; from the uropygial gland to the eggshell. We also found evidence of the environment (i.e. study year and reproductive event) influencing level of nestedness of bacterial communities of hoopoes.

These results indicate that bacterial communities of eggshells and body parts of female hoopoes are conditioned by the symbiotic community in the uropygial gland, which therefore cast direct effects of bacterial symbionts restricting colonization of eggshell by pathogenic bacteria.

The meta-community approach used here allowed us inferring the direction of bacterial colonization in hoopoes, which is essential for understanding host-microbial mutualism functioning.

INTRODUCTION

Host species receive a multitude of benefits from their microbial symbionts such as enhanced nutrition and protection from enemies reviewed in Bosch and McFall-Ngai (2011). Understanding how microorganisms are established and maintained within their hosts is a leading question in biology that is being explored from different perspectives such as molecular biology, behavioral ecology, community ecology and evolutionary game theory (Bright and Bulgheresi 2010, Archie and Theis 2011, Ezenwa et al. 2012, Scheuring and Yu 2012, McFall-Ngai et al. 2013). Mainly for horizontally acquired symbionts, authors have traditionally dealt with this question by considering antagonistic characteristics of bacterial strains driving competitive exclusion within bacterial communities (Scheuring and Yu 2012).

From a community ecology perspective, acquisition of symbionts by hosts as well as beneficial effects of symbionts on hosts can be seen as results of the interaction between bacterial communities. Those communities are not isolated from each other and sometimes achieve direct contact due to their expansion or because of migration of some species or strains with particular antagonistic characteristics between them (Long and Azam 2001, Prasad et al. 2011, Long et al. 2013). Such interactions would influence functionality (i.e. antibiotic production and resistance) of bacterial communities as a whole (Cordero et al. 2012). In this scenario, hosts can acquire neutral bacteria from the surrounding communities and recruit them into the mutualistic ones, which produce antimicrobials that impede or limit proliferation of pathogenic strains at particular body locations. Thus, identifying the degree of connection among different bacterial communities of animal or plant hosts in a meta-community framework would help to understand mechanisms by which particular symbionts protect their hosts. This exercise, which is lately approached within frameworks derived from network theory, has recently

been applied to ecological studies of several mutualistic systems including those of plants and mycorrhizals (Chagnon et al. 2012, Montesinos-Navarro et al. 2012, Jacquemyn et al. 2015). However, it has largely been ignored in studies exploring mutualistic associations between bacteria and animal hosts.

Some mutualistic symbionts or their produced antimicrobial chemicals protect ants' gardens, wood galleries of beetles and embryos of shrimp, lobsters, squid, wasps, salamanders and birds from pathogenic bacteria and/or competitor fungi (Gil-Turnes et al. 1989, Barbieri et al. 1997, 2001, Currie et al. 1999, Kaltenpoth et al. 2005, Cardoza et al. 2006, Scott et al. 2008, Banning et al. 2008, Martín-Vivaldi et al. 2014). Microbial communities growing in ants' gardens or on embryos coverings in these systems, should be interconnected with, and at least partially determined by, the mutualistic ones inhabiting the body or even particular special glands in host individuals. In some cases symbiotic bacteria or their chemical products do not reach sites where they are expected to function (i.e. egg coverings) directly. This is for instance the case of hoopoes (*Upupa epops*) harboring beneficial bacteria with high antimicrobial potential in their uropygial gland (Martín-Platero et al. 2006, Soler et al. 2008, Martín-Vivaldi et al. 2010, Ruiz-Rodríguez et al. 2012, 2013, 2014). In this species, incubating females collect the uropygial secretion with the beak and, then, use it to either preen feathers (Ruiz-Rodríguez et al. 2009) including those of the belly, or to directly smear the eggshells (Martín-Vivaldi et al. 2009, 2014, Soler et al. 2014). In this way, the bacteria hosted in the female uropygial gland can reach the eggshell indirectly by means of the secretion on the beak surface, or during incubation by means of secretion impregnated on belly skin and feathers. Thus, in this case, the bacterial community on the eggshells should be conditioned by those on the beak and/or belly; which in turn should depend on those in the uropygial secretion of females.

We know that some of the bacterial strains detected in the uropygial gland are also detected on the beak, brood patch and eggshell of hoopoes, and that finding some of them in one of these sites (i.e. uropygial secretion) increases the probability of detecting the same bacteria in some other site (i.e. eggshells) of the same female (Martínez-García et al. 2015). However, because there are many sources of microbes for the eggs, brood patches and beaks other than uropygial secretion (bird's skin microflora, nest materials, food, etc.), we do not know whether similarities between microbial communities of such floras are caused by characteristics of that of the uropygial secretion. Finding evidence of such a hypothetical route of effects (i.e. hierarchized bacterial communities from the gland to the eggshells) would suggest a causal explanation for the bacterial community living on the eggshell of hoopoes and would contribute to understand functionality of symbionts.

One useful approach to detect interactions affecting the distribution pattern of multiple species across multiples localities is nestedness analysis (Almeida-Neto et al. 2008, Almeida-Neto and Ulrich 2011, Traveset et al. 2014). The nestedness concepts originated in the context of explaining insular biotas as the result of colonization by a source pool of species from the mainland. Better dispersers are expected to colonize the majority of islands, including the most distant ones, whereas poor dispersers would be restricted to the less isolated island, which result in a nested pattern of species occurrence on islands (Ulrich and Almeida-Neto 2012). Nestedness analysis detects non-random patterns of variability of species composition along environmental gradient and, in meta-communities, the presence of strong nestedness is a clear indication of coupled gradients of site environmental characteristics and species traits (Ulrich et al. 2009). Nested patterns are also common in ecological networks of interacting species (Bascompte et al. 2003, Fortuna et al. 2010) but has rarely been explored in bacterial communities

(Poisot et al. 2011, Aguirre-von-Wobeser et al. 2014). Knowledge of the nestedness of symbiotic meta-communities will help to the comprehension of the dynamic and stability of microbial communities of animals.

Here, we study nestedness characteristics of bacterial communities living in the uropygial secretion, beak, belly and eggshells of hoopoes. As we mentioned above, before establishment on the eggshells, symbiotic bacteria from the uropygial gland should be detected in the beak and/or the belly of females. Thus, if symbiotic bacteria in the uropygial gland determine bacterial communities on the beak, belly and eggshells of hoopoes, these bacterial communities should be nested from the gland to the eggshell. Finding statistical support of bacterial communities of hoopoes being nested in that direction would suggest that some of the bacteria in the beaks, belly and eggshell of hoopoes came from those in the uropygial gland, which otherwise determine bacterial community on the eggshell.

There are strong experimental evidence suggesting that environmental conditions such as resource availability, temperature, pH, etc. (Grossart et al. 2004, Long et al. 2005, 2013) may drive the outcomes of interactions among bacterial communities and, therefore, the distribution patterns of multiple bacterial strains within hosts different habitats. We here explored possible environmental effects on nestedness estimated by considering possible influences of year, breeding attempt and breeding conditions (captivity *vs* wild) of breeding hoopoes.

MATERIAL AND METHODS

The fieldwork was performed during the breeding seasons 2010-2011 in a wild population located in the Hoya de Guadix (37°18′N, 38°11′W), southern Spain, where hoopoes breed in crops, forests and gullies within nest-boxes

placed in trees or buildings (Martín-Vivaldi et al. 2009). In 2011 hoopoes were also sampled in two captive populations; one in the Hoya of Guadix in Granada, and the other one in the Finca Experimental La Hoya, in Almería (36º50´N, 2º28´W). Breeding pairs of hoopoes were housed in independent cages at least 3m x 2m x 2m installed in the open, scattered and isolated to avoid interactions among pairs. Cages had access to soil and provided with live food (crickets, vitamin-enriched fly larvae) and meat (beef heart) *ad libitum*.

The European hoopoes distribute throughout Europe, Asia and Africa. They mainly breed in open woods or open areas with scattered trees, walls or buildings where they breed in holes (Martín-Vivaldi et al. 2014). Hoopoe females usually lay two clutches of 6-8 eggs along the breeding season, between February and July (Martín-Vivaldi et al. 1999). Incubation lasts 17 days and starts with the first or second egg, followed by complete hatching asynchrony in which eggs hatch at 24 h or even greater intervals (Cramp 1985).

Bacterial sampling

Incubating females were sampled 14 days after laying the first egg. We wore new latex gloves cleaned with ethanol during the whole sampling process. Incubating females were caught from the nest box, feathers around the gland were separated and washed with ethanol to avoid contamination, and 5 µl of uropygial secretion were collected with a micropipette directly from within the uropygial gland. The secretion was introduced in a sterile 1.5 mL microfuge tube and stored at 4 ºC. Afterwards, we sampled the complete beak and belly (brood patch) of the females and the eggshells of the whole clutch. Each sample was collected by cleaning the surfaces with a sterile swab slightly wet with sterile sodium phosphate buffer (0.1 M, pH 7.1) (see Peralta-

Sánchez et al. 2012). The swabs were preserved in 1.5 mL microfuge tubes with 1.2 ml of buffer at 4 ºC. Gloves were cleaned with ethanol after collecting each of the samples and, within 12 hours after collection, all samples were stored at -20 ºC until the molecular analyses.

Laboratory work

Given the viscosity of the uropygial secretion, bacterial DNA from these samples was extracted with a commercial KIT (The FavorPrep™ Blood Genomic DNA Extraction Kit). Bacterial DNA from swabs kept in phosphate buffer was extracted by following Chelex-based DNA isolation protocol, recently proposed by Martín-Platero et al. (2010).

Bacterial communities were characterized following the well-established ARISA (Automated rRNA Intergenetic Spacer Region) protocol (Fisher and Triplett 1999). Briefly, we amplified the 16S/23S intergenic spacer region by using the primer pair ITSF and ITSReub consisted of 5´-GTCGTAACAAGGTAGCCGTA-3´ (forward primer sequence) and 5´-GCCAAGGCATCCACC-3´ labelled fluorescently with 6-FAM (reverse primer sequence) (Cardinale et al. 2004). The primer ITSReub was labelled fluorescently with 6-FAM. Amplifications were performed in 50 µl reaction volumes containing ultrapure H_2O, 20 µl of 5 PRIME MasterMix (2.5x) including 1.5mM $Mg(OAC)_2$, 200 µM dNTPs, 1.25 U Taq DNA polymerase 0.2 µM of primers and 5µl of diluted DNA 1:10. PCRs were conducted in the Eppendorf Mastercycler Nexus Family. Fragments were amplified under the following conditions: initial denaturation at 94 ºC for 2 min, followed by 30 cycles with denaturation at 94 °C for 45 s, annealing at 52 °C for 45 s and extension at 72 °C for 1 min, with a final extension at 72 ºC for 5 min. Amplified PCR products were diluted 1:10 and denatured by heating in Formamide. Fragment lengths were determined by means of automated

fluorescent capillary electrophoresis on 3130 Genetic Analyzer and electropherogram peak values were calculated after interpolation with an internal size standard named GeneScan™ 1200 LIZ dye Size Standard (both Applied Biosystems). These analyses were realized in the ING unity (Genetic Information) of CIC (Scientific Instrumentation Center) of the University of Granada.

Resulting fragment lengths were analyzed with Peak Scanner v 1.0 (Applied Biosystems) by the "Microsat G5" method. We considered peaks with values of relative fluorescence intensity higher than 0.09% and fragments above a threshold of 50 fluorescence units, ranging between 100 and 1,000 bp. Operational Taxonomic Units (OTUs) were established by calculating the best binning frame of different fragment lengths considering a window size (WS) of 3 bp and a distance between two consecutive binning frames (Sh) of 0.1. This exercise was carried out in "R" environment (http://cran.r-project.org/, R.2.12.2 (R Development Core Team 2010)) following scripts by Ramette (2009) at https://www.mpi-bremen.de/en/Software_2.html. We identified 145 OTUs that appeared with different frequencies in different hoopoes bacterial communities.

Sample sizes and statistical analyses

We collected 468 bacterial samples from 81 females, but we failed to amplify bacterial DNA of 21 samples from uropygial gland, beak, brood patch or eggshells coming from 10 females. We, thus, considered 71 individual females with complete information of bacterial communities of the secretion, beak, brood patch, and eggshells. Of these females, 20 were sampled twice, 18 during the same season (i.e. two consecutive breeding attempts) and 2 during their first breeding attempt of the two study years. Two more females were sampled three times; one of them during consecutive breeding events in

2011, and the other one was sampled once during the first breeding attempt of 2010 and twice during 2011. The remaining 49 females were only sampled during their first breeding attempt. We performed 27 samplings in 2010 and 78 in 2011.

Nestedness estimations

We organized presence absence matrices for each sampling event (individual females during a single reproductive event and study year) as including all bacterial strains (OTUs) detected in samples from secretion, beak, brood patch or eggshells. Sites of bacterial communities were in columns ordered following the expected direction of nestedness (secretion, beak, brood patch or eggshells). OTUs identities were therefore organized as rows. The network between OTUs and sampled bacterial communities was built with the "cca" method of the "plotweb" function in the library Bipartite (Dormann et al. 2008) of the statistical software R.2.12.2 (R Development Core Team 2010).

As index of nestedness, for each female and sampling event, we calculated the metric based on overlap and decreasing fill (NODF) (Almeida-Neto et al. 2008, Almeida-Neto and Ulrich 2011) as implemented in the user-friendly web interface NeD (http://purl.oclc.org/ned) by Strona et al. (2014). NODF can be estimated for columns and rows and does not depend on number of rows and columns considered (Almeida-Neto et al. 2008). NODF for columns would therefore inform of nestedness of communities among sampling places, while NODF for rows will determine whether the rarest OTUs are present in the sampling place that also have the most common (Almeida-Neto et al. 2008). NODF is dependent on the arrangement of columns and rows which allow testing hypothesis about the cause of nestedness (i.e. direction of colonization) by ordering columns and rows

according to criteria representing different hypotheses (Almeida-Neto et al. 2008, Ulrich et al. 2009, Almeida-Neto and Ulrich 2011). To test our hypothesis we thus arranged columns following the predicted colonization sequence from the uropygial gland through the beak and brood patch to the eggshell and estimated NODF of columns, while rows (OTUs identity) were arranged from those detected in all sites to those detected in only one or none. The significance of NODF values was assessed against 50 randomization using the Equiprobable row total – Fixed column totals (EF) null model that maintain observed column totals (i.e. OTUs richness) but allow row total (OTUs occurrence frequencies) to vary randomly. NeD (Strona et al. 2014) computes Z-values as

$$Z = {(NIr - \overline{NIs})}\Big/{\sigma(\overline{NIs})}$$

Where NIr is the NODF index of the matrix under examination, $\overline{NIs}$ is the average value of the set of index values for the null matrices generated by the program and $\sigma(\overline{NIs})$ is the standard deviation. Z-values > 1.64 indicate significance at P = 0.05.

We estimated NODF and Z-values with matrices built for each individual sampling considering the four kinds of bacterial communities, but also excluding community of brood patches because hoopoes may directly smear uropygial secretion on the eggshells with the beak. In all cases communities were arranged according to the hypothesis tested. We later estimated average effect size of nestedness (i.e. NODF index) of bacterial communities of hoopoes and of Z-values, and tested for possible effects of breeding attempt, study year and captivity on the strength of communities' nestedness. Statistical significance of average NODF values was inferred from

the 95% CI of Z-values (i.e. whether or not it includes the threshold value of 1.64).

Statistical models

Captive populations were only sampled in 2011 and thus the effect of study year on nestedness of bacterial communities of hoopoes was explored only considering samples from the wild population. The statistical General Linear Model (GLM) included the NODF values as dependent variable, year, breeding attempt and their interaction as fixed effects, and female identity nested within study year and its interaction with breeding attempt as random factors. Similarly, for exploring the effect of captivity on NODF values, we only used information from 2011, the only study year with samples from captive and wild nests. In this case the GLM model included breeding condition (captivity *vs* wild), breeding attempt and their interaction as fixed effects, and female identity nested within breeding condition and its interaction with breeding attempt as the random factor. GLM analyses were performed in Statistica 10.0 (StatSoft 2006).

Since the bacterial community of the secretion may access eggshells directly from the beak (e.g. Path: Secretion – Beak - Egg; hereafter SBE) or indirectly throughout the contact of beak with the brood patch (e.j., Path: Secretion – Beak - Brood Patch - Egg; hereafter SBPE) (Martín-Vivaldi et al. 2014), we performed the above analyses for NODF values estimated for SBE and SBEF bacterial meta-communities.

RESULTS

We identified 145 different OTUs in the bacterial communities of hoopoes. 124 of these OTUs were detected in the uropygial secretion, 101 in

the beak, 96 in the brood patch and 95 in the eggshell bacterial communities (Fig 1). The OTU richness observed per sampled nest ranged from 11 to 60 (N = 97, Mean (SE) = 33 (1.1), Mode = 40). In general, sampled bacterial communities of hoopoes did result in nested from the uropygial gland to the eggshells (Fig. 1) independently of considering (NODF = 53.08, SE = 1.83) or not (NODF = 48.23, SE = 2.05) the bacterial community of brood patch in the expected hierarchy of communities (Fig 2, Appendix 1).

