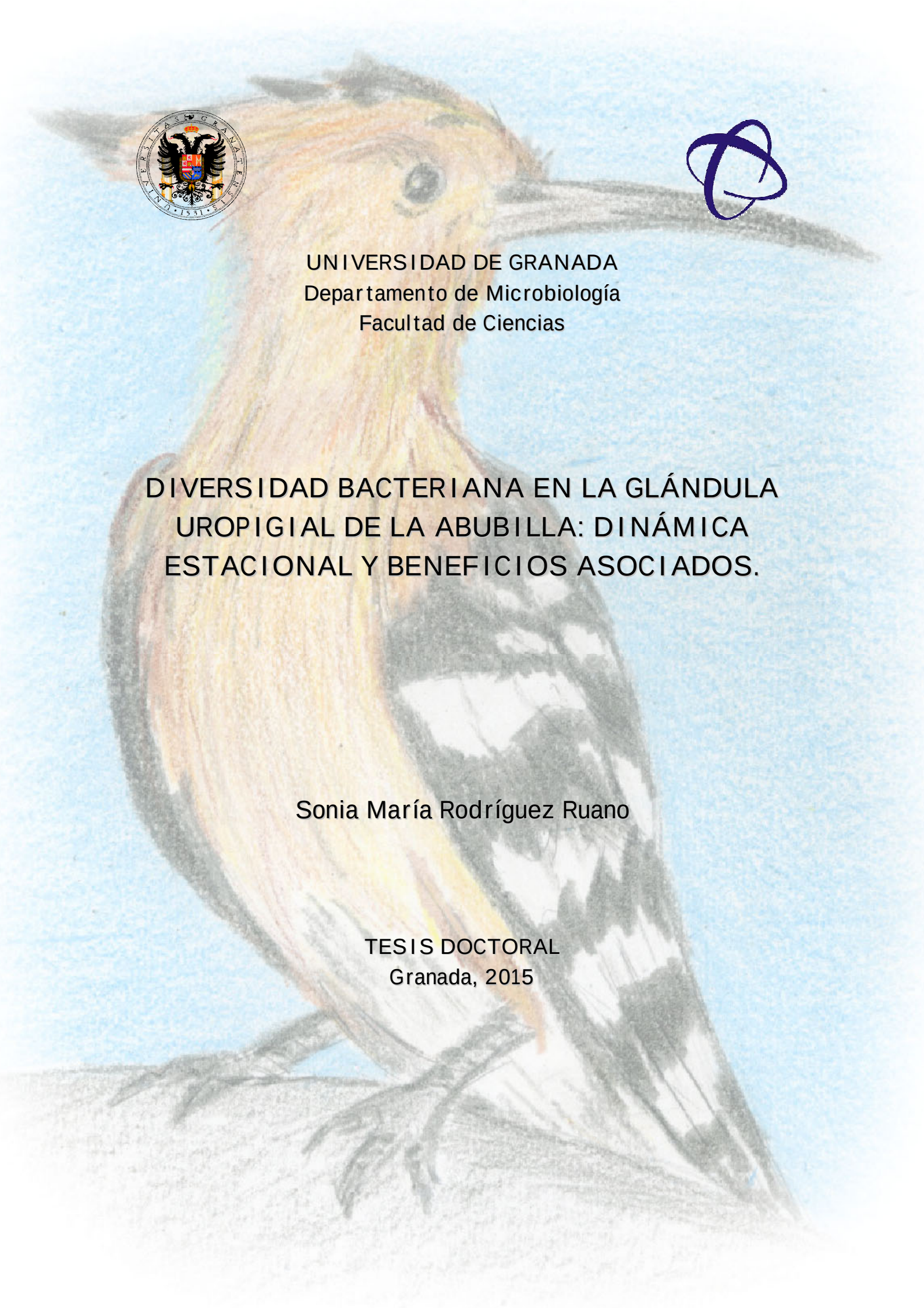

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Sonia María Rodríguez Ruano

TESIS DOCTORAL
Granada, 2015

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Memoria presentada por la licenciada en Biología Dña. Sonia María Rodríguez Ruano para optar al Grado de Doctor en Ciencia Biológicas por la Universidad de Granada con mención internacional.

Esta tesis ha sido dirigida por Manuel Martínez Bueno, Catedrático de la Universidad de Granada y Manuel Martín-Vivaldi Martínez, Profesor Titular de la Universidad de Granada.

Vº Bº de los Directores

Manuel Martínez Bueno

Manuel Martín-Vivaldi Martínez

La doctoranda

Sonia María Rodríguez Ruano

Esta Tesis Doctoral ha sido realizada en el Departamento de Microbiología (Facultad de Ciencias) de la Universidad de Granada durante los años 2011-2015 dentro del grupo de investigación Antagonismo microbiano y biodiversidad (BIO-160).

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- **Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA.** Tema: Determinación de las variaciones estacionales, interanuales, y asociadas al ciclo reproductor del hospedador que afectan al microbioma de la glándula uropigial de las hembras de abubilla. Duración: 113 días (09/09/2013-30/12/2013). Financiación: Ayudas para la realización de estancias breves en otros centros de I+D (FPI) 2012, Ministerio de Ciencia e Innovación.

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La doctoranda, Sonia María Rodríguez Ruano, y los directores de la tesis, Manuel Martínez Bueno y Manuel Martín-Vivaldi Martínez, garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

En Granada, a 5 de Noviembre

Directores de la Tesis

Fdo.: Manuel Martínez Bueno Fdo.: Manuel Martín-Vivaldi Martínez

Doctoranda

Fdo.: Sonia María Rodríguez Ruano

Portada: Encarnación Ruano Quesada

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A la suerte, por no quitármelo.

Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.

(Antonio Machado)

ÍNDICE

RESUMEN

La glándula uropigial de la abubilla europea (*Upupa epops*), es una glándula holocrina encerrada dentro de una cápsula de tejido conectivo que deposita su secreción en un tubo colector común. Las aves esparcen esta secreción sobre la superficie de su piel y de su plumaje de manera periódica, en un proceso conocido como *"preening"* o acicalamiento. A esta secreción se le han atribuido distintas propiedades, tales como protección e impermeabilización de las plumas, o el mantenimiento de la higiene frente a patógenos y parásitos. En el caso de la abubilla, tanto su volumen como composición química se han relacionado con factores estacionales y sexuales, produciéndose una secreción más oscura y maloliente en hembras reproductoras y en pollos durante el período de estancia en el nido, mientras que en machos y en hembras no reproductoras la secreción producida es blanquecina e inodora. Además de las diferencias encontradas en su composición química entre ambos tipos de secreciones, a partir de las marrones se han aislado bacterias pertenecientes mayoritariamente al género *Enterococcus*. Sin embargo, las observaciones mediante microscopia electrónica de transmisión de la glándula, han puesto de manifiesto una gran heterogeneidad de tipos morfológicos microbianos lo que sugiere la existencia de una diversidad mucho más compleja de la inicialmente propuesta.

En esta memoria de Tesis Doctoral se ha determinado la comunidad microbiana de la secreción de la glándula uropigial de la abubilla mediante utilización de métodos independientes de cultivo, como es el establecimiento de huellas genéticas de la comunidad o la secuenciación masiva

(pirosecuenciación y secuenciación por síntesis). También se ha descrito la dinámica poblacional de esta comunidad a lo largo del ciclo reproductor del ave y su relación con otros factores ambientales. Por último se han demostrado experimentalmente los efectos beneficiosos que podrían representar para el animal la presencia de esta comunidad de bacterias comensales y/o mutualistas, evaluando su transmisión vertical desde la glándula a los huevos y su posible relación con el éxito de eclosión.

Un primer enfoque sobre la diversidad microbiana de la secreción uropigial se ha realizado mediante el análisis de librerías de los ADNs ribosomales y/o secuenciación directa de sus amplicones. Estas técnicas han puesto de manifiesto una compleja comunidad bacteriana formada principalmente por especies de microorganismos anaerobios estrictos y facultativos incluidos en el filo *Firmicutes* (clases *Clostridia* y *Negativicutes*) y en el filo *Bacteroidetes*. También con menor frecuencia se han identificado taxones pertenecientes a los filos *Actinobacteria* y *Proteobacteria*. Mediante estudio del perfil genético de la comunidad realizado a través del análisis de las regiones intergénicas entre los genes ADNr 16S y 23S o de electroforesis en gradiente temporal de temperatura, se ha puesto de manifiesto la gran homogeneidad existente entre los individuos de esta comunidad sobre la base de su similitud de los perfiles de bandas obtenidos para cada una de las muestras objeto de estudio.

El microbioma uropigial se ha obtenido mediante métodos de secuenciación masiva, estando claramente representado por microorganismos anaerobios estrictos incluidos en el grupo de los clostridios (filo *Firmicutes*, clase *Clostridia*), aunque también aparecen ciertos representantes de otros filos

como *Proteobacteria*, *Bacteroidetes* y *Actinobacteria*. El hecho de que la composición predominante de las comunidades uropigiales incluya bacterias que también se encuentran con frecuencia en el hábitat del tracto gastrointestinal (tGI), sugiere una posible colonización de la glándula a partir del tGI del animal de al menos parte de la comunidad bacteriana. Mediante el uso de herramientas como la PCR cuantitativa y FISH se ha determinado la carga microbiana tanto de las secreciones de las hembras y los pollos durante su estancia en el nido, las cuales muestran una concentración mucho más elevada que las secreciones de las hembras en invierno y fuera de su periodo reproductor. En este último caso es similar a la de los machos.

Se ha encontrado que los únicos factores que modifican la composición del microbioma de la secreción uropigial de abubilla son el ciclo reproductor, la cría en cautividad y en ocasiones el año de muestreo. Sin embargo su composición no parece estar relacionada con factores ambientales ni geográficos. Los cambios más importantes que suceden durante el periodo reproductor de las hembras se refieren al cambio de una secreción blanquecina en la que predominan las clases *Gamma* y *Betaproteobacteria* (filo *Proteobacteria*), *Flavobacteria* (filo *Bacteroidetes*), *Bacilli* y *Clostridia* (filo *Firmicutes*) en menor proporción, a una comunidad claramente dominada por la clase *Clostridia*. Además de estos cambios a gran escala, como es lógico ocurre una sucesión de OTUs que aumentan (o disminuyen) relativamente su abundancia en la secreción uropigial a lo largo de los periodos muestreados. Dichas OTUs no son siempre las mismas, y esto hace pensar que podría haber cierta influencia ambiental en la composición de la glándula secreción uropigial.

Por último se ha demostrado experimentalmente que la microbiota de la secreción uropigial de la abubilla contribuye a la composición de la comunidad bacteriana establecida en la superficie de la cáscara de los huevos, de forma que a partir de cierto umbral, esta contribución puede resultar beneficiosa en cuanto que se relaciona con una disminución en el nivel de colonización del huevo por bacterias potencialmente patógenas provenientes de una fuente experimental de contaminación externa.

INTRODUCCIÓN

Los estudios ecológicos y evolutivos, que tradicionalmente se han centrado en explicar la variación individual y los factores que determinan el éxito reproductivo asociado, pueden beneficiarse hoy en día de las nuevas perspectivas que consideran por un lado las características fenotípicas directamente ligadas al genoma del individuo (por ejemplo los órganos) y por otro las que están más relacionadas con el microbioma (la perspectiva del holobionte, Bordenstein y Theis, 2015). Esto se debe a que la comunidad de microorganismos simbiontes y los factores que afectan su estabilidad pueden tener consecuencias drásticas en el fenotipo del hospedador y su supervivencia (Flórez *et al.*, 2015; Peterson *et al.*, 2015). Un primer paso necesario para entender estos efectos es describir la composición y la variación de las comunidades microbianas y explorar los factores ambientales que las afectan, además de identificar miembros claves que desarrollan funciones esenciales (Rajilić-Stojanović, 2013). Además, explorar la variación entre comunidades simbiontes en función de as características del hospedador y su ciclo vital puede ser útil para entender la función de la microbiota para el hospedador, y sus consecuencias para los procesos coevolutivos entre ambos (Moran, 2006; McFall-Ngai, 2006; Scheuring y Yu, 2012).

En esta tesis se utiliza esta aproximación, mediante el uso de métodos moleculares, para caracterizar la comunidad de simbiontes alojados en la glándula uropigial de la abubilla, lo que nos permite avanzar en la comprensión de un sistema en el que ya se ha comprobado la presencia y la importancia de un grupo particular de bacterias, los enterococos.

En los siguientes apartados se explican distintos aspectos de las relaciones entre seres vivos, particularmente las que implican a las bacterias, del sistema de estudio: la glándula uropigial de la abubilla y de los avances metodológicos que permiten analizar la composición de comunidades complejas, para mostrar la importancia de abordar la comprensión completa de la dinámica del microbioma de la glándula uropigial de esta especie de ave.

1. Mutualismo

El mutualismo se produce cuando distintos organismos interaccionan de forma que el éxito reproductor, potencial o real, de cada uno incrementa por la acción de los demás miembros del sistema (Janzen, 1985). Aunque puede darse entre miembros de una misma especie (mutualismo conspecífico, cooperación y altruismo), en general se habla de mutualismo refiriéndose al mutualismo aloespecífico, o entre organismos de distintas especies. Es el caso de las relaciones que se establecen entre algunos rumiantes y las aves insectívoras que se alimentan de sus parásitos, o entre las hormigas que se alimentan de sustancias producidas por pulgones o cochinillas a los que protegen y facilitan el acceso a su alimento vegetal (Figura I.1).

Figura I.1. Hormigas colocando cochinillas de los cítricos sobre un limonero. Por Katja Schulz [CC BY 2.0].

De hecho, este tipo de asociación mutualista se establece más frecuentemente entre individuos muy distantes filogenéticamente, a menudo de distintos reinos biológicos (Leigh Jr, 2010). La consolidación de este tipo de interacción se puede explicar evolutivamente de dos formas: por combinación de habilidades muy distintas, o por baja competencia directa (Leigh Jr, 2010). En el primer caso, individuos muy diferentes poseen habilidades muy distintas que pueden suponer un gran avance cuando se combinan, lo que significaría una gran ventaja evolutiva que favorecería la consolidación del mutualismo. Esto es lo que ocurre, por ejemplo, cuando un hongo y un alga se combinan para dar lugar a un liquen, éste es capaz de aprovechar el agua y la luz de forma mucho más eficiente que sus constituyentes por separado. En el segundo caso, la competencia directa entre individuos muy distantes es menor que aquella que ocurre entre individuos muy próximos filogenéticamente, de forma que los efectos negativos que podrían desequilibrar el resultado positivo del mutualismo, y provocar su desaparición, son menores. En el caso de las plantas y sus polinizadores (Figura I.2), la competencia por recursos como los

nutrientes o el espacio no suponen un problema para su relación mutualista, puesto que ambos organismos tienen formas de vida muy diferentes.

Figura I.2. Polinización de una flor por un insecto. Por Beatriz Moisset [CC BY-SA 2.5].

El mutualismo, tal y como se define teóricamente, no es fácil de estudiar por varias razones. La primera es que a veces los beneficios para alguno de los participantes no son claros, problema que se acentúa cuando en lugar de una interacción uno a uno tenemos interacciones múltiples entre más de dos organismos. Además, la propia definición de mutualismo implica que los resultados de la interacción sean siempre favorables, cosa que puede no ser cierta en todo momento (Janzen, 1985). De este modo cuando hablamos de interacciones simbióticas, en las que varios organismos interaccionan muy estrechamente, hay que ser cautos al definir las fronteras entre mutualismo (con efectos positivos para todos los miembros), comensalismo (sin efecto para alguno de los miembros), y parasitismo (con efectos negativos para uno de los miembros). Cuando las circunstancias en que se desarrolla una determinada relación varían, teniendo en cuenta que todas ellas comparten su origen evolutivo y se basan en unos mecanismos similares, es probable que se

produzca un cambio cuantitativo o cualitativo que dé lugar a otro tipo de interacción (Ochman y Moran, 2001; Pérez-Brocal *et al.*, 2013).

2. Simbiosis mutualistas con bacterias

La simbiosis fue definida inicialmente en 1879 por de Bary como la convivencia de organismos diferentes ("*the living together of unlike organisms*"). Aunque esta acepción no implica que la interacción sea positiva o negativa, comúnmente se suele asociar el término simbiosis a relaciones mutualistas (Sapp, 2004).

Los microorganismos en general, y las bacterias en particular, han sido claves en muchos procesos ecológicos y evolutivos, ya que están presentes en todos los ambientes de nuestro planeta (Moran, 2002; Pace, 1997). Estos procesos en los que intervienen las bacterias involucran también al resto de seres vivos (incluidos protistas, hongos, plantas y animales; Figura I.3) mediante interacciones simbióticas (Moran, 2006; Wernegreen, 2012), a través de las cuales estos microorganismos han sido determinantes de muchos cambios que han favorecido la aparición y diversificación de los seres pluricelulares (Moran, 2002; Sapp, 2004). Por esta razón, hoy en día no es posible considerar las teorías de la ecología y la evolución sin tener en cuenta las interacciones de los seres vivos con los microorganismos asociados a ellos, y el estudio de la biología actual debe basarse en la consideración de todos los organismos como sistemas hospedador-microorganismos, los llamados holobiontes (Bordenstein y Theis, 2015).

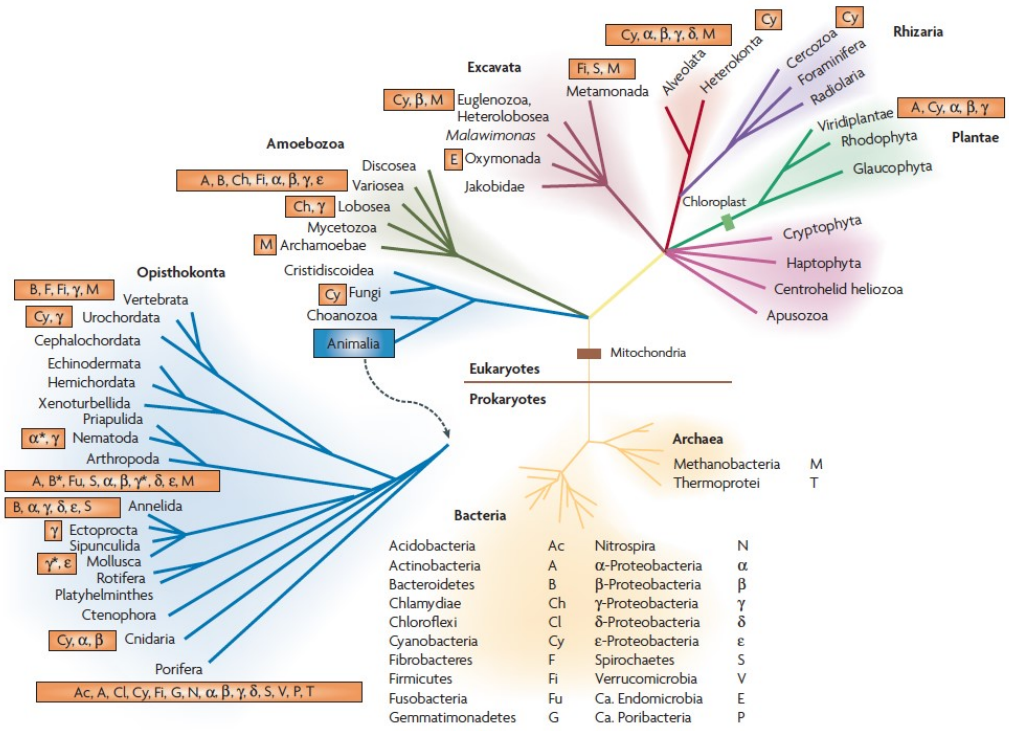


Figura I.3. Distribución de los distintos grupos de bacterias encontrados en simbiosis con el resto de seres vivos, y en especial con animales (Moya *et al.*, 2008).

En la actualidad pueden encontrarse numerosos trabajos centrados en los múltiples beneficios que las bacterias aportan a los organismos que las hospedan. Esta nueva tendencia se debe en gran parte a los avances que la biología molecular y la genómica han sufrido en los últimos años (Moran, 2006; Nikolaki y Tsiamis, 2013). De esta forma, se ha pasado de estudiar relaciones coevolutivas entre organismos y cepas bacterianas concretas, muchas veces transmitidas verticalmente (como las descritas en Baumann, 2005; Ehinger *et al.*, 2014; Moran *et al.*, 2008), a entender la simbiosis como un proceso mucho más flexible y complejo, en el que simbiontes secundarios, o incluso comunidades bacterianas completas, interaccionan con un hospedador (Baumann, 2005; Dunlap *et al.*, 2007; Hussa y Goodrich-Blair, 2013). El reciente descubrimiento de la generalidad de las simbiosis complejas en la

naturaleza ha dado lugar a toda una serie de nuevas teorías acerca del mantenimiento de estos sistemas a lo largo de la evolución. De esta forma, se acepta que la estabilidad de estos sistemas depende del aporte de distintos beneficios por parte de los distintos simbiontes, o de su coadaptación en términos de abundancia relativa o complementación de rutas metabólicas en caso de que sus beneficios sean similares (Hussa y Goodrich-Blair, 2013). Entre los posibles beneficios se pueden incluir las funciones relacionadas con la obtención y asimilación de nutrientes (Gustafsson, 1959; Rajilić-Stojanović, 2013), la tolerancia a factores ambientales extremos o la adaptación a nuevos nichos ecológicos (Bárzana *et al.*, 2014; Morse *et al.*, 2012; Russell y Moran, 2006), la señalización social y sexual (Theis *et al.*, 2013), el desarrollo adecuado del sistema inmune y la regulación metabólica y genética (Kasubuchi *et al.*, 2015; Kelly *et al.*, 2007), así como la defensa frente a patógenos y depredadores (Jones y Nishiguchi, 2004; Lindquist *et al.*, 2005; Shnit-Orland y Kushmaro, 2009; Skarin y Sylwan, 1986; Tsuchida *et al.*, 2010; Vallor *et al.*, 2001). En este último caso, las simbiosis han demostrado ser fundamentales para la protección de muchos animales mediante el aporte de sustancias defensivas, entre otros mecanismos, por parte de sus simbiontes (Figura I.4) (revisado en Flórez *et al.*, 2015).