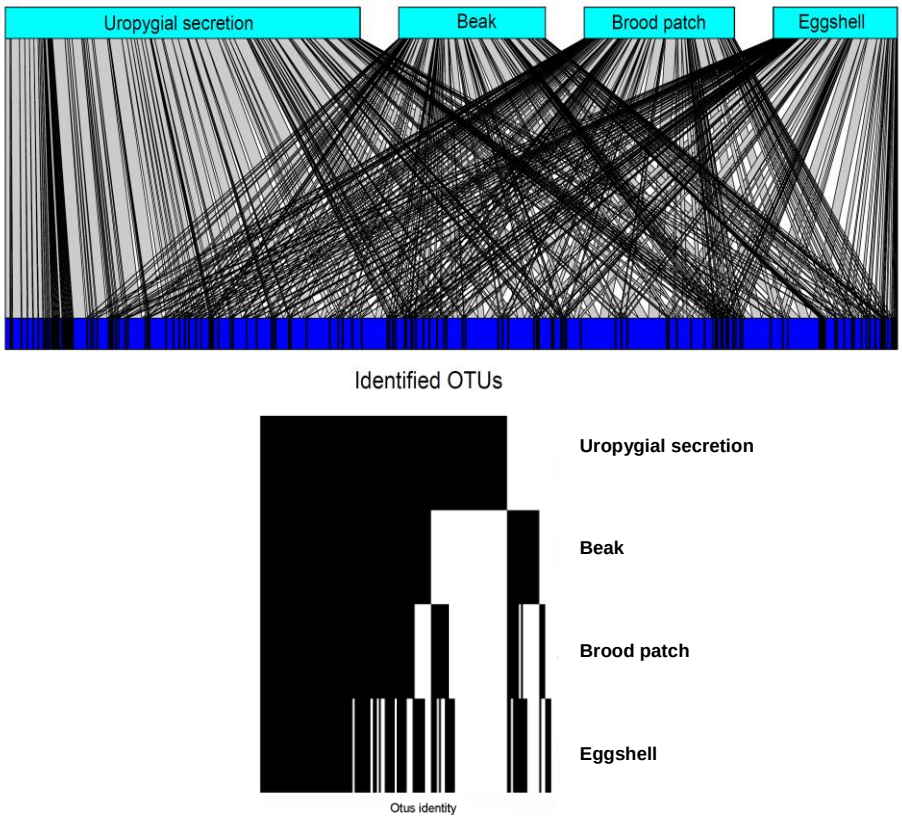

Figure 1. The network built from the matrix data showing prevalence of each OTU in bacterial samples from the uropygial secretion, beak, brood patch and eggshell of hoopoes. The width of the rectangles of sites and OTUs are proportional to their prevalence in the matrix. Similarly, width of polygons connecting OTUs and bacterial communities reflect frequencies of such interactions. OTUs were arranged minimizing the number of crossing, which facilitate visualization of the interactions. Simple heatmap showing nestedness of the matrix is also showed.

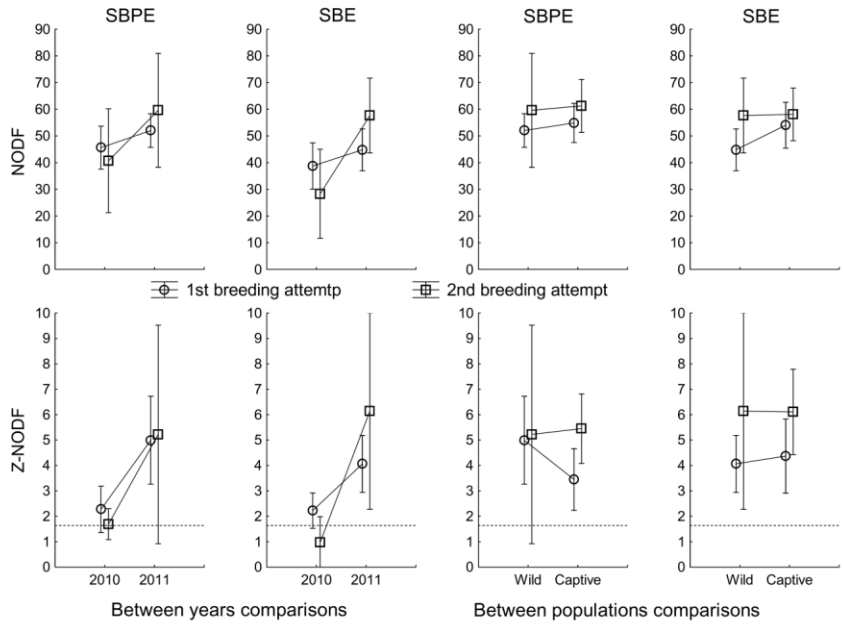

Figure 2. Mean ± 95% CI of nestedness index (NODF) of bacterial communities of uropygial secretion, beak, brood patch and eggshells (SBPE) of hoopoes, and of those of the secretion, beak and eggshell (SBE). We provide values considering all samples together, but also for different years, different breeding attempts, and for captivity and wild hoopoe populations.

Nestedness of hoopoe's bacterial communities varied significantly between study years (Fig 2, Appendix 1), being stronger in 2011 than in 2010 (Fig 2, Appendix 1). Moreover, whether or not the sampled nests were from captivity or from wild populations did not significantly affect nestedness strength (Table 1). Further, NODF estimates for second breeding attempt tended to be higher than these for first clutches (Table 1), although confidence intervals of Z-values for second breeding attempt did include the threshold value of 1.64 and therefore were not significant (Appendix 1). In addition, the effect of female identity did not reach statistical significance (Table 1) in any of the statistical models indicating that within-females variance is not significantly lower than the variance among nests of different females.

Table 1. Results from General Linear Models explaining variation in nestedness index (NODF) and of statistics reflecting the strength of nestedness of every considered matrix (Z-values) in relation to study year, whether or not the study nest was in captivity or in natural conditions and breeding attempt (i.e. first or second clutches). Since the population in captivity was only studied in a single year, the effects of year and of captivity were explored in different models. Female identity nested within year or captivity was included in the model as random factor (R) to account for the within females nest design of the data set. Interaction between the fixed (F) factors was included in the statistical model, whereas that between breeding attempt and the random factors is the error term of the model. Statistically significant effects are highlighted in bold.

		Effects of year and breeding attempt						Effect of captivity and breeding attempt					
		NODF index			Z-values			NODF index			Z values		
		df	F	P	df	F	p	df	F	p	df	F	p
GBPE													
Year (1) / Captivity (1)	**F**	**1,49.8**	**5.91**	**0.019**	**1,13.8**	**6.48**	**0.023**	1,61.9	0.42	0.520	1,64.4	0.22	0.642
Breeding attempt (2)	F	1,4.0	1.45	0.295	1,4.0	0.17	0.703	1,18.0	1.17	0.293	1,18.0	0.12	0.732
(1) x (2)	F	1,4.0	3.72	0.126	1,4.0	0.01	0.936	1,18.0	0.11	0.749	1,18.0	2.22	0.153
Female id (Year) (3)	R	46,4.0	1.82	0.301	46,4.0	0.18	0.999	47,18.0	1.12	0.407	47,18.0	0.80	0.739
(2) X (3) (error term)	R	4,0.0						18,0.0			18,0.0		
GBE													
Year (1) / Captivity (1)	**F**	**1,49.0**	**10.81**	**0.002**	**1,33.0**	**20.38**	**0.000**	1,62.1	0.73	0.395	1,62.0	0.00	0.970
Breeding attempt (2)	F	1,4.0	0.06	0.823	1,4.0	0.46	0.537	**1,18.0**	**4.51**	**0.048**	**1,18.0**	**5.45**	**0.031**
(1) x (2)	**F**	**1,4.0**	**18.67**	**0.012**	1,4.0	1.23	0.329	1,18.0	0.89	0.359	1,18.0	0.07	0.790
Female id (Year) (3)	R	46,4.0	4.48	0.076	46,4.0	0.51	0.884	47,18.0	1.10	0.431	47,18.0	1.11	0.416
(2) X (3) (Error Term)	R	4,0.0			4,0.0			18,0.0			18,0.0		

Finally, NODF estimates for groups of bacterial communities including or not that of brood patch provided similar results, but tended to be higher for the SBPE group suggesting that eggshell bacterial community was better nested in that of the brood patch than in the bacterial community of the beak. All these results suggest that the bacterial community of the hoopoe eggshell is nested within that of the brood patch and/or beak; and that these bacterial communities are nested within that of symbiotic bacteria in the uropygial gland (Fig 1). These results therefore support the hypothetical pathway of symbiotic bacteria from the uropygial gland to the egg surface.

DISCUSSION

Our results show a general nested pattern of bacterial communities of hoopoes from the uropygial gland to the eggshell, which is consistent across all individual females. The level of nestedness of hoopoe bacterial communities varied among study years and reproductive events, indicating environmental influences on the estimates. Average value of nestedness index (NODF) were almost two-folds of those obtained for recently detected nested patterns of non-native invasive floras on tropical islands. These results therefore show that bacterial communities of eggshells and body parts of female hoopoes are conditioned by the symbiotic community in the uropygial gland. Below we discuss this interpretation and the importance of estimating nestedness of bacterial communities for understanding mechanisms (i.e. structure of bacterial communities) and inferring effects (i.e. causality) of microbial symbionts on bacterial communities of hoopoes that could be extended to other mutualistic systems.

Hoopoes harbor antibiotic producing bacteria in their uropygial gland that prevent feather degradation (Ruiz-Rodríguez et al. 2009) and trans-shell contamination of embryos (Soler et al. 2008, Martín-Vivaldi et al. 2014).

Previous explorations of the bacterial community hosted in the uropygial gland of adult females and nestling hoopoes was performed by means of traditional culture techniques and mainly detected few species of the genus *Enterococcus* (Soler et al. 2008, Ruiz-Rodríguez et al. 2012, 2013, 2014). Modern molecular techniques allowed detecting a more complex bacterial community in the uropygial secretion of females with 145 different OTUs (fragment size of the 16S/23S intergenetic space region varying between 103 and 999 bp). Bacterial community of the uropygial secretion was even more diverse than those of the beak, brood patch and eggshells, see Results in Martínez-García, et al. (2015). The higher diversity of the uropygial community, together with the known antimicrobial activity of secretion (Soler et al. 2008, Martín-Vivaldi et al. 2010) and of some of their bacterial symbionts (mainly enteroccocci (OTU307 and OTU407 for *Enterococcus faecalis*, Martín-Platero et al. 2006, Ruiz-Rodríguez et al. 2012, 2013) opened the possibility of detecting evidence of nestedness among hoopoe bacterial communities at places that directly or indirectly became in contact with the uropygial secretion (i.e. beak, feathers, brood patch, and eggshells).

The antimicrobial activity of uropygial secretion will kill non-resistant bacterial strains at these sites, whereas most of the bacteria in the uropygial secretion will colonize beak, feathers, brood patch, and eggshells. Because of the detected nestedness direction, but also because of differences in environmental conditions experienced by bacteria in the uropygial gland and on other sampled sites (i.e. anaerobic vs aerobic, and chemical substrate for bacterial growth: e.g., fatty acids vs keratin), bacterial communities of the hoopoe's beak would include resistant bacteria to the antimicrobials of the uropygial secretion (migrants or residents) plus those from the uropygial secretion that were able to grow in aerobic conditions by using secretion or food remains or keratin for growth. Similarly, bacterial communities of brood

patch and eggshell would include resistant bacteria and those from the uropygial secretion that resist beak environmental conditions.

Environmental factors may also affect composition of bacterial communities. It is known for instance that resource availability and temperature influence antagonistic activity of different bacterial strains (Rypien et al. 2010, Prasad et al. 2011) and thus abiotic and biotic factors will drive the outcomes of interactions among bacterial communities. We have detected significant variation in nestedness of hoopoe bacterial communities in relation with year and breeding attempt. Thus, the distribution patterns of multiple bacterial strains within host different habitats (i.e. nestedness) may be partially explained by associated changes in environmental conditions affecting within-communities antagonistic activity. In previous work, we have also detected strong environmental effects on the acquisition of enterococci bacterial symbionts (Martín-Vivaldi et al. 2009, Ruiz-Rodríguez et al. 2014) that strengthen a possible effect of the environment determining bacterial community of the uropygial secretion and, thus, characteristics of the symbiotic relationship between hoopoes and bacteria.

An alternative non-ecological explanation worth to discussing here is the possibility that the detected nestedness was the consequence of considering dead or non-active bacteria in sites outside of the uropygial gland. Molecular techniques detect active and dead bacteria, and therefore, characterized communities may include inactive OTUs from the uropygial secretion that may be randomly dragged towards the eggshells. Simply because of random processes, bacteria from the secretion that do not resist environmental conditions at the beak of hoopoes, will also be transported and thereby detected by molecular methods in samples from the brood patch and eggshells. Obviously, because dead bacteria will pass from the beak to the eggshell but will not continue dividing, they will be detected at lower rates in

samples from the eggshells than in those from the beak or the brood patch. Besides, the ARISA approach detects just the dominant members of the community making unlikely the detection of the so called rare-biosphere or low abundant bacteria such as those in a dormant state. Although we cannot completely reject this possibility, using traditional culture techniques, we have previously found a positive relationship between densities of symbiotic bacteria (i.e. enterococci) on the eggshells and in the secretion of hoopoes (Martín-Vivaldi et al. 2014) indicating that, at least, some of the symbionts in the uropygial secretion colonize the eggshell.

The meta-community approach used here has as far as we know never been used to characterize the mutualistic communities protecting hosts, but opens new interesting possibilities to investigate effects of symbionts on hosts. From an ecological perspective, symbionts that for instance protect embryos from pathogenic infections are in fact influencing or determining bacterial communities of egg covers. The beneficial effects may be achieved by either/both, (i) directing antimicrobial chemicals from symbionts to the eggshells and/or, (ii) transporting symbionts to the egg covers where they grow and protect embryos. The former possibility would result in a microbial community of resistant microbes, whereas the later would be detected by nested patterns of communities. Interestingly, it may be even possible that some bacteria producing antibiotics within the hosts (i.e. glands) were not able to grow outside, but their chemical products facilitated colonization of eggs cover by other symbionts. We are still having very limited knowledge of mechanisms of microbial symbionts protecting hosts. The characterization of relationships (i.e. nestedness) between communities including pathogenic and/or symbiotic microorganisms, and the detection of geographical or temporal changes in species composition and/or interaction in the context of network (Poisot et al. 2011, 2012, 2014) or within classical meta-community

theory (Costello et al. 2012, Pillai et al. 2014) will definitely most likely help to understand mechanisms of host-microbial mutualism functioning.

Our results show a hierarchical relationship between the bacterial community in the uropygial gland of hoopoes and that of the eggshell, where symbionts and/or their antibiotic chemicals act preventing trans-shell bacterial colonization (Martín-Vivaldi et al. 2014). Therefore, some bacterial strains from the uropygial secretion that are present in the eggshells may directly affect pathogens joining the bacterial community. Although this possibility should be further tested, the meta-community approach used here allows us to infer the direction of bacterial colonization, which is the basic prediction of the hypothesis of symbiotic bacteria functioning on the eggshells of hoopoes. We hope these results encourage further research in this and other host-microbial mutualistic systems.

Authorship statement

JJS and MM-V designed the study with considerable assistance from MM-B. AM-G and SRR performed all molecular analyses and binning with considerable assistance of AMM-P. AM-G and JMPS performed most of the field work with assistance by MM-V and JJS. JJS performed all statistical analyses and wrote and first version with help of MM-V; all other authors substantially contributed to the final version.

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CAPÍTULO III

Nest bacterial environment affects microbiome of hoopoe eggshells, but not that of the uropygial secretion

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ABSTRACT

The study of associations between symbiotic bacterial communities of hosts and those of surrounding environments is particularly important because it would help to understand how bacterial assemblages are acquired, and how they are transmitted from one location to another location (i.e. symbiotic bacteria acquisition by hosts). European hoopoes (*Upupa epops*) (hereafter hoopoe) smear their eggshells with uropygial secretion (oily secretion produced in their uropygial gland) that harbors antibiotic producing bacteria. Trying to elucidate a possible role of nest material and gut microbiota in determining the bacterial community of the uropygial gland and the eggshells of hoopoes, we characterized bacterial communities of nest material, cloaca, uropygial gland and eggshells by the ARISA fingerprinting technique. We also manipulated nest material by adding commercial crushed olive stones with scarce bacteria and antimicrobial properties, and explored its effects on microbiomes of the uropigial secretion and of the eggshells. Our experimental modification of nest material of nest-boxes occupied by hoopoes did not influence the microbiome of the uropygial secretion of females, but influenced that of the eggshells. This is the first experimental evidence indicating that nest material influences that bacterial community of the eggshells and, therefore, probability of embryo infection. Moreover, we found consistent differences among the bacterial communities studied, that of the uropygial secretion being the most diverse. Some of the bacterial strains detected in the secretion were also in the bacterial communities of the nest material and of the female cloaca. However, occurrence of these strains in the uropygial secretion was not associated with that in samples from nest material or cloaca suggesting that the gut microbiota are not sources of symbiotic bacteria for the first. We discuss possible scenarios that reconcile our results with the possible role of nest environments of hoopoes as reservoirs of symbiotic bacteria.

INTRODUCTION

Exploring the influence of bacteria on animal health and evolution is nowadays of central importance for life sciences (McFall-Ngai et al. 2013). The most known impacts of bacteria are the detrimental effects of some strains, but animals may also benefit from others. Most bacteria produce defensive compounds that inhibit antagonistic competing microorganisms (Riley and Wertz 2002), and some of these may be host pathogens (Soler et al. 2010). Apart from the effects of individual bacterial strains, the diversity and structure of the complete bacterial communities may also have important implications for health of hosts (Clemente et al. 2012). Thus, not only exploring the effects of particular bacteria on animal fitness, but also characterizing bacterial communities related to animal life style is of prime importance to understand evolutionary and ecological associations between animals and bacteria.

Detecting associations between symbiotic bacterial communities of hosts and those of surrounding environments is particularly exciting because it would help to understand how bacterial assemblages are acquired and how they are transmitted from one location to another location (i.e. bacteria acquisition) (Brandl et al. 2014). Similar bacterial strains may have beneficial or detrimental effects for hosts depending on the bacterial community (i.e., location) to which they are incorporated. Pathogenic bacteria for embryos may for instance have no detrimental effects when included within the gut microbiota of animals, but those on eggshells would increase probability of embryo infection (Barrow 1994, Bruce and Drysdale 1994). Similarly, bacteria that have negative effects for hosts in some body locations (i.e. feather degrading bacteria in adult's primary feathers (Shawkey et al. 2003)) may have beneficial effects when included in some other bacterial communities (i.e. nest lining feathers or on eggshells (Soler et al. 2010,

Peralta-Sanchez et al. 2010)). Thus, exploring the associations between bacterial communities in a broad ecological framework allows study colonization by microorganisms of hosts (infection or symbiont acquisition). This approach is essential not only for understanding of the ecological processes for pathogens contamination of hosts, but also to know how beneficial mutualistic bacteria are acquired, and their effects on host bacterial communities (McFall-Ngai et al. 2013). Despite the importance of that approach it has rarely been used in these lines of research.