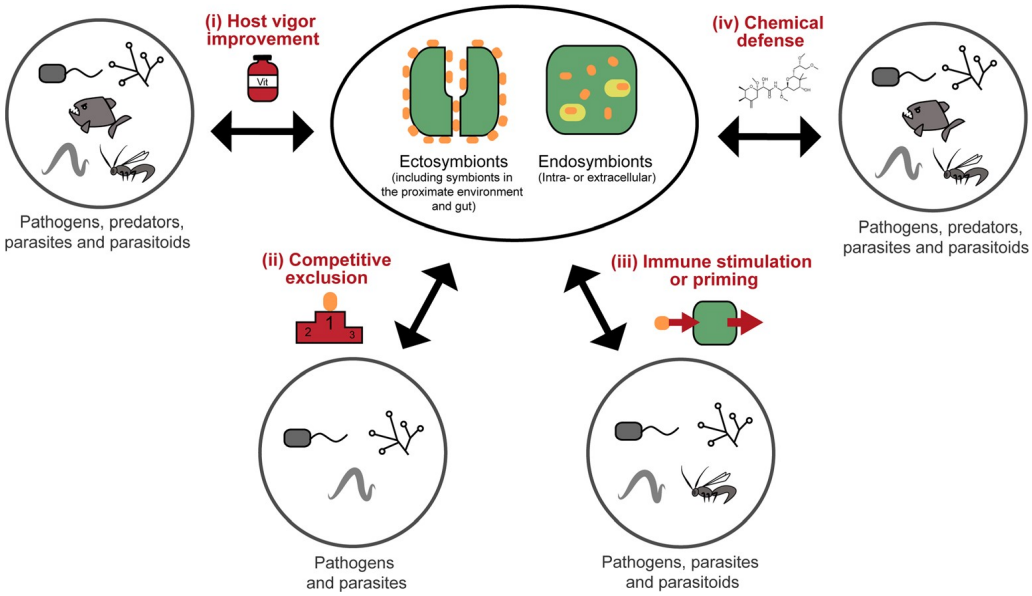


Figura I.4. Distintos mecanismos mediante los que los microorganismos pueden proteger a sus hospedadores frente a depredadores, parásitos, parasitoides y patógenos. Se incluye la mejora del vigor del hospedador (i), la exclusión competitiva de patógenos (ii), la estimulación del sistema inmunitario (iii) y la producción de sustancias defensivas (iv) (Flórez *et al.*, 2015).

Según describen Flórez y colaboradores (2015), las simbiosis defensivas presentan un patrón menos estable que otros tipos de simbiosis, como por ejemplo las asociadas con la nutrición del hospedador. En muchas ocasiones la transmisión de simbiontes defensivos se produce de forma horizontal o está determinada por factores ambientales, lo que favorece una adquisición más flexible, que permite una rápida adaptación de los hospedadores frente a sus antagonistas en la carrera coevolutiva. Además, en lugar de limitarse a sustancias concretas, la función defensiva de los simbiontes recae en una batería completa de productos metabólicos que protegen al hospedador frente a un amplio rango de patógenos, dificultando a su vez la aparición de resistencias en los mismos (Flórez *et al.*, 2015). Este tipo de simbiosis ha sido ampliamente estudiada en algunos organismos marinos como esponjas, cnidarios, briozoos, nemertinos, moluscos, crustáceos o tunicados y también en

diversos invertebrados terrestres como nemátodos e insectos. En vertebrados sin embargo, este tipo de interacciones se han descrito en menor grado, fundamentalmente por la mayor complejidad en el estudio de estos sistemas, que ha dado lugar a una menor cantidad de información disponible (Flórez *et al.*, 2015). No obstante, las simbiosis defensivas en vertebrados pueden ser una fuente importante para el descubrimiento de nuevas sustancias antimicrobianas de utilidad para el ser humano, en constante lucha contra la aparición de resistencias frente a los antibióticos clásicos y quimioterápicos de uso clínico (Shallcross *et al.*, 2015), y además de su relevancia ecológica, pueden tener aplicaciones interesantes a largo plazo.

3. La glándula uropigial de la abubilla

Las abubillas dedican bastante tiempo al acicalamiento de su plumaje (Figura I.5). Se trata de un comportamiento común en las aves, que les permite recolocar el plumaje, eliminar parásitos y flexibilizar e impermeabilizar las plumas mediante el uso de su secreción uropigial. Esta secreción proviene de la glándula uropigial (también llamada glándula de acicalamiento) y puede tener funciones adicionales como la defensa frente a la depredación y frente a la infección por patógenos o actuar como una señal sexual de calidad (Reneerkens *et al.*, 2006).

Figura I.5. Abubilla acicalando su plumaje con la secreción de su glándula uropigial. Por AntoGros [CC BY 2.0].

La glándula uropigial es una glándula holocrina presente en el intertegumento de las aves, que aparece en la región sinsacrocaudal en posición dorsal media y consta de dos lóbulos separados por un septo. Los lóbulos contienen el tejido secretor y un sistema de conductos que desembocan en la parte caudal en una papila en la cual se acumula la secreción. En muchas especies, como sucede en la abubilla, la papila se abre al exterior mediante una abertura rodeada por un penacho de plumas suaves (Figura I.6). En el caso de la abubilla la papila es cilíndrica y está cerrada por un esfínter (Jacob y Ziswiler, 1982).

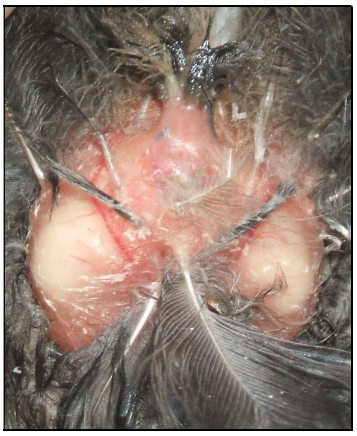
A  B 

Figura I.6. Glándula uropigial de la abubilla: (A) posición bajo la cola y salida de secreción desde la papila; (B) estructura lobular y penacho de plumas en la apertura (Martín-Vivaldi *et al.*, 2009).

La secreción de la glándula uropigial es recogida por el ave con el pico, bien a través de las gotas que surgen de la abertura de la papila o bien a partir de las acumulaciones de fluido en el penacho de plumas que la rodea. En la abubilla, la abertura de la papila es única y amplia, por lo que el ave puede extraer la secreción directamente de la papila con el pico. La composición de esta secreción es variable y suele incluir mono y diésteres de ácidos grasos y alcoholes alifáticos, además de esteroles, triglicéridos y carbohidratos en algunos casos (Jacob y Ziswiler, 1982). Esta composición puede responder a las diversas funciones que se han propuesto para la secreción: desde la impermeabilización y flexibilización de las plumas, a la protección frente a depredadores e infecciones, pasando por la señalización sexual o la actividad feromonal (Reneerkens *et al.*, 2006).

Las abubillas, así como las abubillas arbóreas (*Phoeniculus purpureus*), poseen una secreción uropigial con características atípicas tales como un color oscuro, un olor desagradable o la presencia de compuestos poco comunes en

el resto de aves (Burger *et al.*, 2004; Martín-Vivaldi *et al.*, 2010). Concretamente, en el caso de la abubilla estas características de la secreción se asocian sólo a los individuos que permanecen en el nido durante la estación reproductora (es decir, las hembras y sus pollos), mientras que tanto los machos adultos como las hembras fuera del periodo reproductor presentan secreciones claras e inodoras, más parecidas a las del resto de aves (Figura I.7). Estos cambios en la secreción están además acompañados por un incremento de hasta 8 veces en el tamaño de la glándula, y de un aumento del volumen de secreción producida de hasta 30 veces en las hembras (Martín-Vivaldi *et al.*, 2009). La composición de las secreciones claras y oscuras también es diferente, ya que las últimas presentan, además de los ácidos grasos y monoésteres propios de las secreciones claras, una serie de compuestos volátiles (del tipo ácidos orgánicos de cadena corta y sus derivados benzólicos e indólicos) (Martín-Vivaldi *et al.*, 2010).

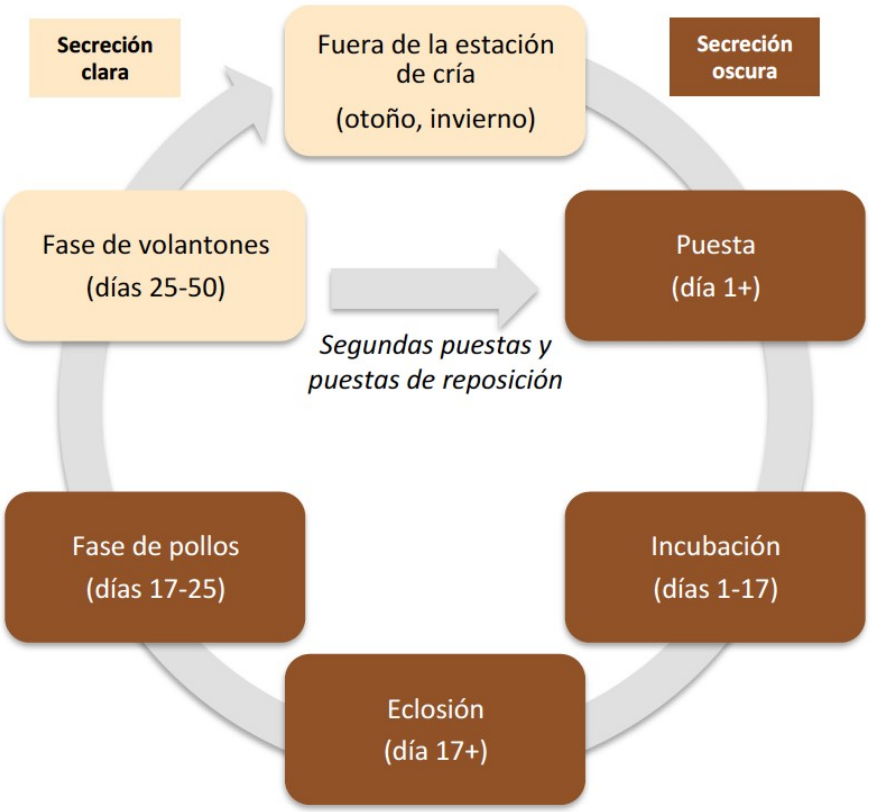


Figura I.7. Esquema del ciclo reproductor de la abubilla, indicando los cambios que se producen en el aspecto de la secreción de la hembra.