The avian nest is an appropriate environment for performing such studies because, among other reasons, the spatial focus of the study is easily delimited. The bacterial communities inhabiting the nest environment of wild birds can be easily defined: those in the nest cup, on the eggshells, on the skin of adult and nestlings, and in the gut. The close spatial interactions of these components of the system should cause most of these communities being related to each other, and the strength of the association would depend on particularities of bacterial communities and on factors affecting transmission among locations (Brandl et al. 2014). Moreover, nest materials (Peralta-Sánchez et al. 2014), as well as bird physiological (i.e. feces (Ibáñez-Álamo et al. 2014)) and behavioral (i.e. feeding (Møller et al. 2015)) activities are important sources of bacteria in avian nest environments. Several climatic conditions (i.e., temperature and humidity (Ruiz-de-Castañeda et al. 2011, Horrocks et al. 2014)), life history characteristics (Peralta-Sánchez et al. 2012), some behavioral defensive traits (i.e. incubation (Cook et al. 2005a, Shawkey et al. 2009, Lee et al. 2014), or the use of material with antimicrobials (Clark 1991, Mennerat et al. 2009, Peralta-Sanchez et al. 2014)) are also known as determinants of nest bacterial communities and, therefore, would affect relationships among nest microbiomes.

As far as we know, only Brandl et al. (2014) have explored bacterial assemblage present on multiple nest components within the same nests of reed warblers (*Acrocephalus scirpaceus*). They paid special attention to the effects of incubation determining the relationship between bacterial communities of the nest cup and eggshells. They convincingly showed that some bacteria are transmitted from one to another nest location (nest material to the eggshell), and that incubation affects the microbiome on the eggshell (see also, Shawkey et al. 2009, Lee et al. 2014). This research was particularly interested in groups of pathogenic bacteria, although this approach should also be useful for exploring acquisition and transmission of beneficial bacteria among communities within the nest of birds.

Here, we characterized the bacterial communities inhabiting nests of hoopoes (*Upupa epops*). Nesting females and chicks of this species harbor antibiotic producing bacteria in their uropygial gland (Martín-Platero et al. 2006, Martín-Vivaldi et al. 2010, Ruiz-Rodríguez et al. 2013), and females paint their eggs with uropygial secretion containing symbiotic bacteria (Soler et al. 2014). Hoopoes do not build nests, but use cavities that frequently had been used by conspecifics or other bird species for breeding. Thus, nest materials from old nests or remains (feathers, feces, etc.) from previous reproduction events (hereafter, nest materials) are common in hoopoe nests, which may be a source of microorganisms for bacterial communities of new active nests.

Our aims here are two folds. The first is to explore the associations between the bacterial community of the uropygial secretion and those of nest materials and cloaca. A positive association between these communities would suggest that nest-material remains and/or gut microbiota are sources of symbiotic bacteria for the uropygial gland. The second aim is to study the relationship between eggshell bacterial community and those of the nest

material and cloaca. In the case of nest material we will test its contribution in an experiment comparing the effect of materials with and without typical hoopoe nest bacterial communities. We already know that some of the bacteria of the uropygial secretion are transmitted to the eggshell (Soler et al. Unpublished data). Finding evidence of the associations explored here would suggest that nests remains and/or gut microbiota also contribute to the eggshell bacterial community of hoopoes.

MATERIAL AND METHODS

Study specie and study area

The hoopoe is distributed throughout Europe, Asia and Africa, inhabiting open woods or open areas as steppes, grasslands, pastures, semi-deserts, or crops whenever they have scattered trees, walls or buildings providing holes for nesting and soil without tall vegetation for feeding (Rehsteiner 1996, Barbaro et al. 2008, Schaub et al. 2010). The uropygial secretion of hoopoe females but not that of males experiences apparent seasonal changes (Martín-Vivaldi et al. 2009). Uropygial secretion of nesting females and nestlings are malodourous, of brown-greener coloration, and contains a large amount of bacterial symbionts that produce antimicrobial substances (Martín-Platero et al. 2006, Soler et al. 2008, Martín-Vivaldi et al. 2009, 2010, Ruiz-Rodríguez et al. 2012, 2013). Female hoopoes besmear the eggshells with the antimicrobial secretion which accumulates in special crypts (Martín-Vivaldi et al. 2014), turning the egg color from pale-blue to brown greenish (Soler et al. 2008, Martín-Vivaldi et al. 2014) and protecting the embryo from trans-shell pathogenic infection (Soler et al. 2008, Martín-Vivaldi et al. 2014). Females lay one or two clutches of 6-8 eggs along the breeding season, between February and July (Martín-Vivaldi et al. 1999). Incubation lasts 17

days and starts with the first or second egg, which results in eggs hatching asynchronously at 24 h or even greater intervals (Cramp 1998).

The study was performed in 2011 in a population maintained in captivity since 2008. The captive pairs were distributed in two localities with appropriate facilities; one in the Hoya of Guadix (37°18′N, 38°11′W, Granada province, southern Spain) and the other in Almería (36º50´N, 2º28´W, Finca Experimental La Hoya, EEZA-CSIC). All females were ringed with numbered aluminum and plastic color rings for individual recognition. Breeding pairs were housed in independent cages at least 3m x 2m x 2m installed in the open, scattered and isolated to avoid interactions between pairs and ensure successful breeding. Cages had access to soil and provided with live food (crickets, vitamin-enriched fly larvae and meat (beef heart)) *ad libitum*, and were visited daily from mid-February to the end of July.

Experimental design and sampling

Before reproduction started, we collected nest material from 15 nest-boxes that hoopoes used for reproduction during 2010 in the Guadix wild population. The material from each nest-box was individually stored in labeled bags at room temperature until their use in our captive population. Experimental breeding pairs were randomly assigned to control and experimental treatments. All nest-boxes used in cages had never been installed or used by any bird species for breeding. Each control pair received a nest-box filled with a 3 cm layer of material collected from one nest box of our wild population. Experimental pairs on the contrary received a nest-box filled with a similar amount of commercial crushed olive stones. This kind of material is not expected to harbor the typical bacterial community living within hoopoe nests. Moreover, it is known that olives contain substances (i.e. oleuropein) with high antimicrobial activity (Fleming et al. 1973, Aziz et al.

1998, Cruz-Peragón et al. 2006). To test for the antimicrobial properties of the experimental nest material, we performed antagonistic tests with crushed olive stones against several indicator bacteria. We found high grow inhibition capacity for most tested bacterial strains (Appendix 1). Thus, we expected that the microbial community present in experimental nest material greatly differ from that of control nests.

Control and experimental nest-boxes were fastened to cages one day before the experimental hoopoe pairs were released inside. The experiment involved 30 nest boxes (15 for each treatment), but for six of them the genetic analyses failed for at least one of the considered communities (nest material, secretion, cloaca and/or eggshells). Since we were interested in within nest association of bacterial communities, we only considered the 24 nests (12 of each experimental treatment) with complete information for statistical analyses

Nest material was sampled the same day that it was introduced in nest-boxes (day 0). We collected samples of nest materials by hand with sterile latex gloves and stored them in Falcon tubes with 15 ml of sterile sodium-disodium phosphate buffer (Na_2HPO_4 0.1 M and NaH_2PO_4 0.1 M, pH 7.1). Bacterial communities of the uropygial gland, the digestive tract of females, and the eggshells were sampled 14 days after the first egg was laid. Incubating females were caught by hand and after sampling bacterial communities were released again within the nest box to reduce disturbance. We wore new sterile latex gloves cleaned with 96% ethanol for the whole process of sampling to avoid bacterial contamination among nests.

Before sampling the uropygial gland, the circlet and surrounding skin of the uropygial gland were softly washed with a cotton swab soaked in 96% ethanol to reduce the risk of contamination of the secretion with external

bacteria. After evaporation of the alcohol, a sterile micropipette tip (1-10 µl micropipette Finpipette) was introduced in the gland papilla after opening the circlet of feathers that cover the gland entrance. The papilla was pressed softly with a finger to collect the entire secretion available. The secretion was transferred to a sterile microfuge tube and, afterwards, 5 µl were separated in a different sterile microfuge tube for the analyses. The gut microbiota was sampled by introducing and repipetting three times 500 µl of sterile phosphate buffer in the cloaca. We used sterile tips and automatic pipettes (100-1000 µl micropipette Finpipette). Samples were stored in sterile microfuge tubes (for further information on this methodology see Ruiz-Rodríguez et al. (2009)). Bacterial samples of eggshells of the entire clutch were collected by completely cleaning the surfaces of all eggs with the same sterile swab slightly wet with sterile phosphate buffer. These samples were individually stored in sterile Eppendorf tubes within 1.2 ml of buffer solution (see Peralta-Sánchez et al. 2012). All samples were kept cool (i.e. 1-3º C) until storing them in the lab at -20º C in the same day of sampling for further molecular analyses.

Laboratory work

We used different bacterial genomic DNA extraction protocols depending on the type of sample. DNA from nest material samples was extracted from 0.25g of homogenized nest material per sample using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA), following the manufacturer's instructions. The viscous secretions of the uropygial gland and digestive tract samples were extracted with FavorPrep™ Blood Genomic DNA Extraction Kit (Favorgen Biotech Co., Ping-Tung, Taiwan). Finally, eggshells DNA was extracted with a specific procedure for obtaining genetic material from swaps named Chelex-based DNA isolation (Martín-Platero et al. 2010).

Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Fisher and Triplett 1999) was used to characterize the composition of bacterial communities. ARISA amplifies an intergenic transcribed spacer (ITS) region between the prokaryotic 16S and 23S rDNA. This region is highly variable both in size and sequence between species, offering higher taxonomic resolution than other techniques (Danovaro et al. 2006). The ITS was amplified using the primer pair ITSF (5´-GTCGTAACAAGGTAGCCGTA-3´) and ITSReub (5´-GCCAAGGCATCCACC-3´) (Cardinale et al. 2004). The primer ITSReub was labelled fluorescently with 6-FAM. Amplifications were performed in 50 µl reaction volumes containing ultrapure H_2O, 20 µl of 5 PRIME MasterMix (2.5x) including 1.5mM $Mg(OAC)_2$, 200 µM dNTPs, 1.25 U Taq DNA polymerase 0.2 µM of primers and 5µl of diluted DNA 1:10. PCRs were carried out in an Eppendorf Mastercycler Nexus Family. Fragments were amplified under the following conditions: initial denaturation at 94 °C 2 min, followed by 30 cycles with denaturation at 94 °C 45 s, annealing at 52 °C 45 s and extension at 72 °C 1 min, with a final extension at 72 °C 5 min. Amplified PCR products were diluted 1:10 and denatured by heating in formamide. Fragment lengths were determined by mean of automated fluorescent capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystems). Electropherogram peak values were calculated after interpolation with an internal size standard named GeneScan™ 1200 LIZ dye Size Standard (Applied Biosystems). These analyses were performed at the Scientific Instrumentation Center of the University of Granada.

Statistical analysis

Peak Scanner v1.0 (Applied Biosystems) was used to determine fragment lengths identifying different bacterial Operational Taxonomic Units (hereafter, OTUs) within each sample. Scripts in R-environment [http://cran.r-

project.org/]) available at http://www.ecology-research.com, were used for binning DNA fragment lengths from different samples. Binning exercise was performed by stablishing a window size of 4 base pairs (bp) and a distance of two consecutive binning frames (i.e. shift) of 0.1. We only considered peaks with values of relative intensity of fluorescence larger than 0.09% and fragments above a threshold of 50 fluorescence units that ranged between 100 and 1,000 bp (Ramette 2009). We used the presence-absence matrix generated after binning process for the analyses. Molecular fingerprinting techniques are highly reproducible, robust, and have been proven useful for comparative analysis of microbial community structure (Loisel et al. 2006, Bent and Forney 2008).

Because of the possible differential experimental effects on microbial communities of each sampled site, they were described (i.e. richness, OTU's prevalence, nestedness) and compared to each other by only using control nests. We used general linear models (GLMs) to explore the effects of sampled sites (nest material, cloaca and uropygial gland and eggshells) and experimental treatments (experimental *vs* control) on bacterial richness (i.e. number of OTUs per sample) and nestedness.

We are particularly interested in detecting sources (i.e. nest materials or gut microbiota) of symbiotic bacteria that install in the uropygial gland of hoopoes. Hence, for these analyses we only considered OTUs that were detected in the bacterial community of the uropygial secretion. In addition, to explore the within individual association in OTUs prevalence among different sampled sites, we built 2x2 contingence frequency tables for every OTU and pair of sampled sites. Nestedness estimations for each sampling event were explored by calculating the NODF index (nestedness based on overlap and decreasing fill) (Almeida-Neto et al. 2008, Almeida-Neto and Ulrich 2011) with web the interface NeD (http://purl.oclc.org/ned) developed by Strona et

al. (2014). We organized matrices for each sampling event (individual females during a single reproductive event) as including all bacterial strains (OTUs) detected in the sampled sites. Sampled sites of bacterial communities were in columns ordered following the expected direction of nestedness, and OTUs identities were therefore organized as rows (Almeida-Neto et al. 2008, Almeida-Neto and Ulrich 2011, Traveset et al. 2014). To test for the possible origins of bacteria in the uropygial gland, we explored whether the bacterial community of the uropygial secretion was nested within those of nest materials, and/or cloaca. We have previously shown that the bacterial community of the eggshells of hoopoes is nested within those of the brood patch and/or beak, and those are nested within the bacterial community of the uropygial secretion of hoopoes (Soler et al. Unpublished data). Here, to explore the possibility that the community on the eggshells also receive strains from nest materials and/or the cloaca, we tested for its nestedness within those sampled sites. Briefly, we estimated NODF for columns for each of the study nest, which inform of nestedness of communities among sampling places (Almeida-Neto et al. 2008). Z-values estimated of NODF indicate the existence of nested if value is higher than 1.64 ($p < 0.05$) (Strona et al. 2014). We later estimated average effect size of nestedness (i.e. NODF index) of bacterial communities of hoopoes and of Z-values, and tested for possible influences of study area and treatments on the strength of communities' nestedness. Statistical significance of average NODF values was inferred from the 95% CI of Z-values (i.e. whether or not it includes the threshold value of 1.64). The statistical General Linear Model (GLM) included the NODF values as dependent variable and geographic area, experimental treatment and the interaction as fixed effects. Since the performed experiment modified the bacterial community of nest materials, nestedness of communities including this microbiome were estimated only for control nests. Moreover, since microbiome of nests material of hoopoe nests was experimentally manipulated, we did not compare NODF values for pairs

of bacterial communities that included nest material of experimental and control nests.

Finally, for multivariate analysis of communities directed to explore similarities or differences among bacterial communities of hoopoe nests we did not consider rare OTUs (those that appeared in less than 4 samples in any of the bacterial communities considered) or nests with experimental materials. These analyses were performed by means of NPMANOVAs based of similarity matrices of Jaccard´s distance (Zuur et al. 2007) and 9999 permutations. Similarities among bacterial communities were shown in three dimensional figures from Principal Coordinates Analysis (PCO, PCoA)) (Legendre and Legendre 1998). Both analyses were conducted by PAST Paleontological Statistics Software (Hammer et al. 2001).

Study area did not explain significant proportion of variance of any of the analyzed dependent factor ($P > 0.1$) and was therefore not considered in the final statistical models.

RESULTS

Microbiomes of nest material, gut, uropygial gland, and eggshell of hoopoes

We identified a total of 142 different OTUs (sizes between 100 bp and 819 bp); 101 of them were present in the uropygial gland, 58 in the gut microbiome, 91 in material of control nests, and 65 on the eggshell. On average, the number of OTUs detected per sampled nest differed among sampled sites (only control nests considered; $F = 10.82$, $df = 3, 44$, $p < 0.0001$). The richest community was that of the uropygial secretion followed by those of the nest material, the eggshell, and the digestive tract (Table 1).

Table 1. Average ± Standard Error (SE) of richness of microbiome of nest material (NM), uropygial secretion (US), gut (G) and eggshell (ES) of hoopoes. We also show average values (±SE) of degree of nestedness (NODF index) between pairs of microbiomes, excluding those with nest material (see Material and Methods). All these values were estimated for all studied nests (N = 24), and separately for nests with control (N = 12) and experimental (N = 12) materials. Results from statistical comparisons between control and experimental nests are also shown.

	All nests	Control	Experimental	Statistical test	
	Mean (SE)	Mean (SE)	Mean (SE)	F(1,22)	P
Microbiome Richness					
Nest material (NM)	9.46 (2.04)	16.50 (2.67)	2.42 (1.12)	23.72	0.0001
Uropygial secretion (US)	22.17 (1.87)	22.83 (2.92)	21.50 (2.47)	0.12	0.7305
Gut (G)	6.71 (1.10)	6.17 (1.60)	7.25 (1.55)	0.24	0.6323
Eggshells (ES)	7.04 (4.67)	9.08 (1.64)	5.00 (0.60)	5.49	0.0286
Microbiome Nestedness					
NODF [US(G)]	24.32 (5.81)	17.15 (6.47)	31.5 (9.50)	1.56	0.2246
NODF [ES(US)]	28.23 (4.33)	30.14 (6.78)	26.32 (5.64)	0.19	0.6689
NODF [ES(G)]	43.12 (8.15)	37.3 (10.45)	49 (12.76)	0.50	0.4910

From the 101 OTUs detected in uropygial glands, 44 were detected in the cloaca samples, and 57 in samples from materials of control nests. Detecting these OTUs in the bacterial communities of the uropygial gland did not depend of their presence in the nest materials or in the digestive tract of the same nests (all $\chi^2 < 0.29$, $p > 0.19$). When we took into consideration only the 39 OTUs present in more than 30% of some sampled sites, 6 OTUs were exclusive of uropygial secretion, 2 of nest material and no one was exclusive

of the gut microbiota. OTU 307 appeared with highest prevalence in the gut microbiota (70.83%) (Fig. 1), whereas OTUs 407 and 467 were the most commonly detected in samples of the uropygial secretion (89.5% and 79.17%, respectively) (Fig. 1). For nest material microbiome, the most prevalent OTUs (>50%) were the 283 and 311 (Fig 1). Bacterial communities of the uropygial gland, gut and nest material did separate significantly from each other in a multidimensional scale analysis (only control nests considered) (NPMANOVA, $F = 4.23$, $df = 2,31$, $p < 0.001$, Fig 2).

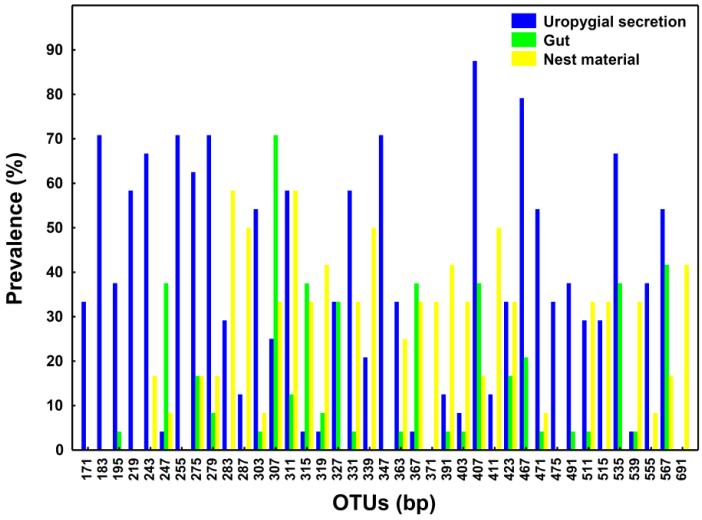

Figure 1. Prevalence (%) of different bacterial OTUs (named by their length in base pairs (bp)) found in more than 30% sampled uropygial glands (N = 24). We also show prevalence of these OTUs in the gut microbiota (N = 24) and in that of control nest material (N = 12).

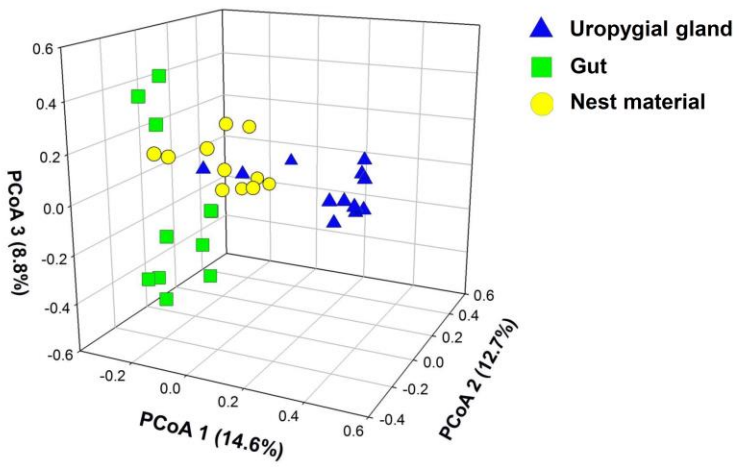

Figure 2. Multidimensional space representation (PCoA) based on similarities of communities harbored in female hoopoe uropygial gland, gut and nest material of control nests. Variance captured by each of the three axes is shown in parenthesis. The analysis was performed including only the OTUs present in uropygial secretion that were detected in at least 4 samples of any of the bacterial communities considered.

Finally, no evidence of nestedness of the microbiome of the uropygial secretion on that of the nest material (only control nests considered, all OTUs included, NODF(SE) = 15.01(8.51), Z(±CI) = -0.85 – 2.57, N = 12) or gut (Table 1, Z(±CI) = 0.78 – 4.04, N = 24) were detected, but the community of the eggshells was nested within the community of the secretion (Table 1, Z(±CI) = 2.08 – 8.47, N = 24). The eggshell bacterial community was not nested within those of the nest material (only control nests considered, all OTUs included, NODF(SE) = 15.51(4.62), Z(±CI) = -0.64 – 3.31, N = 12) or gut (Table 1, Z(±CI) = 0.18 – 3.26, N = 24). Finally no evidence of nestedness among the gut microbiota and nest material communities were detected (only control nests considered, all OTUs included, NODF(SE) = 9.28(4.10), (Z(±CI) = 0.77 – 4.52, N = 12). Consequently, no result supports the possibility that the microbiome of the nest material or that of the gut supplied

the bacterial community of the uropygial secretion, but the uropygial secretion supply the bacterial community of the eggshell of hoopoes.

Experimental effects of nest material on nest microbiomes (uropygial secretion, digestive tract and eggshells)

In accordance with the assumption of the experimental protocol, bacterial communities of nest material of experimental and control nests significantly differed in richness (Table 1) and composition (NPMANOVA, F = 2.34, df = 1,22, P = 0.007). However, the experimental nest material did not affect richness (Table 1) or composition of the microbiome of the gut (NPMANOVA, F = 1.97, df = 1,22, P = 0.510), or that of the uropygial secretion (NPMANOVA, F = 0.61, df = 1,22, P = 0.895) of nesting hoopoes (Fig. 3).

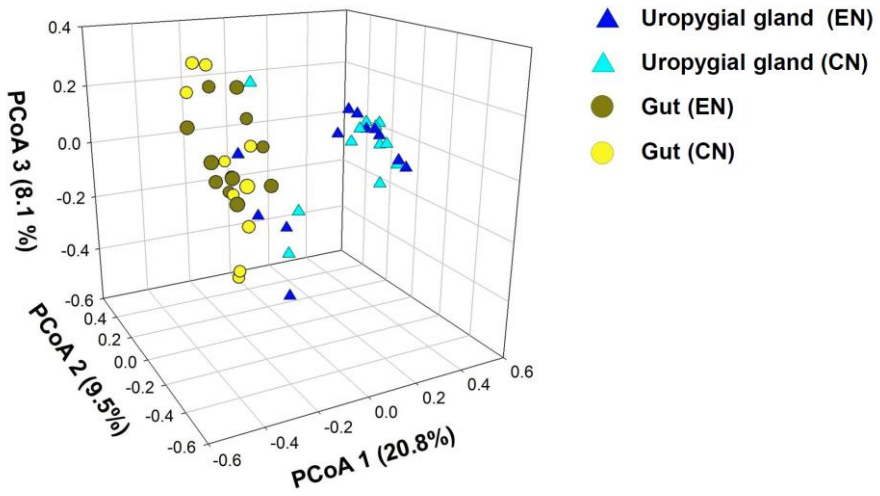

Figure 3. Multidimensional space representation (PCoA) based on similarities of most frequent bacteria in communities harbored in uropygial gland and cloaca in experimental (EN) and control nests (CN). Variance captured by each of the three axes is shown within the axis legends in parenthesis.

The only bacterial community that our experimental modification of nest material did affect was that of the eggshells. Hoopoe eggshells in experimental nests harbored poorer bacterial community (Table 1) that differed in composition from those of control nests (NPMANOVA, F = 3.63, df = 1,20, P = 0.005, Fig. 4). Our experimental manipulation of nest material did not affect estimates of nestedness among bacterial these communities (Table 1).

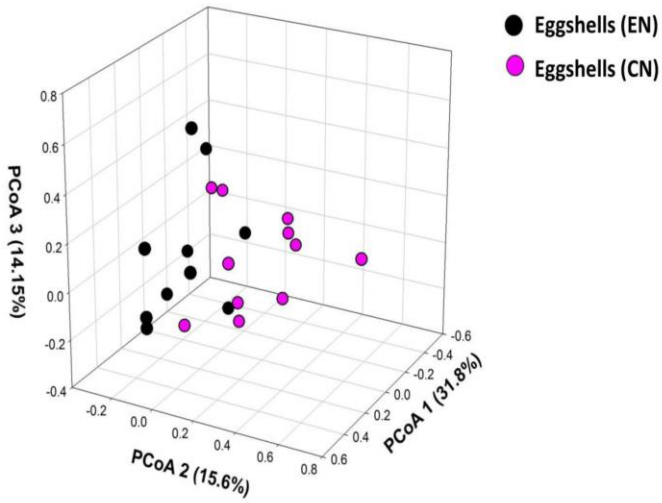

Figure 4. Multidimensional space representation (PCoA) based on similarities of the composition of bacterial communities harbored on eggshells of experimental nests (EN) and control nests (CN). Variance captured by each of the three axes is shown within the axis legends in parenthesis.

DISCUSSION

We found consistent differences in the bacterial communities harbored in different components of the hoopoe nests. Bacterial community of the female uropygial secretion was the richest, and some of the bacterial strains detected there were also present in the nest material and the female gut. However, the detection of one of these strains in the uropygial secretion did not depend on

its presence in the nest material, or female gut microbiota of the same nest. These results suggest that the community living within the female hoopoe uropygial gland is not the result of transmission from the digestive tract or the nest material. Finally, we found experimental evidence suggesting an influence of the bacterial community of the nest materials on that of the eggshells of hoopoes. Below we discuss the importance of such results for understanding the association among microbiomes of avian nests in general, and the symbiotic association between hoopoes and the bacteria living in their uropygial gland in particular.

In accordance with a recently published work by Brandl et al. (2014) where they described complex bacterial assemblages within nests of reed warblers (*Acrocephalus scirpaceus*) with unique bacterial signatures detected for microbiomes of nest materials, gut, and eggshells, we found similar results in hoopoe nests. Although microbial communities of nest materials, gut and uropygial secretion of hoopoes shared some bacterial strains, overlaps among them are rather limited. These authors also showed correlative evidence suggesting that bacterial transmission across the nest component is likely to occur. Here we tested this hypothesis experimentally and found support for an association between the microbiomes of the nest materials and of eggshells. The bacterial communities of eggshells incubated in nests with experimental nest materials (i.e., with very poor bacterial communities) differed from those of eggshells incubated in control nests. Although it is assumed in the literature that nest lining materials and nest sanitation behavior affect the bacterial environments where offspring develop (see Introduction), the influence of nest materials on eggshell bacterial community had never been tested experimentally. Eggshell bacterial load is commonly used as a proxy of probability of trans-shell embryo infection (Cook et al. 2005b, Soler et al. 2011), and, therefore, our results are the first experimental evidence of nest materials influencing the microbiome experienced by avian embryos.

The detected influence of nest materials on eggshell microbiome is particularly important for hoopoes. The females of this species smear eggshells with uropygial secretion containing symbiotic bacteria that produce antimicrobial substances (Martín-Vivaldi et al. 2014, Soler et al. 2014), and here we found evidence suggesting that the microbiome of the eggshell is nested within that of the uropygial secretion oil. Thus, our experimental results suggest that bacteria from nest materials, or their antimicrobial properties, interact with bacteria from the uropygial secretion to determine the microbiome on the eggshells. We used crushed olive stones as experimental nest material, which demonstrated considerable antimicrobial properties against several indicator bacterial strains (Appendix 1). It is likely that the antimicrobial activity of this material in the nest of hoopoes affected the strains of the uropygial secretion that were able to grow on the eggshell and, therefore, could affect the protective effects of these symbiotic strains impeding trans-shell pathogens penetration. However, we did not find any effect of the experiment on hatching success (Martínez-García et al. Unpublished data), and further research is needed to know particularities of the interactions between symbiotic bacteria from the uropygial gland and nest material properties affecting egg viability.

Explaining how microbiomes are established and maintained in their animal hosts is a central question in biology, especially for those with beneficial effects (Scheuring and Yu 2012, McFall-Ngai et al. 2013). Hosts seem able to choose the right bacterial partners from a huge number of surrounding candidates. Some of the bacterial symbionts are vertically transmitted (Ezenwa et al. 2012), whereas in some others systems, the symbiotic microbiome is the result of interference competition among recruited bacteria from the nearby environment (Scheuring and Yu 2012). In the case of hoopoes, symbiotic bacteria are only detected in uropygial gland secretion of nesting females and chicks (Soler et al. 2008) and, thus, bacterial

acquisition is likely related to the nest environment. Previous studies have suggested that the acquisition of gut microbiota by nestling birds may have both genetic and environmental components (Mills et al. 1999, Ruiz-Rodríguez et al. 2009, 2014, González-Braojos et al. 2012) and that, within nests, bacteria are transmitted from one to another environments (i.e., nest material – gut – eggshells) (Brandl et al. 2014). Thus, we explored the possibility that bacteria in the uropygial gland of incubating females were recruited from those in the nests material or in their digestive tract.

Despite detected similarities among the microbial communities of nest materials, gut and uropygial secretion of hoopoes might suggest that bacteria from the gut and/or the nest material colonize the uropygial gland, we did not find support for the expected association. The detection of a particular bacterium in the uropygial secretion of females did not depend on its presence in nest materials or gut of the same females. Furthermore, the experimental manipulation of the microbial community of nest materials did not influence the microbiome of the uropygial secretion, which further supports that microbiomes of nest materials and of uropygial secretion are isolated from each other.

There are several possible explanations for the absence of evidence of inter-connection among the bacterial community of the uropygial secretion of hoopoes and those of nest materials and/or gut. We have previously shown evidence of a genetic component explaining the enteroccoci strains detected in the uropygial secretion of nestling hoopoes (Ruiz-Rodríguez et al. 2014). Thus, it is possible that stockpiles of symbiotic bacteria that come from their mothers were responsible for the bacteria in the uropygial secretion of reproducing females. This bacterial reservoir may be located in particular sites of the gut inaccessible for our cloacal samples.

Another possible explanation is related to properties of interference of different bacteria that is known to depend on environmental factors as resource availability, chemical environments, and bacterial community (Poisot et al. 2011, Pérez-Gutiérrez et al. 2012, Scheuring and Yu 2012). Long et al. (2012) argued that if hosts fueled interference competition in particular environments (i.e. cuticle crypts of fungus growing ants (Currie et al. 2006) or uropygial gland of hoopoes (Martín-Vivaldi et al. 2014)) by for instance providing bacteria with abundant resources, hosts would be able to recruit bacterial strains with high antimicrobial capabilities. These strains may therefore be at a high density in host provided environments (the secretion), despite being rare in other interacting environments, which could serve as a source (i.e. nest materials and/or digestive tract), but where their low density makes their detection difficult by ARISA techniques (Ramette 2009).

Thus, although we did not find evidence of the expected associations between hoopoe acquired bacteria for their uropygial gland and those in samples collected from the intestine or from nest materials from previous reproduction further research is needed to robustly reject the hypothesis of a possible role of nest materials and digestive tract as reservoir of symbiotic bacteria of the uropygial gland of hoopoes.

Authorship statement

JJS and MM-V designed the study with considerable assistance from EV. AM-G and SRR performed all molecular analyses and AM-G carried out all binning and statistical analyses. AM-G and JMPS performed the field work. AMG wrote a first version with help of JJS and MM-V; all other authors substantially contributed to the final version.

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CAPÍTULO IV

Nest of origin shapes the microbiome of the uropygial secretion of hoopoes

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ABSTRACT

One of the main issues in evolutionary biology is to uncover mechanisms explaining how symbiotic microbiomes are established and maintained in their animal hosts. These mechanisms may include genetically determined traits of parents, as those related to vertical transmission from mother to offspring (maternal effects). Horizontal transmission of symbionts may also depend on genetically determined characteristics of offspring that enhance the probability of acquiring from the environment symbionts that are identical to those hosted by parents. Offspring may also acquire these symbionts horizontally from the environment shared with parents but in the absence of genetic factors. By cross-fostering experiments, 'sibling-sibling' and 'mother-offspring' comparisons of bacterial communities of hoopoe (*Upupa epops*) uropygial glands, we here disentangle nest of origin and nest of rearing explaining microbiome variability. Bacterial assemblages of cross-fostered nestlings were significantly explained by nest of rearing, which suggests that hoopoes incorporated from the environment bacteria that were not present in the secretions of their biological mothers or genetic sibling, but in that of their stepmothers and stepsibling (i.e. environmental component). Moreover, nest of origin explained a larger amount of variance than nest of rearing, and the microbiome of cross-fostered nestlings was more similar to those of their mothers and siblings than to the bacterial communities of their stepmother and stepsiblings. These last results suggest an important component of nest of origin governing the microbiota of nestling hoopoes mediated by either, early vertical transmission from mother to offspring (i.e. indirect genetic effects of mothers), or by heritable variation in morphological, physiological and/or chemical characteristics of the uropygial gland of nestlings that select for particular bacterial communities. Further studies are necessary to infer the direct influence of nestling phenotype determining the symbiotic microbiome.

INTRODUCTION

Mutualistic relationships between animals and microorganisms may for instance allow the former to exploit otherwise inaccessible niches (Minic and Hervé 2004, Moran 2006, Janson et al. 2008, Sachs et al. 2013) or avoid parasites and/or predators (Gil-Turnes et al. 1989, Arnold et al. 2003, Jaenike et al. 2010). Knowing the particularities of such relationships, including those related with the acquisition of microbial symbionts (Bright and Bulgheresi 2010, McFall-Ngai et al. 2013), is a prime task for evolutionary biologists and essential for understanding animal evolution (McFall-Ngai 2002, Zilber-Rosenberg and Rosenberg 2008, McFall-Ngai et al. 2013). Hosts may acquire symbionts directly by vertical transmission from parents to offspring (Moran and Wernegreen 2000, Darby and Douglas 2003), or by horizontal transmission from the environment (Nyholm and McFall-Ngai 2004). Modes of transmission influence rates of coevolution between counterparts (Dillon and Dillon 2004), and may determine the effectiveness of the mutualistic relationship (Douglas 1998). Fitness of vertically transmitted symbionts is closely related to that of their hosts (Herre 1993, Frank 1996, Douglas 1998, Poulsen et al. 2003), i.e., symbionts enhancing hosts' reproductive success will directly benefit their own performance, which favors intimate coevolutionary processes (Cafaro and Currie 2005). For horizontally transmitted symbionts the fitness of counterparts is not so intimately related and, thus, the coevolutionary relationship would be in general more diffuse (Douglas 1998). Therefore, exploring microbial acquisition is extremely valuable in shedding light on the coevolution in symbiotic relationships.