4. Simbiosis mutualista en las glándulas uropigiales de las abubillas

El primer estudio sobre la abubilla europea y su posible relación con bacterias simbiontes alojadas en su glándula uropigial fue publicado en 2006. Ese año, Martín-Platero y colaboradores describieron por primera vez una bacteriocina (péptido de síntesis ribosómica, bajo peso molecular y con actividad antimicrobiana, Riley y Wertz 2002) denominada MR10-3, producida por una cepa de enterococo, *Enterococcus faecalis* MRR10-3, aislada de la secreción uropigial de un pollo de abubilla (Martín-Platero *et al.*, 2006). Unos años antes, en 2003, se había descrito una especie nueva de enterococo (*E. phoeniculicola*) aislada también de la glándula uropigial de la abubilla arbórea

(*Phoeniculus purpureus*), una especie de ave próxima a la abubilla en la que también se han descrito propiedades similares de su secreción uropigial (Law-Brown y Meyers, 2003). Estas dos especies, a pesar de ser próximas filogenéticamente (Feduccia, 1975; Mayr, 2000), muestran modos de vida diferentes que se asocian con diferencias en la forma en que su simbiosis con bacterias se desarrolla a lo largo del ciclo vital del ave. La abubilla arbórea (Figura I.8) mantiene una asociación simbiótica con enterococos a lo largo de toda la vida de los individuos, (Law-Brown, 2001). En el caso de la abubilla europea sin embargo, la aparición de una secreción uropigial parecida a la de la abubilla arbórea (oscura y maloliente) ocurre solo en el periodo reproductor y solo en hembras y pollos, mientras que en los machos adultos y el periodo no reproductor de las hembras la secreción es más parecida a la del resto de aves (blanca e inodora, (Martín-Vivaldi *et al.*, 2009). Varios experimentos en los que se inyectó un antibiótico (amoxicilina) en las glándulas de pollos y hembras durante su estancia en el nido mostraron la influencia de las bacterias simbiontes alojadas en ellas sobre los cambios que experimentaban a lo largo del ciclo reproductor, incluyendo su tamaño, el color y su composición química (Martín-Vivaldi *et al.*, 2009, 2010).

Figura I.8. Abubilla arbórea (*Phoeniculus purpureus*). Por Bernard Dupont [CC BY-SA 2.0].

Al igual que se ha comprobado en las simbiosis de los calamares, hormigas, avispas o salamandras con bacterias alojadas en sus glándulas (referencias de los estudios de estos sistemas), las bacterias que habitan las glándulas uropigiales de las abubillas proporcionan ventajas al ave aparentemente mediadas por su actividad antimicrobiana. Los enterococos son cocos Gram positivos, anaerobios aerotolerantes , catalasa-negativos y pertenecientes al filum *Firmicutes*, que generalmente habitan el intestino de algunos vertebrados (Giard *et al.*, 2001) y cuyos beneficios simbióticos derivados de la producción de péptidos antimicrobianos (enterocinas) se han demostrado para la abubilla y otra aves en investigaciones previas (Moreno *et al.*, 2003; Ruiz-Rodríguez *et al.*, 2009; Soler *et al.*, 2008). De hecho las secreciones con bacterias de las dos especies de abubillas tienen propiedades antibióticas (Burger *et al.*, 2004; Soler *et al.*, 2008). Además las cepas de enterococos aisladas de la glándula uropigial de la abubilla europea con elevada frecuencia producen sustancias antimicrobianas de naturaleza proteica (Martín-Platero *et al.*, 2006; Ruiz-Rodríguez *et al.*, 2009, 2012, 2013). En el caso de la abubilla europea, Soler y colaboradores (2008) demostraron que la presencia de sustancias antimicrobianas en la secreción uropigial podía relacionarse con la acción de las bacterias que residían en la glándula, ya que solo se detectaron bacterias en las secreciones oscuras y estas secreciones eran las únicas que inhibían el crecimiento de *Bacillus licheniformis,* utilizada como indicadora de la actividad antimicrobiana (Soler *et al.*, 2008). Dado que el tratamiento de las secreciones con proteasa inhibía su capacidad antimicrobiana el principal candidato a mediar esa actividad eran las sustancias proteicas del tipo de las bacteriocinas. Un análisis detallado de la capacidad

antimicrobiana de las cepas de enterococos aisladas de glándulas de abubilla mostró una elevada prevalencia de las que mostraron esa actividad frente a una colección de 11 bacterias indicadoras patógenas (más del 70 % fueron activas frente a una de las cepas indicadoras al menos) aunque el espectro de actividad y su intensidad fueron muy variables (Ruiz-Rodríguez *et al.*, 2012). En un trabajo posterior se establecía que más de la mitad de las cepas de enterococos aisladas de la secreción uropigial de pollos y hembras en reproducción eran productoras de al menos una bacteriocina y la mayoría de ellas presentaban los genes para las bacteriocinas MR-10 y AS-48 (Ruiz-Rodríguez *et al.*, 2013).

Los beneficios concretos para la abubilla que han podido favorecer la evolución de la simbiosis con las bacterias productoras de sustancias antimicrobianas podrían tener que ver con la protección frente a bacterias degradadoras de plumas, ya que la secreción uropigial es usada principalmente para extenderla sobre el plumaje (Jacob y Ziswiler, 1982), o con la protección de los huevos y pollos frente a los patógenos que pudieran afectarles en el interior del nido, ya que la secreción especial se desarrolla sólo durante la estancia de las hembras en él (Martín-Vivaldi *et al.*, 2009). Se ha demostrado experimentalmente en condiciones de laboratorio que tanto la presencia de *Enterococcus faecalis* MRR10-3 como de la bacteriocina que produce es capaz de reducir significativamente la degradación de queratina pura y plumas completas por parte de *Bacillus licheniformis*, un patógeno potencial de aves que se ha relacionado con la degradación del plumaje (Ruiz-Rodríguez *et al.*, 2009). Sin embargo, en un estudio para comprobar si el uso de la secreción por la hembra durante su estancia en el nido afectaba al estado de sus plumas, no

se encontró un efecto de impedir experimentalmente el uso de la secreción sobre los niveles de degradación medidos en ellas (García-Olivares, 2014), por lo que la protección de las plumas no parece ser el factor principal que explique la evolución de la asociación con bacterias.

Varias evidencias sí apoyan que la simbiosis con estas bacterias proporciona ventajas en la lucha frente a patógenos de los huevos. En primer lugar, la inhibición de las bacteriocinas presentes en la superficie de los huevos con proteasas disminuyó su éxito de eclosión (Soler *et al.*, 2008). Por otra parte, las hembras aplican la secreción directamente sobre los huevos (Martín-Vivaldi *et al.*, 2014) lo que provoca un cambio de su color, inicialmente azulado, que se vuelve pardo durante la incubación debido al aporte de secreción uropigial (Soler *et al.*, 2014). Adicionalmente se ha encontrado que en la cáscara de los huevos existen criptas especiales no presentes en otras especies que facilitan la retención de esta secreción (Fig. I-9, Martín-Vivaldi *et al.*, 2014). Cuando se ha estudiado la influencia de las bacterias presentes sobre la superficie de los huevos en el éxito de eclosión, se ha encontrado que la densidad de enterococos se relaciona positivamente con el éxito de eclosión. Esto es principalmente debido a la presencia de enterococos que llegan a través de la secreción de la hembra, pues cuando se impidió experimentalmente su llegada hasta los huevos, esa relación desapareció y se incrementó el número bacterias patógenas en el interior de los huevos (Martín-Vivaldi *et al.*, 2014).

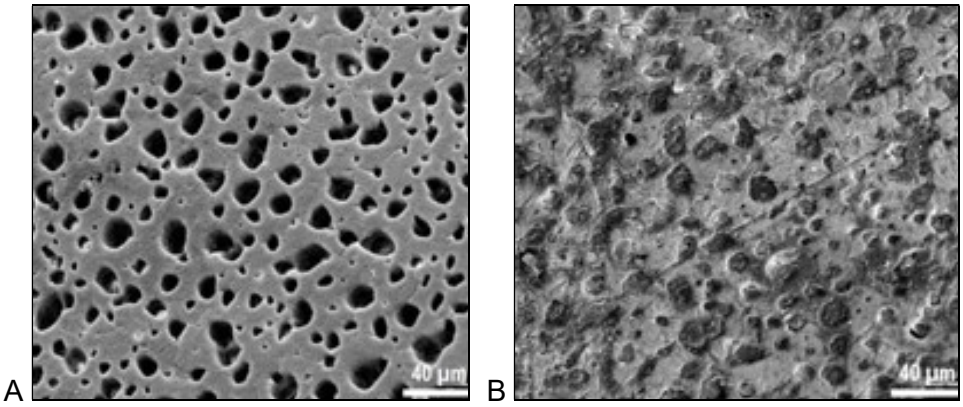


Figura I.9. Apariencia de las criptas encontradas en la superficie de la cáscara de los huevos de abubilla: (A) criptas de un huevo recién puesto, vacías; (B) criptas de un huevo al final de la incubación, llenas de secreción uropigial (Martín-Vivaldi *et al.*, 2014) .

Todos estos resultados han puesto de manifiesto que el sistema formado por la abubilla europea y los enterococos presentes en su glándula uropigial constituye una simbiosis mutualista en la que las bacterias parecen proveer algunas de las sustancias antagonistas producidas en la glándula, y por tanto ofrecer protección a su hospedador frente a los patógenos, principalmente de huevos.

Diversidad y dinámica del microbioma de la glándula

Los estudios dependientes de cultivo han mostrado que las poblaciones microbianas cultivables de las glándulas uropigiales de la abubilla estáno representadas casi exclusivamente por enterococos (Soler *et al.*, 2008). La tipificación de las cepas de enterococos aisladas de la secreción uropigial de pollos y hembras en reproducción mediante amplificación aleatoria de ADN polimórfico o RAPD (*Randomly Amplified Polymorphic DNA*) y posterior identificación de cada uno de los grupos genómicos encontrados ha puesto de

manifiesto la presencia de siete especies diferentes entre las que fueron más abundantes las de *E. faecalis*, *E. faecium* y *E. mundtii* (Ruiz-Rodríguez *et al.*, 2012; Soler *et al.*, 2008).

Sin embargo, cuando se intentó comprobar mediante técnicas independientes del cultivo si existían en las glándulas otras cepas bacterianas, se encontró que el microbioma era más complejo del inicialmente propuesto. Los perfiles de ARISA (Automated Ribosomal Intergenic Spacer Analysis, Fisher y Triplett, 1999) de las secreciones mostraban la existencia de hasta 124 OTUS (*Operational Taxonomic Units*) diferentes, con un núcleo de 27 de ellos con una prevalencia igual o superior al 30 % (Martínez-García, 2015). Aunque la técnica de ARISA no permite la identificación taxonómica de los OTUS detectados, su utilización claramente demuestra que la simbiosis entre abubillas y bacterias de la glándula no es un sistema simple, y que los cambios en las propiedades de la secreción y su utilidad para el ave deben estar relacionados con una dinámica compleja de la comunidad del simbionte (Bordenstein y Theis, 2015; Hussa y Goodrich-Blair, 2013).

Las propiedades especiales de las secreciones de las abubillas (coloración oscura, mal olor, carga bacteriana y elevada actividad antimicrobiana) sólo se dan estacionalmente durante la estancia de hembras y pollos en el nido (Martín-Vivaldi *et al.*, 2009; Soler *et al.*, 2008). Esto sugiere que la simbiosis es establecida sólo temporalmente y por tanto que debe adquirirse periódicamente. En lo que respecta a los enterococos, mediante un experimento de cría cruzada o *cross-fostering* en el que algunos pollos recién eclosionados (con 3-4 días, antes de desarrollar la glándula uropigial) eran

intercambiados con pollos de otros nidos, se comprobó la adquisición horizontal de determinadas cepas durante el desarrollo, aunque también se detectó cierto componente genético que determinaría en algún grado la colonización de la glándula antes de su completo desarrollo (Martín-Vivaldi *et al.*, 2014). Lo mismo sucede en el caso de las hembras ya que un mismo individuo puede cambiar su comunidad de enterococos entre distintas puestas (dentro de una temporada o en diferentes años, Ruiz-Rodríguez *et al.*, 2014) lo que sugiere la posibilidad de incorporación de enterococos a la glándula uropigial a lo largo de toda la vida del animal. Con estos resultados, se demostraba la importancia de los factores ambientales en la conformación de la población de enterococos presente en la glándula uropigial de la abubilla. Algo similar se ha encontrado con posterioridad al analizar las influencias ambientales y genéticas en la composición de la comunidad detectada por ARISA. De nuevo se ha establecido la existencia de efectos ambientales y genéticos, y por tanto la existencia de una base sobre la que la selección natural puede actuar, en la composición bacteriana de la glándula uropigial de los pollos en nuevos experimentos de cría cruzada en los que los pollos eran intercambiados de nido a una edad más avanzada (8 días), cuando empieza a producirse la secreción uropigial (Martínez-García, 2015).

Dado que las cepas bacterianas presentes en las glándulas parecen ser, en parte adquiridas del ambiente que rodea al individuo o por transmisión horizontal en el desarrollo de la glándula (secreciones de individuos con los que comparte el nido, material del nido u otros ambientes) y en parte determinadas por transmisión vertical, predisposición genética y efectos maternos, el estudio de la procedencia de esos simbiontes es importante para poder entender su

dinámica estacional. Los intentos realizados para comprobar dicha procedencia, mediante la comparación de las comunidades bacterianas caracterizadas por ARISA entre la secreción y otras localizaciones del ave han puesto de manifiesto la transmisión de determinadas cepas desde la glándula al pico, la placa incubadora y la cáscara de los huevos (Martínez-García, 2015; Martínez-García *et al.*, 2015), pero no aparece ninguna conexión clara con la comunidad de la cloaca; una posible fuente de bacterias mantenida todo el año, o el material del nido, una posible fuente de bacterias usada estacionalmente (Martínez-García, 2015). Sin embargo sí se ha confirmado que el material del nido puede afectar a las comunidades bacterianas de la superficie de los huevos, determinando así su probabilidad de infección por bacterias patógenas (Martínez-García, 2015).

Las aproximaciones metodológicas utilizadas hasta el momento en el estudio de la comunidad de bacterias simbiontes de la glándula uropigial de la abubilla, han permitido caracterizar muy bien la relación del ave con los enterococos de su secreción. Sin embargo, para poder entender realmente la importancia de este grupo en el sistema y lo que aporta el resto de la microbiota que forma parte de él, es necesaria una caracterización taxonómica de los otros grupos presentes, lo que permitiría la detección de otros grupos clave, y la deducción del papel que sus capacidades metabólicas y de síntesis pueden suponer a nivel del microbioma completo (Flórez *et al.*, 2015; Hussa y Goodrich-Blair, 2013). Ello es posible gracias al gran desarrollo de las herramientas moleculares que permiten el estudio de comunidades completas que se ha producido en los últimos años (Moran, 2006; Nikolaki y Tsiamis, 2013).

5. Herramientas para el estudio de la biodiversidad bacteriana

Desde los inicios de la microbiología como una disciplina independiente hasta la fecha actual, los métodos empleados para estudiar la diversidad microbiana han evolucionado enormemente. Las primeras observaciones microscópicas de Robert Hooke y Antoni van Leeuwenhoek en el siglo XVII dieron lugar a una primera etapa de descripción morfológica de los microorganismos (Gest, 2004). Esta etapa fue sucedida en el siglo XIX, con la aparición de los métodos de cultivo y la obtención de cultivos puros, por una etapa centrada en la descripción ya no solo morfológica sino también fisiológica de los microorganismos, permitiendo que Robert Koch estableciese por primera vez la relación entre bacterias y enfermedades concretas (Blevins y Bronze, 2010). Muchas de esas técnicas se siguen empleando hoy en día en los laboratorios de microbiología a nivel de rutina, especialmente en clínica y ciencias alimentarias para la identificación de patógenos, y aún se siguen desarrollando nuevas técnicas basadas en este enfoque (Almeida *et al.*, 2014). Sin embargo, a partir del siglo XX se empezó a poner de manifiesto que estas técnicas, llamadas dependientes de cultivo, no reflejaban la diversidad real existente en muchas de las muestras estudiadas (Jannasch y Jones, 1959), llegando en algunos casos a menospreciar más del 99% del total de los microorganismos presentes en muestras ambientales (Amann *et al.*, 1995). La alternativa, las llamadas técnicas independientes de cultivo, son capaces de revelar la mayoría de los microorganismos tanto cultivables como no cultivables, y su aplicación y utilidad en estudios de diversidad microbiana es hoy indiscutible (Fisher y Triplett, 1999; Muyzer *et al.*, 1993; Smalla *et al.*, 2007).

Estos métodos, también llamados moleculares, se basan generalmente en la reacción en cadena de la polimerasa o PCR (*Polimerase Chain Reaction*), que permite obtener un elevado número de copias o amplicones a partir de regiones concretas del ADN de cualquier ser vivo sin necesidad de cultivarlo para generar réplicas *in vivo* (Mullis *et al.*, 1986).

Entre las técnicas para el estudio de la diversidad microbiana basadas en la PCR se encuentran la generación de patrones característicos (huellas genéticas o *fingerprinting*) y la secuenciación (mediante librerías de clones y posteriormente de forma masiva) a partir de marcadores genéticos determinados (Nikolaki y Tsiamis, 2013). La tabla I.1 recoge algunas de las técnicas moleculares más empleadas en la identificación de la diversidad de comunidades bacterianas hasta la actualidad. Muchas de ellas, especialmente las más recientes, tienen además otras aplicaciones como la identificación de mutaciones y variaciones genéticas, la secuenciación de genomas y metagenomas (es decir todos los genomas de un ambiente juntos) o la transcriptómica (estudio de la expresión genética mediante secuenciación de ARN).

Tabla I.1. Técnicas moleculares más empleadas para la descripción de comunidades bacterianas.

	Técnica		Metodología	Referencias
Fingerprinting	DGGE (Denaturing Gradient Gel Electrophoresis) TTGE (Temporal Temperature Gradient Electrophoresis)[1]		Amplificación del ADNr 16S y separación electroforética en geles de poliacrilamida de amplicones de igual tamaño pero distinta composición nucleotídica gracias a su desnaturalización parcial aplicando un gradiente de agentes químicos (urea, formamida) o físicos (temperatura[1])	(Muyzer *et al.*, 1993) (Thatcher y Hodson, 1981) (Ogier *et al.*, 2002)
	RISA (Ribosomal Intergenic Spacer Analysis)[2] ARISA (*Automated* RISA)[3]		Amplificación de la región intergénica 16S-23S del ADNr y separación de amplicones de diversos tamaños mediante electroforesis en geles de poliacrilamida[2] o electroforesis capilar[3]	(Borneman y Triplett, 1997) (Fisher y Triplett, 1999)
Secuenciación	Librerías de clones y secuenciación clásica (Sanger)		Amplificación del ADNr 16S (u otro marcador), clonación en vectores (librerías) y replicación en *E. coli* para su posterior secuenciación individual por Sanger	(Giovannoni *et al.*, 1990) (Sanger *et al.*, 1977)
	Secuenciación de segunda generación (CGS, "*Current Generation Sequencing*")	Pirosecuenciación (454 GS FLX y 454 GS *Junior* de Roche)*	Amplificación del ADNr 16S añadiendo adaptadores específicos y secuencias identificadoras (*barcodes*) que permiten la combinación de diferentes muestras en un solo proceso de secuenciación o *run*, seguida de una PCR en emulsión y su posterior pirosecuenciación. En una sola lectura permite obtener las secuencias más largas (hasta 500-1000 pares de bases)	(Margulies *et al.*, 2005)
		Secuenciación por síntesis (HiSeq y MiSeq de Illumina/Solexa)*	Amplificación del ADNr 16S añadiendo adaptadores específicos y secuencias identificadoras que permiten la combinación de diferentes muestras en un solo proceso de secuenciación, seguida de una PCR en puente y una secuenciación por síntesis con terminadores reversibles. Esta técnica permite solapar las lecturas 5'→ 3' (*forward*) y 3'→5' (*reverse*) para generar secuencias pareadas (*paired-end*)	(Bentley *et al.*, 2008)
		Otros sistemas de secuenciación masiva	• AB SOLiD* y Polonator*: PCR en emulsión y secuenciación por ligación de oligonucleótidos degenerados con marcaje fluorescente (sin polimerasa). • Ion Torrent: PCR en emulsión y secuenciación en semiconductores	(Shendure *et al.*, 2005) (Rothberg *et al.*, 2011)
	Secuenciación de tercera generación (NGS, "*Next Generation Sequencing*")		• HeliScope*: secuenciación de moléculas individuales por síntesis • PacBio[4]* y Nanopore[5]*: secuenciación de moléculas individuales en tiempo real (con detección por fluorescencia[4] o por corriente eléctrica[5]) • Genómica de células individuales (*single-cell genomics*)	(Thompson y Steinmann, 2010) (Eid *et al.*, 2009) (Timp *et al.*, 2010) (Blainey, 2013)

* Una descripción de estas técnicas en mayor profundidad puede encontrarse en las revisiones de Shendure y Ji (2008) y Metzker (2010).

Los marcadores que se han empleado preferentemente para estudios de diversidad bacteriana han sido las regiones conservadas del correspondientes al ADNr 16S (Rosselló-Mora y Amann, 2001), y que permiten el diseño de cebadores universales válidos para amplificar esta región de la práctica totalidad de bacterias (Mori *et al.*, 2014), a la vez que se pueden distinguir taxones incluso si éstos están muy próximos filogenéticamente, especialmente cuando se usan las herramientas de asignación taxonómica adecuadas (Liu *et al.*, 2008). En concreto, el uso del ADNr que da lugar a la subunidad pequeña de los ribosomas (SSU "*Small Subunit*") en bacterias, denominada 16S por su coeficiente de sedimentación, ha sido tradicionalmente la diana predilecta para la identificación bacteriana, como demuestra la disponibilidad de amplias bases de datos de secuencias de referencia para este marcador (Tabla I.2). Muchas de ellas vienen acompañadas, además, de una serie de herramientas que permiten realizar ciertas tareas de manejo y análisis de las secuencias, como los algoritmos de alineamiento (BLAST, "*Basic Local Alignment Search Tool*", Altschul *et al.*, 1990; Clustal Omega, Sievers *et al.*, 2011) y asignación taxonómica (RDP Classifier, Wang *et al.*, 2007) disponibles en los servidores web del NCBI (*National Center for Biotechnology Information*), el EMBL-EBI (*European Molecular Biology Laboratory-European Bioinformatics Institute*) y el RDP (*Ribosomal Database Project*) respectivamente.

Tabla I.2. Bases de datos del del ADNr 16S de uso más común.

Base de datos	Dirección web	Referencia
ENA (European Nucleotide Archive)*	http://www.ebi.ac.uk/ena	(Leinonen *et al.*, 2011)
GenBank*	http://www.ncbi.nlm.nih.gov/genbank/	(NCBI Resource Coordinators, 2013)
Greengenes	http://greengenes.lbl.gov/cgi-bin/nph-index.cgi	(DeSantis *et al.*, 2006)
RDP database	http://rdp.cme.msu.edu/	(Cole *et al.*, 2014)
SILVA	http://www.arb-silva.de/	(Pruesse *et al.*, 2007)

* Estas bases de datos no son exclusivas para el ADNr, contienen también secuencias de otros marcadores y de genomas completos.