Although the vast majority of symbioses described in eukaryotes involve bacteria (Moran and Wernegreen 2000, Lindquist et al. 2005), studies on mechanisms of bacterial transmission are limited to a handful of model systems (Chaston and Goodrich-Blair 2010, Salem et al. 2015). Horizontally

transmitted bacteria are known for squids (Nyholm and McFall-Ngai 2004), tubeworms (Nussbaumer et al. 2006) and mussels (Salerno et al. 2005), while mechanisms of vertical transmission have been detected for instance in ascidians (Hirose et al. 2006), bryozoans (Sharp et al. 2007) and earthworm (Davidson and Stahl 2008). For some other model systems, microbial symbionts are acquired both vertically and horizontally, as it is the case for beneficial gastrointestinal microbiomes of animals (Bright and Bulgheresi 2010).

Independently of the mode of transmission, several mechanisms exist to ensure the acquisition of the correct or most beneficial symbionts (Chaston and Goodrich-Blair 2010). These mechanisms may include genetically determined traits of parents, as those related to vertical transmission from mother to offspring (maternal effects). In addition, horizontal transmission of the appropriated symbionts may also have a genetic component if it is related to characteristics of offspring that are inherited from parents, and that enhance the probability of acquiring from the environment symbionts that are similar to those hosted by parents. Offspring may also acquire these symbionts horizontally from the environment shared with parents in the absence of genetic factors. It has been considered that a mix of genetic and environmental factors determining bacterial communities of symbionts is advantageous because it would guarantee the simultaneous presence of beneficial microorganisms from different environments (Douglas 1998, Currie et al. 2006, Chaston and Goodrich-Blair 2010). In these cases, characteristics of parent or offspring with a genetic basis that favours acquisition and establishment of beneficial symbionts may be of selective advantage and hence promoted by natural selection. Thus, determining the genetic and the environmental component of the symbiotic microbiota as a phenotypic trait of the hosts would allow inferring heritability of mechanisms that allow hosts to acquire proper symbionts.

The system formed by hoopoes (*Upupa epops*) and the bacteria with antibiotic properties hosted in their uropygial gland (Soler et al. 2008) is an interesting model for exploring genetic and environmental factors governing its variability. The symbiotic bacteria have only been detected in incubating females and nestlings, but not in males or in non-reproducing individuals (Soler et al. 2008, Martín-Vivaldi et al. 2009). Thus, symbiotic bacteria are first acquired by all individuals during development and, later, only by females during each reproductive event. Nestlings may obtain symbionts by strict vertical transmission from mothers, but also horizontally from the nest environment (i.e. nest remains of previous reproduction events of con- or hetero-specifics (Ruiz-Rodríguez et al. 2014)). We have already have some evidence suggesting that culturable bacteria, mainly antibiotic producing enterococci, are transmitted from mother hoopoes to offspring soon after hatching (vertical transmission), and that nestlings are able to incorporate new enterococci symbionts from the environment during the development of the uropygial gland (horizontal transmission) (Ruiz-Rodríguez et al. 2014). However, we do not know whether the mode of transmission and the genetic factors governing the non-culturable bacteria of the community follow the same trends. Therefore, studying the whole bacterial community through molecular methods and determining their resemblance between siblings or mothers and offspring would be an important contribution to the study of the coevolutionary relationships between hoopoes and symbionts.

We approached this aim by cross-fostering nestlings from different nests and comparing bacterial communities characterized by mean of the ARISA (Automatic Ribosomal Intergenic Spacer Analysis) molecular technique. We estimated the proportion of variance of bacterial communities of nestlings that was explained by nest origin (i.e., similarity among siblings reared in different nests), which would reflect the genetic component of the

microbiome plus any prior manipulation maternal effects (Falconer 1989, Roff 1997; Merilä 1996, Møller 1990). Moreover, we estimated the proportion of variance explained by the identity of nest of rearing (i.e., similarity among stepsiblings reared in the same nest), which would reflect the environmental influence of bacterial community of nestling hoopoes. The relative contribution of genetic (plus any pre-manipulation maternal effects) and environmental factors were determined by (i) comparing the proportion of variance explained by nest of origin and nest of rearing respectively. We also (ii) compared similarity estimated for cross-fostered siblings reared in different nests with that estimated for step siblings reared in the same nest. Finally, relative contribution of genetic and of environmental factors explaining microbiota of the uropygial secretion was also explored by (iii) comparing similarity among cross-fostered nestlings and biological mother with that among cross-fostered and stepmother.

MATERIALS AND METHODS

Study species, study area and general methods

The hoopoe is distributed throughout Europe, Asia and Africa, inhabiting open woods or open areas as steppes, grasslands, pastures, semi-deserts, or crops whenever they have scattered trees, walls or buildings providing holes for nesting and soil without tall vegetation for feeding (Rehsteiner 1996, Barbaro et al. 2008, Schaub et al. 2010). Females lay one or two clutches of 6-8 eggs along the breeding season, between February and July (Martín-Vivaldi et al. 1999). Incubation lasts 17 days and starts with the first or second egg, which results in eggs hatching asynchronously at 24 h or even greater intervals (Cramp 1998).

The fieldwork was performed during the breeding seasons 2010-2011 in a wild population located in the Hoya de Guadix (37°18′N, 38°11′W), southern Spain, where hoopoes breed in crops, forests and gullies within nest-boxes placed in trees or buildings. In 2011, hoopoes were also sampled in a captive population descendant from our wild population and breeding in captivity since 2008. The captive pairs were distributed in two different subpopulations located in South-eastern Spain, one in installations of the University of Granada in Hoya of Guadix (Granada), and the other one in facilities of the Estación Experimental de Zonas Áridas (CSIC) at the Finca Experimental La Hoya in Almería (36º50´N, 2º28´W). All females and nestlings were ringed with numbered rings and females also with color rings for individual recognition.

Nest-boxes in the wild were visited twice per week from midFebruary to the end of July to record laying date, clutch size and hatching date. Pairs of hoopoes breeding in captivity were housed in independent cages at least 3m x 2m x 2m installed in the open, scattered and isolated to avoid interactions between pairs and ensure successful breeding. Cages had access to soil and were provided with live food (crickets, vitamin-enriched fly larvae and meat (beef heart)) *ad libitum* and were visited daily.

Experimental design and sampling

The cross-fostering experiment was performed in 2010 and 2011 field seasons. The experimental design consisted in exchange of two experimental nestlings among pairs of nests of identical hatching date and similar brood size. The exchange was carried out when the oldest nestling in each nest was 8 days old (i.e., when nestlings start to produce small amounts of secretion containing bacteria). Two of the heaviest nestlings in each nest were exchanged with those from another nest (i.e. with the same age and similar

weight). Experimental nestlings were individually marked by painting their tarsus with permanent innocuous markers. Cross-fostering experiments were performed between wild nests in 2010 and in 2011 between one nest in captivity and the other in wild conditions, but when this was not possible, experimental nestlings were exchanged between two captivity nests, or between two wild nests. This was done so to increase phenotypic variance among cross-fostered nests that allow a more realistic estimation of the genetic and environmental components (Falconer 1989). Transport of nestlings between nests lasted approximately 1 h and was done in a portable incubator at 37 ºC to reduce stress due to temperature change.

Uropygial secretions of females were sampled before hatching date (i.e. 14 days after laying the first egg), whereas those of nestlings were sampled 10 days after nest exchange (i.e. oldest nestlings had 18 days old). Incubating females were captured within the nest-box by hand, quickly sampled, and released again within the nest to reduce disturbance. For each capture, we wore new sterile latex gloves cleaned with 96% ethanol for the whole process to avoid external bacterial contamination. Before collecting samples from uropygial gland, we softly washed the circlet of feathers and surrounding skin with a cotton swab slightly soaked in ethanol to reduce the risk of contamination with external bacteria. After evaporation of the alcohol, a sterile micropipette tip (1-10 µl micropipette (Finpipette)) was introduced in the gland papilla. The papilla was pressed softly with a finger and the uropygial secretion entirely collected was transferred to a sterile microfuge tube. Afterwards, 5µl were separated in a different sterile microfuge tube for the analyses. Nestling hoopoes were sampled with identical protocol than adult females were. All samples were individually stored in 1.2 ml sterile microfuge tubes in a portable cooler (1-3 ºC) until being stored in the lab at -20º C the same day of sampling for further molecular analyses.

We sampled 44 nests and got information for the 44 breeding females and for 165 nestlings; 93 of them did grow in the same nests where they hatched, whereas 72 were moved to foreign nests. Because of different types of problems related to predation of wild nests, sampling, or failed molecular analysis, we finally obtained complete information of siblings that were reared in the same nests of hatching (N = 57) or moved to another nests (N = 44) for 28 nests. Only for 21 of these nests, we got the necessary information to compare the bacterial community of experimental nestlings with that of their foster and genetic siblings on the one hand, and with the bacterial community of their mother and stepmother on the other hand.

Laboratory work

Bacterial genomic DNA for the viscous uropygial-secretion samples was extracted with a commercial KIT (The FavorPrep™ Blood Genomic DNA Extraction Kit, Favorgen). Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Fisher and Triplett 1999) was used to characterize the composition of bacterial communities, which amplifies an intergenic transcribed spacer (ITS) region between the prokaryotic 16S and 23S rDNA. This region is highly variable both in size and sequence between species and, thus, offers higher taxonomic resolution than other techniques (Danovaro et al. 2006). The ITS was amplified using the primer pair ITSF (5´-GTCGTAACAAGGTAGCCGTA-3´) and ITSReub (5´-GCCAAGGCATCCACC-3´) (Cardinale et al. 2004). The primer ITSReub was labelled fluorescently with 6-FAM. Amplifications were performed in 50 µl reaction volumes containing ultrapure H_2O, 20 µl of 5 PRIME MasterMix (2.5x) including 1.5mM $Mg(OAC)_2$, 200 µM dNTPs, 1.25 U Taq DNA polymerase 0.2 µM of primers and 5µl of diluted DNA 1:10. PCRs were carried out in Eppendorf Mastercycler Nexus Family. Fragments were amplified under the following conditions: initial denaturation at 94 °C 2 min,

followed by 30 cycles with denaturation at 94 °C 45 s, annealing at 52 °C 45 s and extension at 72 °C 1 min, with a final extension at 72 °C 5 min. Amplified PCR products were diluted 1:10 and denatured by heating in formamide. Fragment lengths were determined by mean of automated fluorescent capillary electrophoresis on 3130 Genetic Analyzer. Electropherogram peak values were calculated after interpolation with an internal size standard named GeneScan™ 1200 LIZ dye Size Standard (both Applied Biosystems). These analyses were performed at the Scientific Instrumentation Center of the University of Granada.

Statistical analysis

Peak Scanner 1.0 (Applied Biosystems) was used to determine fragment length in terms of base pairs (bp) of each peak that enables the identification of different bacterial strains (i.e. OTUs). For methodological reasons, the estimated length of the same bacterial strain from different samples may slightly differ. Thus, binning DNA fragment lengths from different samples is necessary before comparing bacterial communities. We did so by using available scripts in R-environment (http://cran.r-project.org/) at www.ecology-research.com (Ramette 2009) with a window size of 4 base pairs (bp) and a distance of two consecutive binning frames (i.e. shift) of 0.1. The algorithm rearranges the data and calculates the relative fluorescence intensity (RFI) of each peak by dividing individual peak areas by the total area delimited by all peaks for the respective sample. All peaks with RFI values of < 0.09% were not included in further analyses since they consisted of background peaks. Only fragments above a threshold of 50 fluorescence units and ranging between 100 and 1.000 bp were taken into consideration so as to include the maximum number of peaks while excluding background fluorescence (Ramette 2009). We used the presence-absence matrix generated after the binning process for all subsequent analyses. Molecular fingerprinting

techniques are highly reproducible, robust, and have proven useful for comparative analysis of microbial community structure (Loisel et al. 2006, Bent and Forney 2008).

We described the bacterial community harbored in uropygial secretion of females and nestlings with information obtained from ARISA for all individuals (44 females and 165 nestlings). To explore the differences in bacterial richness (number of OTUs per sample) between adult females and nestlings, we performed ANOVAs with one fixed factor (adult females *vs* nestlings). Moreover, we explore differences in prevalence (i.e., relative frequencies in percentage) of OTUs detected in uropygial secretions of adult females and nestlings, but considering the most frequent OTUs; i.e., those that were detected in more than 30% of females or nestlings uropygial secretion sampled. Pearson correlation coefficients were used to explore whether OTU's prevalence in females and nestling samples were related. We did this analysis with all detected OTUs and also including only those that were present in more than 30% of females or nestlings sampled. Furthermore, we analyzed differences in the composition of bacterial communities hosted in uropygial secretions by females and nestlings by one-way PERMANOVAs analysis (Jaccard´s distance), taking into consideration all females and only non-moved nestlings. Trying to reduce probability of detecting significant differences among females and nestlings due to rare OTUs, we only considered those that appeared in more than 3 samples of females or nestlings. To graphically show variation in bacterial communities of uropygial secretions of females and nestlings, we used classical multidimensional scaling analysis (Multidimensional Scaling (MDS), Principal Coordinates Analysis (PCoA)). This technique represents the objects (communities) on a plot with canonical axes, where the distance between the objects shows their underlying similarity (Legendre and Legendre 1998).

Cross-fostering experiments are a well stablished approach for partitioning phenotypic variance in its genetic and environmental components in mixed statistical models that include the identity of nest of origin and rearing (nested within nest of origin) as random factors (Merilä 1996). Proportion of variance explained by nest of origin reflects the genetic component of the trait plus any pre-manipulation maternal effect, whereas proportion of variance explained by nest of rearing include environmental effect plus any post-manipulation maternal effect (Merilä 1996; Soler et al. 2003). Genetic component was also estimated by means of parent-offspring comparisons. In the case that bacterial communities of experimental cross-fostered nestlings were consistently more similar to those of genetic mothers than to bacterial communities of stepmothers, a relatively larger genetic influence determining bacterial community of nestling hoopoes would be inferred.

The similarity matrix among all bacterial communities of the individuals sampled were based in Jaccard's distance (Zuur et al. 2007). The similarity values were used as the dependent variables of PERMANOVA model using type III estimation of mean squares. This model try to explain similarity among nestlings including two random factors: nest of origin and nest of rearing (nested within nest of origin). For this model we used only the 28 nests for which we have information for moved and non-moved nestlings from the same nest of origin. Finally, for the 21 experiments with all the information (see above), we estimated mean values of similarities among bacterial communities of experimental nestlings and those of their genetic (reared in different nests but genetically related) or foster (reared in the same environment but genetically unrelated) siblings. We estimated for the same nests mean values of similarities among bacterial communities of experimental nestlings and their genetic or foster mothers. Consistent higher similarity values for comparisons of individuals genetically related for each

sampled nests would suggest a relatively higher influence of genetic factors explaining microbial community of the uropygial secretion of hoopoes.

All multivariate analyses and figures trying to explain similarity matrices (PERMANOVAS) were performed with PRIMER v7 (PRIMER-E) software (Anderson et al. 2008). Statistical inferences (e.g., P-values) of all PERMANOVAs were based on 9999 permutations. Statistical tests trying to explain variation in bacterial richness and prevalence of different bacterial strains, as well as those comparing mean values of similarities estimated for genetically related and unrelated individuals, were performed with STATISTICA 8 software (StatSoft 2006).

RESULTS

Description of bacterial communities in uropygial secretions: prevalence, richness, and composition

We detected 143 different OTUs (length of the ITS fragment varying between 100 bp and 847 bp) in the bacterial community of female and nestling hoopoes uropygial secretions (females and nestlings), 141 of which were present in nestlings, and 116 in females. All except two OTUs that were detected in females at very low prevalence (143 bp and 603 bp, 2.22% and 4.44%, respectively) were also present in nestling samples. Prevalence of detected OTUs ranged from 0.61% (OTU with 847 bp) to 84.44% (OTU with 183 bp), and were similar for females and nestlings as shown by the strong positive relationships among their values (Appendix 1, $R^2 = 0.89$, $N = 143$, $t = 34.2$, $p < 0.0001$). This relationship was evident even when only considering the 28 OTUs that were present in more than 30% of samples from females or nestlings (Fig. 1, $R^2 = 0.73$, $N = 28$, $t = 8.44$, $p < 0.0001$). Richness of bacterial community of the uropygial secretions of nestlings (mean (SE) =

22.64 (0.66)) was also similar to that of females (mean(SE) = 21.78(1.37)) (F = 0.34, df = 1, 207, p = 0.55). Additionally, composition of bacterial communities of nestlings and females did not significantly differ (one-way PERMANOVA, F = 1.53, df = 1, 135, p = 0.0572, Fig. 2).

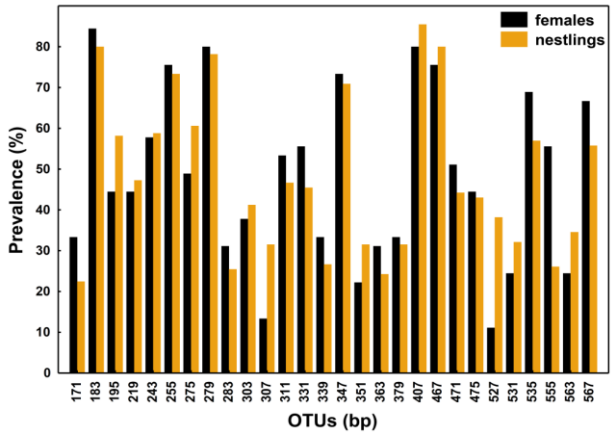


Figure 1. Prevalence (%) of different bacterial OTUs (named by their length in terms of base pairs (bp)) found in more than 30% of samples from uropygial glands of hoopoe nestlings (N = 165) and females (N = 44).