La región hipervariable que se encuentra entre los genes de los ADNr 16S y 23S (también denominada espaciador intergénico ribosómico o ITS, "*Internal Transcribed Spacer*") ha sido igualmente la base de muchos estudios de diversidad bacteriana, aunque la disponibilidad de bases de datos específicas para identificación taxonómica es menor y su utilidad se centra principalmente en estudios comparativos y filogenéticos (Boyer *et al.*, 2001). La aplicación de este marcador en estudios de identificación de eucariotas (fundamentalmente hongos) ha dado sin embargo mejores resultados, promoviendo su uso con las nuevas tecnologías de secuenciación disponibles (White *et al.*, 2013).

Mientras que las técnicas de *fingerprinting* tales como el TTGE o el RISA permiten realizar un escrutinio básico de la diversidad presente en una muestra ambiental, detectando el número de OTUs (*Operational Taxonomic Unit)* presentes y comparar patrones entre muestras mediante software especializado (Benizri *et al.*, 2005; Lear *et al.*, 2008; Liu *et al.*, 2007), las técnicas basadas en la secuenciación permiten, además, identificar

taxonómicamente los organismos presentes en una muestra (López-Fernández *et al.*, 2015). De esta manera los diferentes OTUs (microorganismos cuya secuencia del ADNr 16S tiene al menos un 97 % de similitud, límite artificialmente establecido para especie en estudios de diversidad con este marcador (Schloss y Handelsman, 2005) pueden ser asignados a taxones concretos. Cabe destacar que el *fingerprinting* y la secuenciación no son técnicas completamente independientes, ya que es posible secuenciar directamente bandas aisladas de los patrones de *fingerprinting* (Bosshard *et al.*, 2000). Sin embargo, tanto esta aproximación como la elaboración de librerías de clones son altamente costosas en cuanto a tiempo y esfuerzo cuando se pretende describir una comunidad bacteriana compleja (Huse *et al.*, 2008). De esta forma, el desarrollo de las tecnologías de secuenciación de segunda generación (actualmente denominada CGS, "*Current Generation Sequencing*"; tabla I.1) y su abaratamiento de costes en los últimos años, combinado con el alto rendimiento de información que proporcionan, y una menor inversión de tiempo y esfuerzo requerida (Shendure y Ji, 2008), han hecho de estas aproximaciones el método de elección ya no solo para la descripción de comunidades bacterianas complejas a mayor profundidad, sino también para su seguimiento a lo largo del tiempo (Brazelton *et al.*, 2010; Caporaso *et al.*, 2011a) y su comparación en distintas circunstancias (Rodrigues *et al.*, 2013; Roesch *et al.*, 2007). Por otro lado, la elevada cantidad de datos derivada de estas tecnologías de secuenciación de segunda generación ha propiciado a su vez un avance paralelo en el desarrollo de herramientas bioinformáticas que permitan manejar toda la información ahora disponible (Caporaso *et al.*, 2011b).

La tabla I.3 recoge algunas de las herramientas de procesamiento de datos y compilaciones de las mismas (*pipelines*), de uso público y gratuito que hay actualmente disponibles. Además de los recogidos en la tabla, cuyo uso está ampliamente extendido, pueden encontrarse otros programas y paquetes informáticos para el análisis de secuencias del ADNr 16S con distintos enfoques (Kotamarti *et al.*, 2010; Pandey *et al.*, 2010; Preheim *et al.*, 2013; Soh *et al.*, 2013).

Tabla I.3. Herramientas bioinformáticas para el procesamiento y análisis de secuencias del ADNr 16S procedentes de la secuenciación masiva.

Herramienta	**Dirección web**	**Referencia**
ARB	http://www.arb-home.de/	(Ludwig *et al.*, 2004)
Mothur	http://www.mothur.org/	(Schloss *et al.*, 2009)
QIIME (Quantitative Insights Into Microbial Ecology)	http://qiime.org/	(Caporaso *et al.*, 2010)
RDP pipeline	http://pyro.cme.msu.edu/	(Cole *et al.*, 2014)
USEARCH	http://www.drive5.com/usearch/	(Edgar, 2010)

El procedimiento general que sigue un *pipeline* para el análisis de secuencias del ADNr 16S obtenidas por secuenciación masiva se puede resumir en un esquema básico de procesamiento, que incluye siempre ciertos pasos (Navas-Molina *et al.*, 2013):

1. Procesamiento inicial o preprocesamiento:
    a. Filtrado de calidad de las secuencias.
    b. Asignación de secuencias a cada muestra (*demultiplexing*).

c. Eliminación de fragmentos de secuencia no informativos (adaptadores, cebadores, *barcodes*)
d. Eliminación de quimeras (artefactos procedentes de la amplificación de dos fragmentos de ADN diferentes combinados en uno solo).

2. Procesamiento básico:
   a. Alineamiento de secuencias y agrupamiento (*clustering*).
   b. Determinación de OTUs (*otu picking*).
   c. Asignación taxonómica.
   d. Construcción de filogenias.
3. Análisis de datos (*downstream analysis*):
   a. Rarefacción y cálculos de diversidad (índices ecológicos, alfa y beta diversidad).
   b. Estudio estadístico (análisis multivariantes, análisis de redes, aprendizaje automático o *machine learning*, series temporales).

La metodología empleada en el análisis de los datos procedentes de la secuenciación masiva es clave para obtener resultados fiables. Diseñar adecuadamente los experimentos, ajustar convenientemente los parámetros de cada aplicación a la hora de procesar las secuencias, seleccionar la base de datos de referencia adecuada y recopilar una información de las muestras (metadatos) lo más completa posible para los análisis estadísticos es tan importante como obtener una cantidad representativa de secuencias del ADNr 16S de buena calidad (Aird *et al.*, 2011; Bokulich *et al.*, 2013; Liu *et al.*, 2008; Schloss, 2010). Hoy en día la metodología más empleada en estudios de este

tipo es la dictada por el EMP (*Earth Microbiome Project*), que pretende construir un catálogo global de la diversidad bacteriana de nuestro planeta (Gilbert *et al.*, 2014).

6. Uso combinado de técnicas para comprender la composición y dinámica del microbioma de la glándula uropigial de la abubilla

Las herramientas de secuenciación masiva de segunda y tercera generación realizan la amplificación simultánea de un gran número de secuencias de una o varias muestras, permitiendo obtener una estima de la diversidad global de los sistemas estudiados y cuantificar la abundancia relativa de los distintos grupos en una muestra de ADN bacteriano (Birtel *et al.*, 2015; Quigley *et al.*, 2014). Sin embargo, estos métodos generan un gran número de secuencias y en cierta medida esa progresión hacia el aumento del número obtenido es a costa de obtener secuencias más cortas (Metzker, 2010), existiendo por tanto un compromiso entre la diversidad detectada y la profundidad taxonómica a la que se llegan a identificar los OTUs.

En todo método basado en la detección de secuencias en un pool de ADN existe la posibilidad de sesgos, por ejemplo en la afinidad de las sondas por las secuencias de determinados grupos (Acinas *et al.*, 2005). De esta forma, unos marcadores concretos pueden ser capaces de detectar determinados grupos mejor que otros. Así, aunque el ADNr 16S suele ser el marcador del que más diversidad taxonómica se ha secuenciado (ver Apartado 3), principalmente por ser el primero en desarrollarse para estudios bacterianos, otras regiones como

el ITS podrían producir información complementaria en el estudio de comunidades.

Por último, el uso de los métodos moleculares de secuenciación masiva puede llevar a menospreciar el valor de los taxones más escasos presentes en una comunidad, al dar importancia notable a la cantidad de secuencias detectadas de cada grupo. La abundancia de los taxones en microbiomas complejos no tiene por qué corresponder siempre con la importancia de su papel en el sistema (Dohrmann *et al.*, 2013; Pedrós-Alió, 2012), por lo que los métodos que buscan taxones específicos cuyo papel quiere ponerse de manifiesto, como la amplificación por PCR específica o el marcaje in situ mediante fluorescencia usando sondas específicas (FISH), pueden ser herramientas complementarias y de gran utilidad para comprender la importancia de determinados grupos microbianos.

Con el objetivo global de conocer la composición y la dinámica de la comunidad de bacterias que conforman el microbioma de la glándula uropigial de la abubilla, en esta tesis se han utilizado de manera combinada distintas herramientas moleculares, con el objetivo de tener una visión lo más amplia y completa posible (Capítulos I y II). La eficiencia de los métodos de secuenciación masiva para el procesado de un número grande de muestras será aprovechada para estudiar los factores ambientales que puedan afectar a la comunidad de la glándula centrándonos en aquellos no vinculados a lo que sucede en el interior del nido, que ya ha sido objeto de una tesis doctoral previa (Martínez-García, 2015), como puedan ser el tipo hábitat en el que se ubica el nido, incluido el efecto del mantenimiento de las abubillas en cautividad, las

condiciones meteorológicas que acompañan a los intentos reproductores, el momento de la estación reproductora en que cría cada individuo o la localización geográfica de las poblaciones (Capítulo III).

La obtención de información detallada de la composición completa de esta comunidad y la abundancia relativa de los diferentes taxones nos va a permitir abordar el estudio de la dinámica individual del microbioma a lo largo de la estación reproductora en las hembras (Capítulo IV). Finalmente, y con la misma base del conocimiento de la comunidad completa que permiten estos métodos, utilizaremos una aproximación experimental para tratar de determinar la influencia de la comunidad microbiana de la secreción en el sistema que parece ser central en la evolución de este mutualismo entre la abubilla y sus bacterias (ver Apartado 2): el huevo (Capítulo V).

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OBJETIVOS DEL TRABAJO

La glándula uropigial de la abubilla europea (*Upupa epops*), se trata de una glándula holocrina encerrada dentro de una cápsula de tejido conectivo que deposita su secreción en un tubo colector común. Las aves esparcen esta secreción sobre la superficie de su piel y de su plumaje de manera periódica, en un proceso conocido como *preening* (acicalamiento). A esta secreción se le han atribuido distintas propiedades, como por ejemplo protección e impermeabilización de las plumas, o el mantenimiento de la higiene frente a patógenos y parásitos. En el caso de la abubilla, el volumen y composición química están relacionados con factores estacionales y sexuales, produciéndose una secreción más oscura y maloliente en hembras reproductoras y en pollos durante el período de estancia en el nido, mientras que en machos y en hembras no reproductoras la secreción producida es blanquecina e inodora. Además de las diferencias encontradas en su composición química entre ambos tipos de secreciones, en las secreciones marrones se han aislado bacterias pertenecientes mayoritariamente al género *Enterococcus*. La actividad antimicrobiana de la secreción uropigial se ha atribuido inicialmente, a la naturaleza de sus propios componentes y probablemente también a la presencia de sustancias inhibidoras producidas por bacterias, y entre las que se han incluido las bacteriocinas. Las bacterias presentes en la glándula de las secreciones oscuras, no sólo podrían estar implicadas en las características de la secreción, sino también en sus propiedades antimicrobianas, puesto que en las secreciones blancas –carentes

en todos los casos de actividad antimicrobiana-, tampoco se han detectado poblaciones microbianas cultivables.