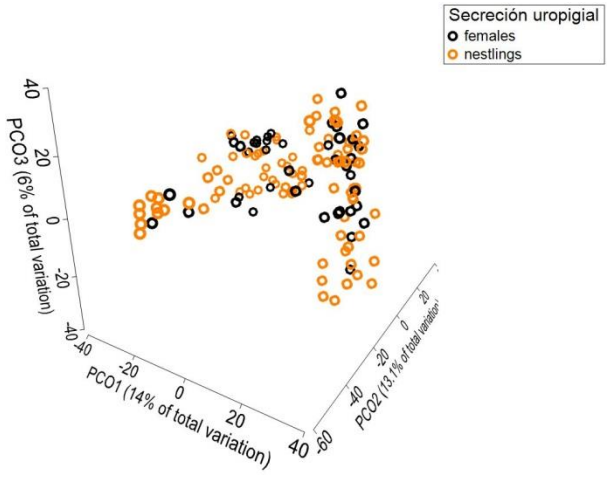


Figure 2. Multidimensional space representation (PCoA) based on similarities of the most frequent OTUs communities harbored in uropygial secretions of hoopoe females and non-moved nestlings. The total variance explained is also shown (captured by the three axes = 33.1%).

Genetic and environmental effects

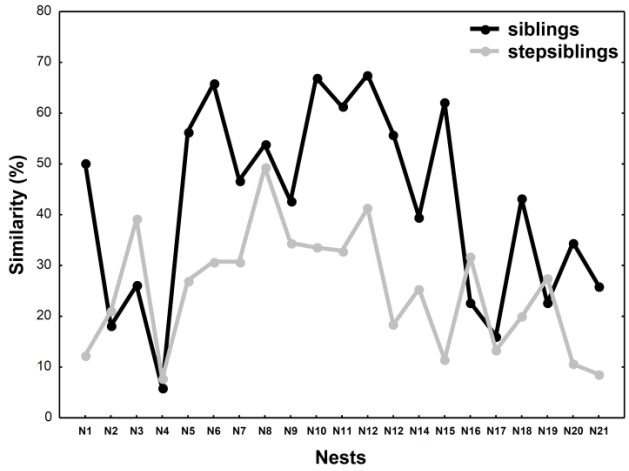
The similarity matrix among bacterial communities of the uropygial gland of experimental nestlings was significantly explained by nest of origin and nest of rearing (Table 1). The proportion of variance explained by the nest of origin was relatively larger than that explained by nest of rearing (Table 1), suggesting a relative larger influence of genetic factors and/or pre-manipulation maternal effects (i.e., vertical transmission) in comparison with environmental influence and post-manipulation maternal effects.

Table 1. Results of a PERMANOVA model explaining matrices of similarity among bacterial communities in the uropygial secretions of hoopoe nestlings. The model includes identity of nest of origin (genetic factor) and rearing (environmental factor) nested within nest of origin. Bold p-values are those lower than 0.05.

Factors	Pseudo-F	df	p	Permutations	% Variance
(a) Nest of origin	3.56	27	**0.0001**	9693	37.0
(b) Nest of rearing (nested in (a))	1.28	27	**0.0127**	9737	14.9

This inference was further confirmed by the significantly larger similarity values of comparisons of siblings reared in different nests than those for comparisons of stepsiblings reared in the same nest (GLM, $F = 19.33$, $df = 1, 20$, $p = 0.0002$; Fig. 3a). Results from comparisons of similarities between bacterial communities of cross-fostered nestlings and those of their foster (mean (SE) = 39.81 (3.91)) and genetic (mean (SE) = 23.20 (3.10)) mothers were also in accordance with a relatively larger influence of genetic factors and/or pre-manipulation maternal effects determining the bacterial community of the uropygial secretion of hoopoe nestlings (GLM, $F = 20.42$, $df = 1, 20$, $p = 0.0002$; Fig. 3b).

a)



b)

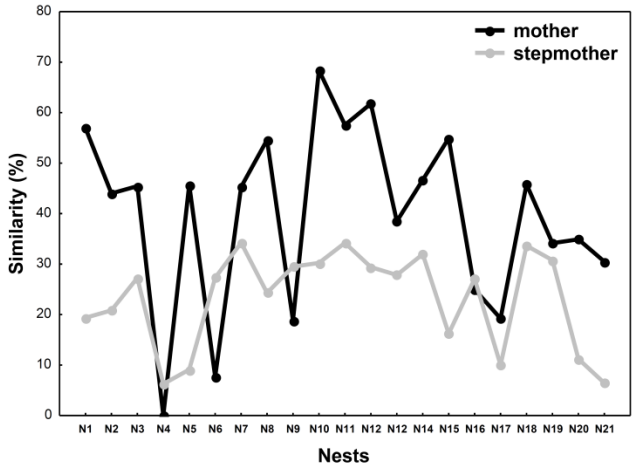


Figure 3. Similarities in composition of bacterial communities (Jaccard's distances in percentage) among samples from cross-fostering experiments. (a) Similarity between uropygial secretions of nestlings that did not grow in their origin nests (experimental nestlings) and genetic siblings reared in their native nests (black lines), or foster siblings (experimental nestlings and nestlings from their rearing nest) (grey lines). (b) Similarities between microbiomes of the experimental moved nestlings and those or their genetic (black lines) or foster (grey lines) mothers.

DISCUSSION

Our main results are two-fold. The first one is that bacterial communities of the uropygial secretion of hoopoe nestlings did not differ significantly from those of adult females. The second group of results pointed out a strong genetic component and/or pre-manipulation maternal effect (i.e., vertical transmission) explaining the composition of bacterial communities in experimental cross-fostered nestlings. We also detected a significant effect of nest of rearing, suggesting that nest environment, including post-manipulation maternal effects, contributes to the microbiome of the uropygial secretion. Below, we discuss the importance of these findings for understanding the mechanisms of acquisition of bacterial symbionts by nestling hoopoes, and the implication for coevolutionary relationships between hoopoes and bacteria of their uropygial secretion.

Previous work has shown that the prevalence of culturable strains in the uropygial secretion of females and nestlings differ (Ruiz-Rodríguez et al. 2014). These differences were at least partially due to the effect of few bacterial strains that appeared at a higher prevalence in samples of females or nestlings (Fig 2; Ruiz-Rodríguez et al. 2014). However, when considering the bacterial community as a whole, differences between females and nestlings did not reach statistical significance, and prevalence of different OTUs in samples from females and from nestlings correlated positively. In terms of bacterial diversity, even when considering the group of enterococci, estimates for females and nestlings did not differ significantly (Ruiz-Rodríguez et al. 2014). Thus, although prevalence of some OTUs in communities of females and nestlings may differ, the microbiome of the uropygial secretion of females and nesting hoopoes is quite similar.

All these results considered together suggest that the bacterial community of the uropygial secretion of nestlings may depend on those of their mothers. Three different scenarios may explain detected similarities between bacterial communities of siblings and adult females: (i) Symbiotic bacteria acquired by adult females before nestlings hatched may be vertically transmitted to offspring. Otherwise, (ii) nesting females may acquire bacteria from the same environment (i.e., nest remains of previous reproduction) as their nestlingsl do during the nestling phase. A mixed possibility is that (iii) females and offspring share phenotypic characteristics of their uropygial gland that favor the selection of particular bacterial strains from the environment, resulting in females and offspring having similar bacterial .

There is strong evidences suggesting that enterococci bacteria from the environment are incorporated into the community of the uropygial secretion of females and nestlings (Ruiz-Rodríguez et al. 2014), and we here have found a significant effect of nest or rearing suggesting that it is also the case for the entire microbial community. These results are in accordance with the second possibility, but do not necessarily exclude the vertical transmission from mother to offspring of bacterial strains, at least partially. Our cross-fostering experiment was performed when nestlings started to produce their own secretion, i.e., cross-fostered nestlings harbored symbiotic bacteria in their uropygial gland when they were cross-fostered among experimental nests. Thus, the effect of nest of rearing confirms that experimental nestlings incorporate new bacteria that are also present in their stepsiblings.

In the previous cross-fostering experiment performed by Ruiz-Rodríguez et al. (2014) nestlings were exchanged before they started to produce secretion (4 days old), which would explain the relatively larger influence of nest of rearing in that early work, but also the strong effect of nest of origin detected here. The effect of both nests included possible

maternal effects that respectively occur before and after the experimental translocation of nestlings (Merilä 1996; Soler et al. 2003). Consequently, it is possible that differences with previous work (Ruiz-Rodríguez et al. 2014) in the estimated effect of nest of origin were explained by differences in maternal effect from mother and stepmother of experimental nestlings.

Vertical transmission of symbionts is by definition a maternal effect that contributes to offspring phenotype, but that is genetically determined in mothers (i.e. indirect genetic effect, (Mousseau and Fox 1998, Wolf and Brodie III 1998, Wolf et al. 1998)). Thus, the detected effects of nest of rearing of the microbial community from nestlings secretions, as well as the relatively high similarities between related nestlings, and between nestlings and mothers, may be explained by direct vertical transmission of symbionts from mother to offspring (first possibility above). However, we knew from previous experimental work (Ruiz-Rodríguez et al. 2014) that direct contacts with mother or nest material is not necessary for hatchling hoopoes to develop normal uropygial glands and acquire enterococci symbionts. Thus, it is unlikely that the strong influence of nest of rearing, and the similarity among bacterial communities of related individuals detected here, were exclusively explained by vertical transmission of symbionts or common environmental influences experienced before the cross-fostering.

A third explanation of the strong genetic and/or maternal component detected is that related hoopoes share characteristics of their uropygial gland and/or secretion (i.e., chemical properties) that influence the composition of the bacterial community established. Bacteria from the environment that were compatible with characteristics of the uropygial gland and secretion of hoopoes would colonize hosts. Within the uropygial gland, competitive ability of different bacterial strains would depend on the particular environment (i.e. chemicals, resources, etc.) provided by hoopoes, which would determine the

stabilized microbiome of the uropygial secretion (see Scheuring and Yu 2012). Disentangling genetic and environmental components of such phenotypic traits are however necessary before reaching firm conclusions.

Independently of the relative contribution of genetic and maternal effects explaining our results, natural selection would work on the genetic component of maternal effects (Mousseau and Fox 1998) and/or on phenotypic characteristics of the uropygial gland and secretion favoring characteristics that select for microbiomes with relatively higher beneficial effects. Moreover, estimated environmental effects explaining the bacterial community of nestling hoopoes refer to variation due to nest environments where hoopoe nestlings develop after the age of the experiment. These environments should also be considered as a kind of parental effect with possible genetic components in adults (Mousseau and Fox 1998, Hansell 2000), where natural selection may act. Environmental and additive genetic effects together explained more that 50% of the variance in composition of the bacterial community of the uropygial gland of hoopoe nestlings (Table 1). Thus, independently of the relative importance of genetic and maternal effects, the bacterial community of the uropygial secretion of nestling hoopoes has a considerable genetic background to be modulated by natural selection.

Detecting mechanisms explaining how beneficial microbiomes are established and maintained within their hosts is a major question in evolutionary biology (Prosser et al. 2007, Chaston and Goodrich-Blair 2010). Here, we found a strong nest of origin effect that likely included indirect genetic effects, but also an influence of the environment, explaining the composition of the microbial community in the uropygial secretion of hoopoes, for which evidence of beneficial effects for hosts are accumulating (Soler et al. 2008, Ruiz-Rodríguez et al. 2009, Martín-Vivaldi et al. 2010). We

expect that these results will encourage further research directed to detect physiological and morphological characteristics of the uropygial gland of hoopoes that explain the detected genetic and environmental bases of their bacterial communities.

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Authorship statement

JJS and MM-V designed the study, JJS and AM-G carried out statistical analyses. AM-G together with SRR performed molecular analyses. AM-G, LA and JMPS performed most of the field work. AM-G wrote the first version of the manuscript with substantial contribution from MRR and JJS. All authors contributed to the final version of the article.

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RESUMEN DE RESULTADOS Y DISCUSIÓN INTEGRADORA

En esta tesis se ha caracterizado por primera vez mediante métodos moleculares el microbioma de la glándula uropigial de un ave, la abubilla, que mantiene en este órgano una simbiosis mutualista con bacterias que le ayudan a protegerse de patógenos (Martín-Platero et al. 2006, Soler et al. 2008, 2010, Ruiz-Rodríguez et al. 2009a, 2012, Martín-Vivaldi et al. 2010, 2014). Con el objetivo de entender la dinámica de esta comunidad, así como sus efectos en otras comunidades de microbios relacionadas con el nido, se ha utilizado un enfoque de análisis de meta-comunidades. Además de la comunidad bacteriana de la secreción de la abubilla, hemos estudiado aquellas presentes en el material del nido, en la cloaca, en el pico, en la placa incubadora y en la superficie de sus huevos.

El sistema abubilla – bacterias de su secreción se ha revelado como un interesante modelo biológico en el que estudiar las interacciones mutualistas entre vertebrados y bacterias productoras de sustancias antimicrobianas (Soler et al. 2010). Gracias a la descripción de la composición de estas comunidades, su comparación en un contexto de colonización mediante el análisis de anidamiento, y a la realización de experimentos para detectar interacciones entre ellas; hemos podido responder a varias cuestiones de gran importancia para avanzar en la comprensión del funcionamiento de este sistema. En esta discusión general de los resultados más relevantes obtenidos en los capítulos de la tesis, revisamos las particularidades de las comunidades bacterianas de las diferentes localizaciones, las implicaciones en los mecanismos de adquisición de los simbiontes de las glándulas, o la microbiota del huevo, y la existencia de posibles cepas clave en la simbiosis mutualista entre abubillas y bacterias.

La caracterización de las comunidades bacterianas de la secreción, del pico, de la cloaca y de la placa incubadora de las abubillas, así como de las cáscaras de los huevos y de los materiales del nido, nos permitió poner de manifiesto diferencias y semejanzas entre ellas. La microbiota de la secreción de las glándulas uropigiales fue la más rica (CAPÍTULO I, III) junto con la del material del nido (en nidos reutilizados), mientras que las comunidades de la cloaca, del pico, de la placa incubadora y de las cáscaras de los huevos, presentaron un número de OTUs por individuo inferior y muy similar entre ellas (Fig. DISC-1). Centrándonos en la microbiota de las secreciones uropigiales como uno de los ejes principales de esta tesis, llama la atención que la riqueza de especies fue incluso mayor que la albergada en la cloaca de los individuos, a pesar de que la cloaca forma parte del órgano (tracto digestivo) más biodiverso en las aves (Klasing 1999). Además de ser la más rica, la secreción es la única localización que presenta OTUs que, siendo de los más prevalentes (presentes en al menos el 30% de los individuos), son exclusivos (OTU139, OTU171, OTU219) y no fueron detectados nunca en ninguna de las demás localizaciones (CAPÍTULO I, III).

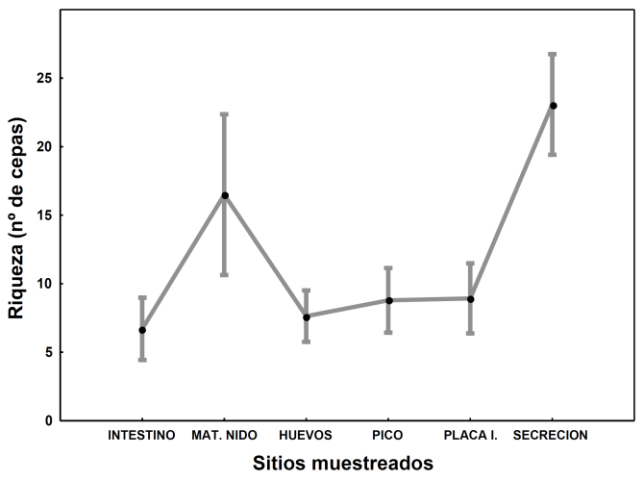

Figura DISC-1. Nº medio de OTUs por individuo (riqueza de cepas bacterianas) según las diferentes localizaciones de estudio de la primera puesta de la población de hembras de cautividad de 2011.

La población de procedencia de las hembras (cautividad frente a libertad en el año 2011) no afectó ni a la composición de la comunidad bacteriana (combinación de OTUs detectados en cada individuo) de las secreciones uropigiales, ni a la riqueza de cepas (nº de OTUs por muestra, (Fig. DISC-2, CAPÍTULO I). Sin embargo, la comunidad sí varió entre las hembras silvestres de 2010 y 2011 (Fig. DISC-2, CAPÍTULO I), indicando un efecto importante del ambiente (clima, temperatura, humedad, etc.) en la microbiota de la glándula uropigial de las hembras.

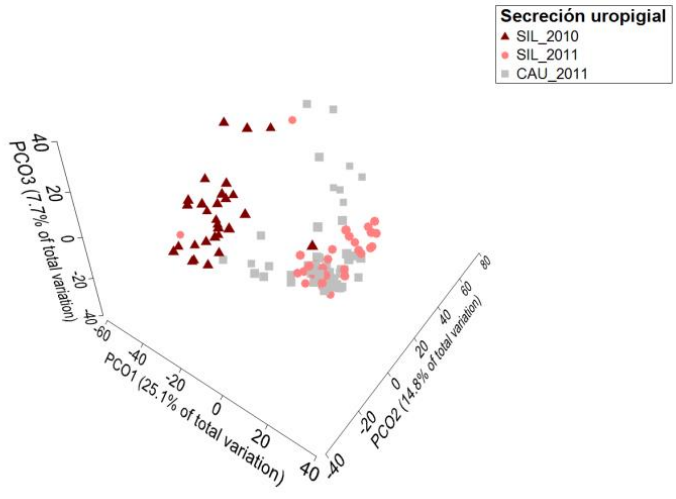

Figura DISC-2. Representación multidimensional (PCoA) basada en las similitudes de las comunidades bacterianas de las secreciones uropigiales de hembras de abubilla. También se muestra la varianza explicada por cada uno de los tres ejes.