Hasta ahora, los estudios relacionados con la caracterización de la microbiota de esta secreción se han llevado a cabo mediante métodos dependientes de cultivo, en las cuales sólo se habían podido aislar especies del género anteriormente mencionado. Sin embargo, las observaciones mediante microscopia electrónica de transmisión de la glándula, han puesto de manifiesto una gran heterogeneidad de tipos morfológicos de microorganismos, y que sugería por lo tanto la existencia de una diversidad mucho más compleja de la que inicialmente se había propuesto. A esto habría que sumar los análisis químicos de la propia secreción de la glándula, donde se han detectado distintos tipos de ácidos orgánicos propios del metabolismo de microorganismos anaerobios estrictos. De todo esto, surge la hipótesis de que podrían existir otras poblaciones microbianas distintas a los enterococos y que no se habrían conseguido aislar probablemente, porque las condiciones de cultivo no hubiesen sido las adecuadas o simplemente se trate de microorganismos no cultivables.

El obtener un perfil completo de la comunidad que habita en la glándula uropigial mediante métodos independientes de cultivo y especialmente en su secreción, es un aspecto fundamental para conocer el progreso del estudio de este sistema ave-bacterias, pues permitirá en el futuro poder realizar comparaciones con las bacterias presentes en otras zonas del cuerpo, estudiar los cambios estacionales que se dan en la comunidad, así como realizar manipulaciones para determinar qué aspectos son responsables del

establecimiento de una comunidad particular o qué beneficios puede aportar ésta a su hospedador.

Actualmente las técnicas moleculares permiten realizar estudios cada vez más precisos y exactos para identificar y cuantificar las poblaciones microbianas de una comunidad sin necesidad de cultivarlas, e incluso establecer las relaciones existentes entre ellas. En este sentido, la pirosecuenciación y la secuenciación por síntesis se ha revelado como las más eficaces para conocer la diversidad microbiana de un ecosistema complejo, principalmente por la reducción de costes y tiempo que se ha logrado en los últimos años y en segundo lugar por el alto número de datos que proporcionan.

Por todo ello, se plantearon varias hipótesis de trabajo en esta Memoria. En primer lugar, la presencia de bacterias en la secreción de la glándula uropigial podría conferir algún tipo de beneficio al ave hospedadora, por lo que resulta enormemente importante establecer los perfiles genéticos de estas comunidades microbianas y al mismo tiempo conocer sus identidades. Adicionalmente, esta comunidad podría estar relacionada con la capacidad antagonista de la propia secreción y al mismo tiempo conferirle características interesantes, especialmente durante la reproducción. Por otra parte, si los factores ambientales y/o el estado hormonal de las hembras tienen algún efecto en la comunidad bacteriana uropigial, tendrían que detectarse cambios estacionales, por ejemplo a lo largo del año o de su ciclo reproductor. Finalmente, si las bacterias uropigiales pueden tener algún efecto beneficioso directo para el ave, dadas las características de este sistema es muy probable

que alguno de ellos se manifieste en la reproducción, por ejemplo en relación con el éxito de eclosión de los huevos.

Los objetivos principales de este trabajo han sido determinar mediante métodos independientes de cultivo, la diversidad microbiana asociada a la secreción producida por la glándula uropigial de la abubilla europea *Upupa epops*, y al mismo tiempo demostrar experimentalmente los efectos beneficiosos que podrían presentar para el animal la comunidad de bacterias comensales y/o mutualistas presentes en su glándula. Para ello se han planteado los siguientes objetivos específicos:

1) Determinación de la huella genética de la comunidad mediante técnicas de tipo *fingerprinting* como por ejemplo la electroforesis en gradiente temporal de temperatura (TTGE) o el análisis de la región intergénica entre los ADN ribosomales 16S-23S, tanto en poblaciones salvajes como mantenidas en cautividad.
2) Estudiar la diversidad microbiana de la secreción uropigial mediante técnicas de secuenciación masiva, como la pirosecuenciación y secuenciación por síntesis.
3) Estudiar la dinámica poblacional estacional de las bacterias de la glándula uropigial de la abubilla comprobando la influencia de factores ambientales, como pueden ser los geográficos, meteorológicos y principalmente los relacionados con su ciclo reproductor.
4) Por último valorar el efecto de la transmisión vertical de bacterias desde la glándula a los huevos durante la incubación y determinar su posible relación con el éxito de eclosión.

MATERIAL Y MÉTODOS

1. Trabajo de campo

1.1. Especie de estudio

La abubilla euroasiática (*Upupa epops epops* Linnaeus, 1758) es la subespecie nominal de la especie politípica *Upupa epops*, perteneciente a la familia *Upupidae* (orden *Coraciiformes*, suborden *Bucerotes*) y cercana a las familias *Phoeniculiae* (abubillas arbóreas) y *Bucerotidae* (calaos). Se trata de un ave de tamaño medio (26-32 cm) con un característico pico fino, largo y curvado ligeramente hacia abajo, y una llamativa cresta eréctil. El plumaje es anaranjado en la cabeza y el cuerpo, pero las alas y la cola presentan un bandeado blanco y negro (Figura M.1) (Kristin, 2001).

Figura M.1. Coloración típica de un macho de abubilla. Por Juan Lacruz [CC BY-SA 3.0].

El dimorfismo sexual es poco acusado, aunque los machos tienen un tamaño ligeramente mayor y el pico proporcionalmente más largo que las hembras y presentan coloración rosácea en el pecho y el vientre, mientras que las hembras presentan un babero blanco bajo el pico, el pecho más anaranjado y el vientre blanquecino con líneas longitudinales oscuras. Los juveniles muestran en general una coloración parecida a la de las hembras, aunque con tonos más apagados, y tienen además el pico más corto y recto en comparación con los adultos (Martín-Vivaldi *et al.*, 2014a).

La distribución geográfica de este ave es bastante amplia, extendiéndose desde la zona subtropical al noroeste de África y del sur de Europa al sureste asiático (Figura M.2). Generalmente habita zonas de campo abierto, en terrenos arenosos, rocosos o limosos, con parches de vegetación baja o pastizal, e incluso terrenos de cultivo siempre y cuando tengan acceso al suelo donde buscan alimento. La abubilla anida en cavidades, por lo que las zonas que habita deben tener disponibilidad de árboles, paredes rocosas o construcciones con huecos en los que poder anidar. En general se trata de una especie solitaria, aunque pueden formarse grupos en época de migración (Cramp, 1985).

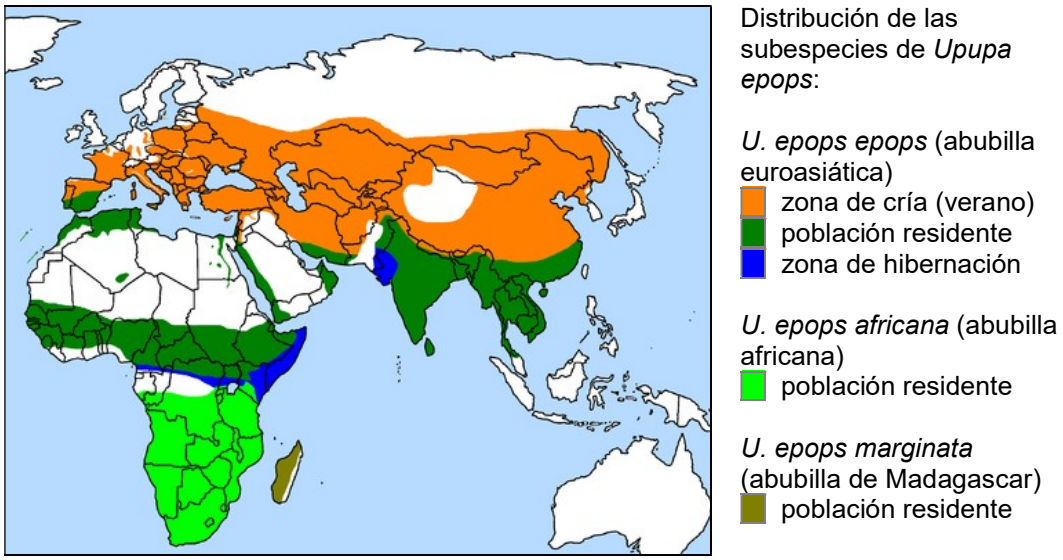

Figura M.2. Mapa de distribución de la abubilla. Adaptado de Ulrich Prokop [GFDL, CC BY-SA 3.0, CC BY 2.5].

La dieta de esta ave consiste fundamentalmente fases inmaduras de insectos (preferiblemente de escarabajos, lepidópteros, saltamontes y cigarras) que buscan introduciendo repetidamente el pico en el suelo, así como insectos adultos con cubiertas esclerotizadas como grillos o escarabajos (Figura M.3). Con frecuencia consumen arañas y escorpiones, y a veces también lagartijas o moluscos (Kristin, 2001).

Figura M.3. Abubilla transportando una presa al nido. Por Artemy Voikhansky [CC BY-SA 3.0].

La abubilla se considera una especie monógama, ya que los individuos reproductores establecen fuertes vínculos de pareja tras el cortejo, aunque éstos se mantienen únicamente durante una estación reproductora. No obstante, hay evidencias que sugieren otros sistemas de emparejamiento como poliandria o poliginandria y en varias poblaciones se ha comprobado que existe cierto porcentaje de paternidad fuera de la pareja (revisado en (Martín-Vivaldi *et al.*, 2014a).

La reproducción en esta especie está principalmente determinada por la disponibilidad de cavidades cerradas en las que poder anidar. Estas cavidades son empleadas como nido año tras año por distintas parejas, que depositan los huevos directamente sobre el material pre-existente en la cavidad, realizando una leve depresión en los restos de madera descompuesta y desechos de otras nidadas previas o remanentes de nidos de otras especies. No se produce, por tanto aporte de material de construcción al nido. Es frecuente que con su uso repetido y la escasas actividad de limpieza que realizan las abubillas, se

acumule en los nidos gran cantidad de restos de presas y excrementos a lo largo de la estancia de los pollos en la cavidad. La escasa sanitación del nido y la capacidad de los pollos de proyectar heces líquidas, además del mal olor de la secreción uropigial de hembras y pollos, se han considerado estrategias defensivas de los individuos que permanecen en el nido y que por tanto están sujetos a mayores riesgos de depredación (Kristin, 2001).

Una hembra realiza una puesta (o dos como máximo) en cada estación reproductora, aunque es frecuente observar puestas de reposición cuando una puesta previa fracasa. La época de cría puede extenderse desde mediados de invierno hasta finales de verano dependiendo de la región geográfica. El tamaño de puesta suele ser de 6 a 8 huevos, pudiendo oscilar entre 4 y 12. Las hembras se encargan de incubar los huevos durante 17 días, a lo largo de los cuales no abandonan el nido y son alimentadas por el macho, que normalmente no entra en el nido. La incubación, realizada solo por la hembra, comienza con el primer o el segundo huevo, de forma que se produce una asincronía total en la eclosión que provoca una jerarquía de tamaño en los pollos, en la que los menores se ven claramente perjudicados en su competencia por el alimento. Tras la eclosión del primer huevo, la hembra continúa en el nido durante unos 8 días más, por lo que el alimento de la progenie es aportado exclusivamente por el macho en este periodo. Posteriormente, hasta que los pollos alcanzan los 24-30 días de edad, tanto la hembra como el macho aportan comida a los pollos. Cuando los pollos abandonan el nido como volantones permanecen cerca de sus progenitores, de los que siguen obteniendo alimento durante las 2-5 semanas siguientes. A lo

largo de este periodo, o incluso antes de que los pollos abandonen el nido, es cuando la hembra puede realizar la segunda puesta, volviendo a permanecer en el nido durante el nuevo periodo de incubación. (Martín-Vivaldi *et al.*, 2014a).