Las comunidades de bacterias de las distintas localizaciones corporales estudiadas en las hembras mostraron un patrón de anidamiento desde la glándula uropigial hasta la cáscara del huevo (CAPÍTULO II). Este resultado indica que el acicalado por parte del ave es responsable de una ruta de colonización bacteriana desde la glándula al huevo (pasando por el pico y la placa incubadora) por la que, junto con la secreción, algunas de las

bacterias simbiontes llegarían a la cáscara de los huevos donde se instalaría en los cráteres, característicos de esta especie. Esas bacterias procedentes de la secreción podrían proteger a los embriones de infecciones, y así aumentar el éxito de eclosión (Martín-Vivaldi et al. 2014). No obstante, la comunidad que se establece en la cáscara de los huevos no depende sólo de la que pueda llegar a ella por el acicalado, sino que demostramos experimentalmente que depende también de la comunidad bacteriana del material del nido. La riqueza de cepas bacterianas en las cáscaras de huevos de abubilla fue mayor en nidales con material procedente de nidos donde las abubillas se habían reproducido el año anterior que en los que estaban en contacto con material de muy baja carga bacteriana (CAPÍTULO III). La influencia de las comunidades bacterianas del material del nido sobre las de las cáscaras de los huevos se ha comprobado recientemente en el carricero común (*Acrocephalus scirpaceus)* (Brandl et al. 2014), siendo nuestros resultados con la abubilla la primera evidencia experimental de este efecto.

Aunque la cantidad total de OTUs distintos detectados en las secreciones de los polluelos fue mayor (141 OTUs) que la de las secreciones de hembras (116 OTUs), ni la riqueza de cepas bacterianas por individuo, ni la composición de la comunidad (Fig. DISC-3), ni la prevalencia de los OTUs más abundantes difirieron significativamente entre hembras adultas y pollos. Además, todos los OTUs detectados en al menos un 5% de las hembras en las que muestreamos su secreción estuvieron también presentes en algunas de las secreciones de pollos (CAPÍTULO IV). Estos resultados evidencian la existencia de una comunidad típica de la glándula uropigial de esta especie, y sugieren la posibilidad de que ésta se transmita de hembras a pollos.

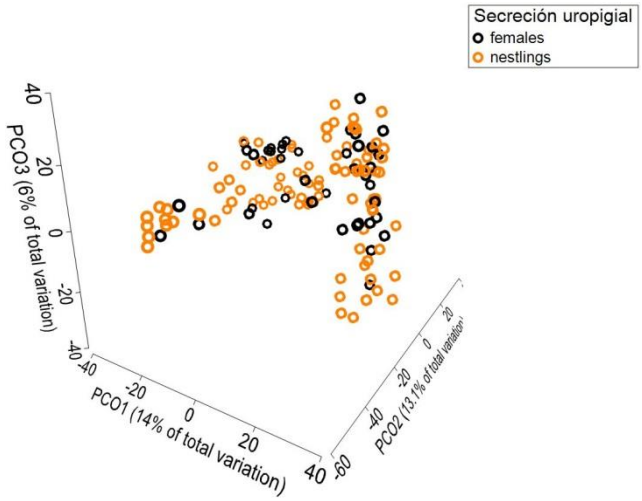

Figura DISC-3. Representación multidimensional (PCoA) basada en las similitudes de las comunidades albergadas en las secreciones uropigiales de hembras y pollos (no movidos en el experimento de intercambio entre nidos) de abubilla. También se muestra la varianza explicada por cada uno de los tres ejes.

En el CAPÍTULO IV mediante un experimento de intercambio de pollos entre nidos ("cross-fostering") ponemos de manifiesto que la comunidad bacteriana de las glándulas de los pollos muestra una mayor influencia del nido de origen que del nido de crianza. La composición de la comunidad bacteriana de los pollos movidos de nido fue mucho más parecida a la de sus hermanos genéticos y a la de su madre biológica, que a la de su madre adoptiva y sus hermanastros con los que crecieron en su nuevo nido. Estos resultados son compatibles con la existencia de una transmisión vertical de las bacterias de madres a hijos. Esta transmisión podría ocurrir por contacto directo entre secreción de madre y glándula de pollos, o por vías indirectas a través de la placa incubadora o la cáscara de los huevos. Otra posibilidad no excluyente que explicaría el efecto del parecido entre madres e hijos, y entre hermanos, es que los pollos heredaran de las madres características de sus glándulas que hicieran más probable la adquisición de unas cepas bacterianas concretas (CAPÍTULO IV). Para comprobar la

importancia relativa de cada uno de esos mecanismos explicando el parecido entre individuos emparentados, sería necesario realizar un experimento manipulando el acceso de las hembras a su secreción uropigial que demuestre si hay transmisión directa desde la glándula de la madre.

A pesar de las evidencias de efectos del nido de origen (i.e., genéticos) en la composición de la comunidad de la secreción, sabemos de experimentos previos de intercambio de pollos entre nidos que también existen efectos importantes del nido de destino (i.e. componente ambiental o evidencia de transmisión horizontal) en la adquisición de las bacterias simbiontes del género *Enterococcus* (Ruiz-Rodríguez et al. 2014). El efecto del nido de destino también se detectó al analizar la variación de la comunidad bacteriana completa de las secreciones de abubillas caracterizadas por métodos moleculares (CAPÍTULO IV). La detección de este componente, implica que la microbiota establecida en los pollos de abubilla es también el resultado de interacciones con el ambiente que les rodea durante el crecimiento.

En todo caso, sigue siendo un enigma el origen de la microbiota que aparece en las glándulas uropigiales de las hembras adultas una vez comienza la época de cría. La secreción tanto de las hembras reproductoras como de los volantones una vez que abandonan el nido se vuelve blanca y carente de bacterias (Soler et al. 2008, Martín-Vivaldi et al. 2009), por lo que la glándula debería repoblarse en el comienzo de cada estación reproductora. Las abubillas, para reproducirse, con frecuencia reutilizan nidos usados previamente por individuos de su misma especie la temporada anterior, por lo que esos nidos podrían servir de fuente de las cepas bacterianas que conforman la comunidad de las glándulas. Sin embargo, este no parece ser el caso pues, aunque hemos puesto de manifiesto que los microbiomas del material del nido y de la glándula comparten algunos OTUs, no detectamos

ninguna asociación entre la presencia de cada cepa en el nido y la glándula del mismo individuo (CAPÍTULO III). Una posibilidad sería que la abubilla mantuviera reservorios de bacterias en otros lugares del cuerpo donde pueden permanecer el resto del año, y que funcionaran como una posible fuente de simbiontes para cada primavera. Dado que sabemos que algunos de los mutualistas típicos de las secreciones de abubillas son enterococos (Martín-Platero et al. 2006, Soler et al. 2008, Ruiz-Rodríguez et al. 2012), y que los enterococos son pobladores habituales de la microbiota intestinal de aves (Moreno et al. 2003, Inger et al. 2003), una posibilidad es que el intestino sirva de reservorio de esas cepas que en primavera ocupan la glándula uropigial. Sin embargo, tampoco encontramos evidencias de la utilización del intestino para inocular la glándula con bacterias simbiontes en las hembras, ya que la presencia de las principales cepas de la comunidad en la glándula no se asoció con su presencia en la cloaca del mismo individuo (CAPÍTULO III). Por tanto, no encontramos evidencias fuertes que nos indiquen la existencia de fuentes o reservorios de estas bacterias simbiontes.

Hay algunos resultados que, indirectamente, sí indican que la cloaca podría estar jugando un papel fundamental en el sistema mutualista de las abubillas y las bacterias de su secreción uropigial. En el CAPÍTULO III, en el que describimos la comunidad de bacterias de la cloaca de las hembras, encontramos que cinco de los ocho OTUs más frecuentes en la cloaca lo son también en la cáscara de los huevos. Esos cinco OTUs son los mismos que resultaron OTUs "clave" en la transmisión bacteriana entre localizaciones corporales durante el acicalado (OTU307, OTU367, OTU407, OTU535, OTU567, CAPÍTULO I). De ellos, tal y cómo se muestra en la Figura DISC-5, el OTU307 y el OTU367, apenas se detectaron en las secreciones uropigiales y además la cepa más abundante en la cloaca (OTU307, 70.83%) también lo fue en los huevos (66.67%, CAPÍTULO III). Todos estos resultados, aparte de sugerir asociaciones directas e indirectas entre cepas de

la cloaca y de la secreción, parecen indicar que algunas cepas bacterianas de las establecidas en las cáscaras de los huevos podrían proceder de la cloaca. La influencia del material del nido en la comunidad de las cascaras de los huevos de las abubillas se puso de manifiesto experimentalmente en el Capítulo III.

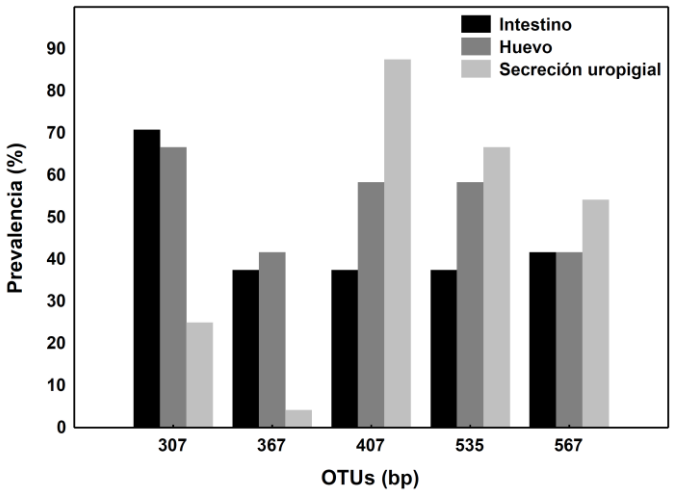

Figura DISC-5. Porcentaje de hembras reproductoras de abubilla (de un total de 24) en las que se ha detectado la presencia de los OTUs más abundantes en más del 30% de las cloacas y a su vez en más del 30% de los huevos o de las secreciones uropigiales (información del CAPITULO III).

La técnica ARISA, utilizada en esta tesis para caracterizar las comunidades bacterianas, no nos permite conocer la identidad taxonómica de las bacterias detectadas, pero proporciona una herramienta muy útil para comparar microbiomas, e incluso poner de manifiesto la existencia de cepas concretas que son clave en el sistema. Ya sabíamos de estudios previos que varias especies de *Enterococcus* están presentes en la comunidad simbionte de la secreción (Martín-Platero et al. 2006, Soler et al. 2008, Ruiz-Rodríguez et al. 2012, 2013), y que juegan un papel fundamental en las características antimicrobianas de la misma (Martín-Vivaldi et al. 2010, Ruiz-Rodríguez et al. 2012, 2013). La secuenciación del ITS de diversas especies del género

Enterococcus obtenidas de la secreción de abubillas, con la misma técnica que la utilizada en este estudio, ha puesto de manifiesto que el tamaño de ITS de este género se identifica en ARISA con dos picos diferentes uno de 306-309pb y otro con 406-409 pb (Martínez-Bueno, Unpublished data). Estos resultados indican que al menos algunos de los OTUs "clave" detectados en esta aproximación del estudio de comunidades completas (OTU307 y OTU407) son enterococos. Por tanto, muchos de los resultados encontrados en trabajos anteriores centrados en las bacterias cultivables (Martín-Platero et al. 2006, Soler et al. 2008, 2010, Ruiz-Rodríguez et al. 2009a, 2012, 2013, 2014, Martín-Vivaldi et al. 2010, 2014) reflejan una parte importante del funcionamiento del sistema abubillas-simbiontes.

Los resultados de esta tesis, que ha abordado el estudio del sistema formado por la abubilla y las bacterias simbiontes que viven en su glándula uropigial desde una perspectiva de meta-comunidades, nos permiten dibujar un posible escenario de funcionamiento de este mutualismo integrado en la dinámica de comunidades microbianas existente en el interior de los nidos de esa especie. La glándula uropigial de los pollos y hembras de abubilla ha resultado ser un ecosistema dinámico que soporta una comunidad clímax formada por unas pocas cepas (Fig. DISC-6) que aparecen con bastante frecuencia (superior al 50%), y un grupo numeroso de taxones menos habituales que puede aparecer en la secreción de forma más o menos esporádica (<50 % de prevalencia). En la parte de la comunidad más o menos estable (i.e., aquella formada por cepas de alta prevalencia), hay varios taxones, entre ellos varios enterococos, que parecen ser clave ("keystone species" sensu Mills et al. 1993). Estos aparecen en todas las comunidades con las que la secreción interactúa CAP.I, CAP.II, CAP.III, y son determinantes en la obtención de ventajas antimicrobianas para el hospedador en su uso de la secreción (Soler et al. 2008, Martín-Vivaldi et al. 2014). Por lo tanto, las relaciones detectadas entre distintas comunidades ayudan a entender

la dinámica de estos enterococos claves en la relación entre abubillas y bacterias.

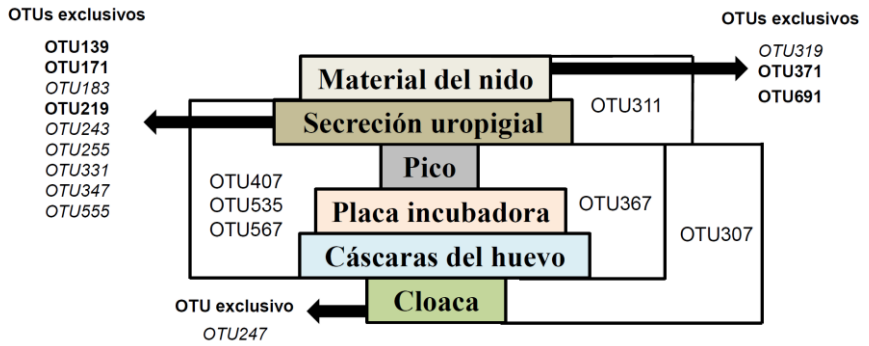

Figura DISC-6. OTUs que se detectan con una prevalencia mayor del 50% en alguna de las localizaciones y a su vez en más del 40% en cualquiera de los demás sitios de estudio. Los OTUs compartidos entre distintas localizaciones se exponen dentro de cajas que abarcan las localizaciones donde aparecen. Además se muestran los sitios que presentan OTUs exclusivos con una prevalencia de al menos 30%. En negrita se muestran aquellos OTUs nunca detectados en otras localizaciones y en cursiva aquellos que aunque se detectaron en otros sitios, la prevalencia en localizaciones distintas a la especificada fue siempre menor del 10%.

La metodología empleada no nos permite conocer la importancia relativa a nivel cuantitativo de estas cepas que han resultado ser las más prevalentes (un objetivo importante a cubrir en estudios futuros con la ayuda de herramientas de secuenciación masiva). Sin embargo, de los resultados obtenidos parece desprenderse que realmente son características de este ecosistema. Por ello, la identificación de las que aún no lo han sido (todas menos los enterococos) aportaría pistas para entender su funcionalidad y su posición en el conjunto de interacciones que se den en la comunidad, así como las cualidades que puedan aportar a la secreción y que se sumen a las ya conocidas de los enterococos (Martín-Platero et al. 2006; Martín-Vivaldi et al. 2010; Ruiz-Rodríguez et al. 2012).

Los resultados de esta tesis también apuntan a que la comunidad de la secreción de las hembras de abubilla es una "comunidad clave" (Mouquet et al. 2013) en el escenario de la meta-comunidad bacteriana existente en los nidos activos de abubilla. Varias de las comunidades bacterianas presentes en el nido (pico y placa incubadora de la hembra, cáscaras de los huevos, glándulas de pollos, CAP.I, CAP.II, CAP.IV) parecen depender en gran medida de la capacidad de transmisión y de la capacidad competitiva de cepas presentes en la secreción. El patrón de anidamiento detectado sugiere que no todas las cepas presentes en la secreción tienen la misma probabilidad de establecerse en el pico y/o en la placa incubadora. De esas, no todas las cepas tienen la misma probabilidad de llegar a la cáscara del huevos, siendo estos enterococos algunos de los que lo consiguen (CAP. II). Estas cepas que logran llegar a la cáscara de los huevos serían por tanto aquellas con mayores capacidades de dispersión entre comunidades, lo cual, a su vez, debería estar explicado por sus capacidades competitivas frente a otras cepas también presentes en el ambiente del nido. Por todo ello, podemos concluir que el uso de la secreción uropigial con simbiontes juega un papel crucial en la dinámica de comunidades bacterianas de los nidos de abubilla, incluidas aquellas que contengan agentes patógenos para las aves. Las bacterias simbiontes, por tanto, en la abubilla se unirían a otros caracteres defensivos frente a patógenos.

Para distintas especies de aves se conocen los efectos de antimicrobianos de su secreción uropigial (Shawkey et al. 2003), del comportamiento de incubación (Cook et al. 2003, 2005a, b, Shawkey et al. 2009, Lee et al. 2014), de los materiales utilizados en la construcción del nido (Clark 1991, Mennerat et al. 2009, Peralta-Sanchez et al. 2010) y de algunas propiedades físicas del nido (Ruiz-de-Castañeda et al. 2011, Horrocks et al. 2014, Peralta-Sánchez et al. 2014). Nuestros análisis descriptivos han puesto de manifiesto asociaciones que nos indican un papel fundamental de la

comunidad bacteriana de la secreción de la abubilla en las comunidades del pico, placa incubadora, y cáscara de los huevos. Esas comunidades pueden ser invadidas por bacterias con efectos negativos para las aves (bacterias degradadoras de plumas, provocadoras de infecciones en la piel, patógenas de embriones, etc.) y nuestro enfoque metapoblacional indica que esas contaminaciones pueden ser controladas por la comunidad de la secreción. El siguiente paso, por tanto, es determinar el papel de las cepas de la secreción con mayores capacidades de dispersión en el control de la estabilidad de las comunidades del huésped y de su infección por patógenos. Su importancia podrá ponerse de manifiesto en futuros estudios a través de manipulaciones de la presencia de esas cepas en dichas comunidades.

CONCLUSIONES

1. En términos de número de Unidades Taxonómicas Operacionales (en inglés OTUs) y de composición de la comunidad, el microbioma de la secreción uropigial difirió del de la placa incubadora, el de las cáscaras de los huevos y el del pico de las hembras de abubilla. Sin embargo, algunos de los taxones más comunes detectados en la secreción uropigial también se hallaron frecuentemente en las demás comunidades estudiadas.

2. La detección de algunas bacterias "clave" en las cáscaras de los huevos se asoció positivamente con su presencia en las muestras de la glándula uropigial, del pico y/o de la placa incubadora de las hembras. Dos de esas cepas bacterianas (OTU307 y OTU407) son enterococos productores de antibióticos que ayudan a las abubillas en su defensa antimicrobiana.

3. La comunidad bacteriana de las cáscaras de los huevos resultó anidada en la de la placa incubadora; ésta en la del pico y la del pico en la comunidad de la secreción. Este patrón de anidamiento sugiere que las hembras de abubilla usan el acicalado para transmitir a las cáscaras de los huevos las bacterias simbiontes de la secreción uropigial ayudando a proteger al embrión de infecciones patógenas.

4. No encontramos ningún apoyo a un hipotético papel de la microbiota del material del nido y de la cloaca como reservorios de bacterias simbióticas de la secreción uropigial de abubillas. Sin embargo, obtuvimos resultados (primera evidencia experimental de ello) que demuestran la influencia de los materiales del nido en la comunidad bacteriana de las cáscaras del huevo.

5. El resultado de la interacción entre las bacterias productoras de antibióticos procedentes de la secreción uropigial y las procedentes de los materiales del nido determinan el microbioma en las cáscaras de los huevos. Por lo tanto, las bacterias simbiontes reducirían la probabilidad de infección de los embriones a través de la cáscara por las bacterias patógenas que colonicen la superficie del huevo.

6. La comunidad bacteriana de la secreción uropigial de los pollos intercambiados entre nidos estuvo explicada tanto por el nido de origen como por el nido de crianza, pero fue más similar a las comunidades bacterianas de sus hermanos y su madre que a las de sus hermanastros y su madre adoptiva. Estas similitudes entre individuos emparentados se podrían explicar por transmisión vertical temprana de simbiontes de la madre a los hijos y/o por la existencia de características de la glándula uropigial de los pollos, heredadas de las madres, que maximicen la probabilidad de adquirir determinadas bacterias simbiontes del entorno (el nido).

CONCLUSIONS

1. The composition of the bacterial community of the uropygial secretion differed from those on the eggshells, beak and brood patch of incubating hoopoe females in terms of number of Operational Taxonomic Units and bacterial assemblage. However, some of the most common taxa detected in the uropygial secretion were also detected at high prevalence within the other studied communities.

2. The detection of some "key bacteria" on the eggshells was positively associated with their occurrence in samples of the uropygial gland, the beak and/or brood patch of the incubating females. Two of those bacterial strains (OTU307 and OTU407) are antibiotic-producing enterococci that help hoopoes in their antimicrobial defense.

3. The bacterial community of the eggshells of hoopoes was nested within the community of the brood patch; the brood patch community was nested within the community of the beak; and the community of the beak within the community of the uropygial secretion. This nested pattern suggests that preening behaviour of female hoopoes with uropygial secretion containing bacterial symbionts is used to transmit them to eggshells to prevent embryo pathogenic infections.

4. We did not find support to the hypothetical role of nest material and gut microbiotas as reservoirs of symbiotic bacteria of the uropygial secretion of hoopoes, but our results are the first experimental evidence of the influence of nest materials on the bacterial community of the eggshells.

5. The outcome of the interaction between antibiotic producing bacteria from the uropygial secretion and those from the nest materials determines the microbiome on the eggshells. Therefore, symbiotic bacteria would reduce the probability of trans-shell infection of embryos by pathogenic bacteria colonizing eggshells.

6. The bacterial community of the uropygial secretion of cross-fostered nestlings was explained both by nest of origin and nest of rearing, but it was more similar to bacterial communities of their siblings and mother than to those of stepsiblings and stepmothers. These similarities among related individuals would be explained by early vertical transmission of symbionts from mother to offspring and/or by particularities of the uropygial gland of offspring, that were inherited from females and enhance probability of acquiring particular bacterial symbionts from the nest environment.

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APÉNDICES

CAPÍTULO I

Appendix 1. Relationships of OTU co-occurrence between pairs of sampled sites (UO *vs.* B, B *vs.* BP, BP *vs.* E, B *vs.* E, UO *vs.* E, UO *vs.* BP) within females, being UO (uropygial oil), B (beak), BP (brood patch) and E (eggshells). The p-values obtained by means of Log-linear analyses were corrected for multiple tests by using FDR methodology. Three of 27 frequent OTUs (139 bp, 171 bp, 219 bp) were specific of uropygial oil (UO) and were not used for this analysis. N represents the number of females in which each OTU was detected in the two sampled sites compared.

	UO *vs* B					**B *vs* BP**					**BP *vs* E**				
OTU	N	$\chi 2$	p	Rs	p	N	$\chi 2$	p	Rs	p	N	$\chi 2$	p	Rs	p
183	11					4					1				
195	10					3					2				
243	1					2					1				
255	2					0					0				
275	14					2					4				
279	22	0.03	0.878	0.02	0.871	13					6				
303	2					2					4				
307	10					37	33.24	0.002	0.56	0.001	44	37.76	0.002	0.6	0.001
311	20	0.78	0.390	-0.09	0.396	16					10				
327	10					11					7				
331	5					2					5				
339	1					0					0				
347	8					2					1				
351	3					6					1				
367	10					41	13.4	0.002	0.37	0.001	49	53.28	0.002	0.7	0.001
407	32	3.02	0.091	0.17	0.103	30	39.07	0.002	0.61	0.001	30	31.23	0.002	0.55	0.001

467	31	4.26	0.045	0.2	0.06	20	15.6	0.002	0.4	0.001	6				
471	8					7					2				
475	15					16					3				
511	4					1					1				
535	47	11.17	0.001	0.34	0.002	51	29.37	0.002	0.54	0.001	40	14.14	0.002	0.37	0.001
555	3					1					3				
563	3					4					4				
567	37	5.51	0.025	0.24	0.024	40	33.02	0.002	0.57	0.001	32	13.48	0.002	0.37	0.001

	B *vs* E					**UO *vs* E**					**UO *vs* BP**				
OTU	N	$\chi 2$	p	Rs	p	N	$\chi 2$	p	Rs	p	N	$\chi 2$	p	Rs	p
183	0					1					7				
195	2					3					4				
243	0					1					2				
255	0					0					3				
275	2					5					6				
279	4					9					19				
303	2					5					5				
307	35	21.85	0.002	0.46	0.001	11					12				
311	16					19					12				
327	7					7					10				
331	1					7					7				
339	0					1					1				
347	0					1					9				
351	1					1					5				
367	41	7.13	0.010	0.27	0.010	12					12				

407	26	21.25	0.002	0.46	0.001	35	4.25	0.045	0.2	0.048	37	7.32	0.009	0.26	0.011
467	3					6					28	1.61	0.217	0.12	0.237
471	2					3					8				
475	5					4					15				
511	1					3					1				
535	38	17.52	0.002	0.42	0.001	39	9.67	0.003	0.31	0.001	53	16.14	0.002	0.41	0.001
555	1					7					4				
563	2					4					8				
567	32	14.89	0.002	0.38	0.001	33	5.16	0.025	0.23	0.001	38	6.28	0.014	0.25	0.016

CAPÍTULO II

Appendix 1. Mean and Standard Error (SE) of nestedness index (NODF) of bacterial communities of uropygial secretion, beak, brood patch and eggshells (SBPE) of hoopoes, and of those of the secretion, beak and eggshell (SBE). Average and SE of the statistic (Z-values) testing significance of nestedness values of each sample, as well as the 95% confidence interval (CI) is also shown together with sample size. Z- values largest than 1.64 are considered to reflect significant nestedness of the analyzed matrices. Confidence intervals do not including this value are in bold. We provide values considering all samples together, but also for different years, different breeding attempts, and for captivity and wild hoopoe populations.

		NODF		Z-NODF			
	N	Mean	SE	Mean	SE	CI-95% (MIN)	CI-95% (MAX)
All samples							
SBPE	97	53.08	1.83	3.96	0.33	**2.81**	**3.74**
SBE	97	48.23	2.05	4.04	0.32	**2.74**	**3.64**
Year variation (SBPE) (only wild nests considered)							
2010	27	44.73	3.36	2.17	0.36	1.43	2.91
2011	27	53.74	2.97	5.05	0.73	**3.55**	**6.54**
Year variation (SBE) (only wild nests)							
2010	27	36.80	3.62	1.99	0.30	1.39	2.60
2011	27	47.68	3.28	4.53	0.55	**3.40**	**5.65**
Breeding attempt variation (SBPE) (only 2011 nests)							
1st	43	48.77	2.49	3.60	0.50	**2.59**	**4.62**
2nd	11	51.04	6.05	3.62	1.04	1.30	5.93
Breeding attempt variation (SBE) (only 2011 nests)							
1st	43	41.71	2.82	3.12	0.34	**2.43**	**3.81**
2nd	11	44.31	6.01	3.79	1.14	1.25	6.34
Population variation (SBPE) (only 2011 nests)							
Wild	27	53.74	2.97	5.05	0.73	**3.55**	**6.54**
Captivity	42	57.48	2.84	4.26	0.46	**3.33**	**5.19**
Population variation (SBE) (only 2011 nests)							
Wild	27	47.68	3.28	4.53	0.55	**3.40**	**5.65**
Captivity	42	55.67	3.10	5.07	0.54	**3.98**	**6.16**

CAPÍTULO III

Appendix 1.

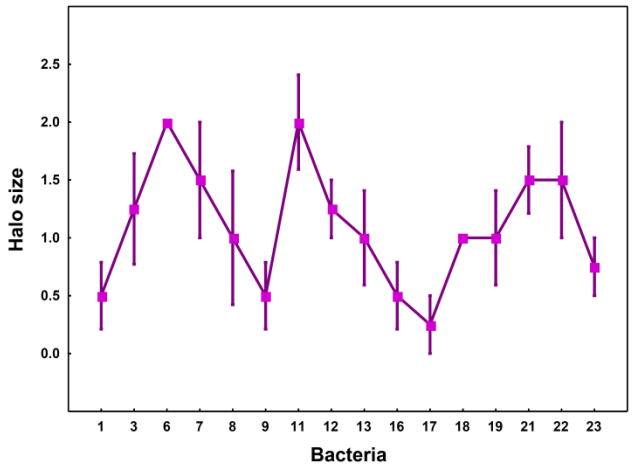
a) Study of the bacterial community of the nest materials used in the experiment.

Approximately the same amount of experimental (mean(SE) = 37.75(2.85)g) and control (mean(SE) = 37.75(2.85)g) material were diluted in 1ml of 0.2M pH7.2 phosphatase saline buffer. Bacterial load was estimated as Number of Colony Forming Units (CFUs) in TSA media and serial dilutions. The estimates were adjusted to the volume of solution used for cultivation and to the slight variation of weight of nest material employed for cultivation. Experimental material harbored bacteria at very low density (log10 transformed values, mean = 0.20,± 95%CI: -0.01 – 0.42, N = 10) in comparison with that of control material (log10 transformed values, mean = 5.10,± 95% CI: 3.79 – 6.41, N = 10; $F = 69.67$, df = 1,18, $P < 0.00001$).

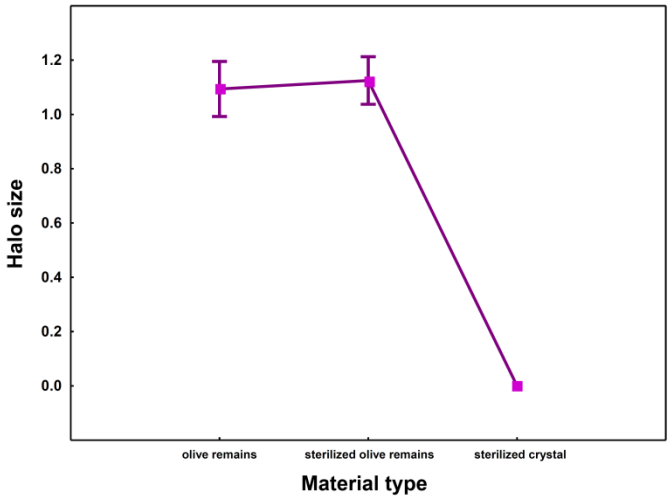
b) Study of the antimicrobial activity of the experimental material used (pellets of olive remains).

Antimicrobial activity of experimental nest material was tested against the following bacterial strains that included known pathogenic and keratinolytic bacteria: *Proteus sp.*, *Escherichia coli*, *Mycobacterium sp.*, *Bacillus licheniformis* D13, *Sthaphylococcus aureus*, *Klebsiella sp.*, *Bacillus megatherium*, *Micrococcus luteus*, *Bacillus thuriguensis*, *Enterococcus faecalis* MRR-103, *Listeria monocytogenes* 4032, *Listeria inocua* CECT 340, *Lactobacillus plantarum* CECT 784, *Enterococcus faecium* 34, *Lactobacillus paracasei* 11-2, *Lactobacillus lactis lactis* LM2301(respectively strains 1, 3, 6, 7, 8, 9, 11, 12, 13, 16, 17, 18, 19, 21, 22, 23 in Fig A1). Inhibitory activity of olive remains differed depending of the bacteria strains tested (Fig. A.1a, $F = 4.33$, df = 15, 144, $P = 0.001$), but was consistently higher than that of control piece of plastic (Fig, A1b, $F = 95.77$, df = 2, 144). Average size of the inhibition halo of sterilized and non-sterilized olive remains did not differ (suggesting that these properties are independent of the bacterial community associated (Fig. A1b).

A.1a)


Halo size
2.5
2.0
1.5
1.0
0.5
0.0
1
3
6
7
8
9
11
12
13
16
17
18
19
21
22
23
Bacteria

A.1b)


Halo size
1.2
1.0
0.8
0.6
0.4
0.2
0.0
olive remains
sterilized olive remains
sterilized crystal
Material type

CAPÍTULO IV

Appendix 1. Prevalence (%) of different bacterial OTUs (named by their length in terms of base pairs (bp)) found in all sampled uropygial glands of nestlings (N=165) and females (N=44). Bold numbers show OTUs that were detected in more than 30% of samples from females or nestlings

	Prevalence(%)	
OTU	Females	Nestlings
100	11.11	9.09
103	2.22	1.82
107	0.00	0.61
111	6.67	10.30
115	4.44	1.21
119	0.00	9.09
123	0.00	3.64
127	15.56	16.97
131	13.33	27.27
135	11.11	15.15
139	28.89	16.36
143	2.22	0.00
147	13.33	22.42
151	4.44	13.33
155	8.89	21.82
159	2.22	7.88
163	6.67	4.24
167	0.00	4.24
171	33.33	2.42
179	2.22	3.03
183	84.44	80.00
187	8.89	10.91
191	17.78	20.61
195	44.44	58.18
199	15.56	13.94
203	4.44	1.21
207	0.00	1.21
211	2.22	3.03
215	4.44	6.67
219	44.44	47.27
223	4.44	3.03
227	8.89	4.85
231	15.56	10.91
235	17.78	2.42
239	11.11	10.91
243	57.78	58.79
247	15.56	6.67
251	2.22	2.42

255	75.56	73.33
259	2.22	6.06
263	20.00	18.79
267	0.00	1.82
271	15.56	18.18
275	48.89	60.61
279	80.00	78.18
283	31.11	25.45
287	6.67	10.91
291	0.00	6.67
295	0.00	1.21
299	22.22	13.33
303	37.78	41.21
307	13.33	31.52
311	53.33	46.67
315	4.44	7.27
319	28.89	28.48
323	4.44	10.91
327	13.33	28.48
331	55.56	45.45
335	11.11	24.24
339	33.33	26.67
343	6.67	15.15
347	73.33	70.91
351	22.22	31.52
355	17.78	13.33
359	2.22	2.42
363	31.11	24.24
367	15.56	16.97
371	0.00	1.21
375	0.00	0.61
379	33.33	31.52
383	0.00	0.61
387	0.00	1.21
391	4.44	5.45
395	2.22	6.06
399	11.11	6.06
403	6.67	5.45
407	80.00	85.45
411	11.11	13.94
415	2.22	1.21
419	8.89	18.79
423	24.44	21.82
427	13.33	20.00
431	0.00	2.42
439	8.89	8.48
451	2.22	4.85
455	4.44	1.82
459	2.22	3.03
463	4.44	3.64
467	75.56	80.00
471	51.11	44.24
475	44.44	43.03
479	15.56	14.55

483	2.22	1.21
487	2.22	2.42
491	24.44	17.58
495	4.44	8.48
499	2.22	5.45
503	0.00	1.21
507	6.67	4.85
511	28.89	25.45
515	17.78	12.73
519	17.78	14.55
523	15.56	16.97
527	11.11	38.18
531	24.44	32.12
535	68.89	56.97
539	11.11	10.91
543	2.22	17.58
547	0.00	2.42
551	6.67	6.06
555	55.56	26.06
559	15.56	8.48
563	24.44	34.55
567	66.67	55.76
571	8.89	12.73
575	2.22	5.45
579	11.11	4.24
583	17.78	18.79
587	13.33	10.91
591	2.22	2.42
595	8.89	4.85
599	6.67	4.24
603	4.44	0.00
611	6.67	4.24
619	4.44	2.42
639	2.22	1.21
647	8.89	4.85
651	0.00	0.61
659	2.22	4.24
667	0.00	0.61
675	0.00	0.61
679	0.00	0.61
699	2.22	9.70
703	0.00	1.82
711	2.22	2.42
715	0.00	0.61
719	0.00	0.61
731	0.00	1.21
755	0.00	0.61
767	4.44	2.42
775	0.00	0.61
779	0.00	0.61
847	0.00	0.61

"Cuando creíamos que teníamos todas las respuestas, de pronto, cambiaron todas las preguntas"

Mario Benedetti

CSIC

Consejo Superior de Investigaciones Científicas