1.2. Zona de estudio

La población de abubillas silvestres objeto de estudio en la presente tesis está ubicada en el sureste de España, concretamente en la Hoya de Guadix (37° 18’ N, 3° 11’ O), un altiplano de aproximadamente 1.000 metros de altitud, situado entre las sierras de Baza, Mágina, Harana y Sierra Nevada (Figura M.4).

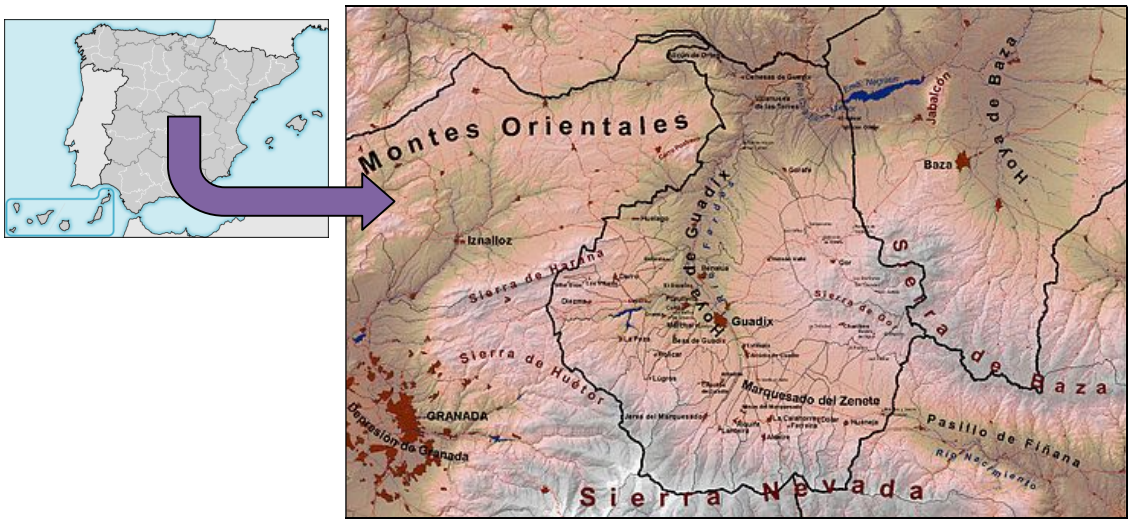

Figura M.4. Ubicación de la población de abubilla sujeta a estudio en la presente tesis. Imagen compuesta: mapa de España por HansenBCN [Public domain] y mapa de la Hoya de Guadix por Juan Pedro Ruiz Castellano [GFDL, CC BY-SA 3.0].

Esta zona es en general abierta, con parches de vegetación aislados que incluyen restos de bosques de encinas, y sobre todo terrenos agrícolas que

albergan cultivos de cereales, almendros, olivos y cultivos de regadío. La población de abubillas de este territorio lleva bajo estudio desde 1992, y ocupa tanto los huecos naturalmente disponibles para anidar, como cajas nido de corcho de 18 x 18 x 40 cm de cavidad interior con orificio de entrada de 5,5 cm de diámetro colocadas por el equipo de investigación en la zona a partir de 2003 y renovadas periódicamente. En el interior de las cajas nido recién instaladas se sitúa un lecho de material particulado fino, ya sea corteza de pino o hueso de aceituna triturado que sirva como sustrato para la nidificación. A partir de ese momento el material que se acumula en las cajas no se retira y sirve para las puestas subsiguientes. La época de cría en esta región puede extenderse desde febrero hasta junio-julio (Martín-Vivaldi *et al.*, 2006, 2014a).

Además, se trabajó con una población de abubillas mantenida en cautividad desde al año 2006. Parte de la población en cautividad se ubicó en instalaciones de la Universidad de Granada en la Facultad de Ciencias (Granada, España) y la Hoya de Guadix (en la misma zona que la población silvestre), y otra parte en las instalaciones de la finca experimental La Hoya perteneciente a la Estación Experimental de Zonas Áridas en Almería (36º 50’ N, 2º 28’ O). En estas zonas se distribuyeron jaulas de 3 m x 2 m x 2 m para albergar parejas en la época de cría, lo suficientemente aisladas unas de otras como para evitar la interferencia entre ellas. Las jaulas se visitaban a diario para alimentar a las abubillas a base larvas de mosca, grillos adultos y corazón de ternera rebozado en pasta de cría para aves insectívoras.

1.3. Diseño experimental y recogida de muestras

Todas las abubillas adultas estudiadas se marcaron con anillas para su reconocimiento individual. Una vez establecidas las parejas en época de cría, los nidos se visitaron a diario hasta la puesta, y luego se siguieron al menos semanalmente para recoger la información necesaria que permitiera establecer tamaño de puesta, fecha de eclosión, éxito de eclosión y número de volantones producidos. En los años 2010, 2011 y 2012 en que se realizaron varios experimentosen la población silvestre para objetivos diferentes a los incluidos en esta tesis, se han utilizado datos provenientes de individuos pertenecientes a los grupos controles de todos ellos.

Para el último capítulo se realizó una aproximación experimental que conllevó muestreos en dos años diferentes (2010 y 2012). En esos años la población de hembras reproductoras de cautividad se dividió al azar en un grupo experimental, un control puro y un control de la manipulación. A las hembras del grupo experimental se les colocó una estructura de tubos sobre la glándula que impedía su acceso a la secreción, mientras que a las del grupo de control de la manipulación se les colocó una estructura similar pero que no bloqueaba el acceso a la glándula (Figura M.5), mientras que a las hembras del control puro no se les hizo manipulación alguna (los detalles del método pueden encontrarse en (Martín-Vivaldi *et al.*, 2014b). Los tubos eran revisados cada tres días y el usado para el almacén de la secreción era reemplazado por uno vacío.

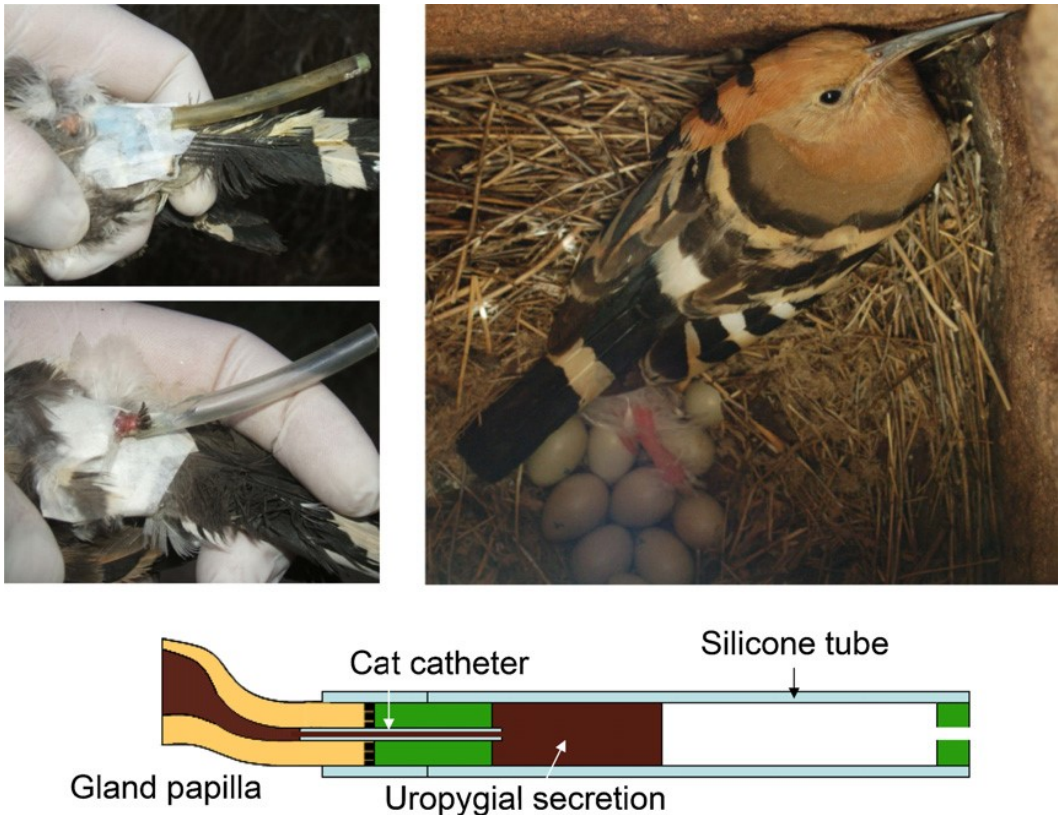

Figura M.5. Aspecto de la estructura de tubos instalados en una hembra experimental (arriba) y una hembra con control de la manipulación abajo) así como un esquema de las partes que componían la estructura completa para recoger la secreción producida por la hembra e impedir su uso. A la derecha aparece en su nido una hembra del grupo control puro que deja ver los huevos de color beige por el uso de la secreción sobre su superficie.

Para incrementar el riesgo de infección de los huevos y comprobar el efecto de la secreción en su defensa, en los nidos de cautividad usados para el experimento en 2012, se untaron dos huevos de la puesta con un homogeneizado del contenido de huevos de gallina que habían pasadodos semanas en el interior de cajas nido del campo que habían sido utilizados por aves en años anteriores. El homogeneizado de huevo de gallina en PBS se incubaba una noche a 37 ºC justo antes de su uso para obtener un inóculo de bacterias potencialmente patógenas. En 2011, además de muestrear a los individuos en la época reproductora (primavera-verano), en las jaulas de cautividad se hicieron seguimientos semanales desde el invierno para la

obtención de muestras fuera del periodo reproductor, necesarias para responder algunas cuestiones planteadas en nuestros objetivos.

Las muestras de secreción uropigial se obtuvieron con guantes lavados con etanol al 96 % y puntas estériles, pipeteando directamente del interior de la ampolla de la glándula tras lavarla ligeramente con etanol al 96 %. Las muestras de las cáscaras de los huevos fueron obtenidas mediante la limpieza con hisopos estériles de toda la superficie de un huevo con PBS estéril. Todas las muestras se conservaron a -20 ºC hasta que eran procesadas en el laboratorio.

Los datos de variables meteorológicas empleados en esta tesis se obtuvieron a través de la Consejería de Medio Ambiente de la Junta de Andalucía:

(http://www.juntadeandalucia.es/medioambiente/servtc5/sica/sima_av.jsp).

2. Trabajo de laboratorio

Los detalles de cada uno de los métodos de laboratorio y análisis empleados se incluyen en los capítulos correspondientes a los diferentes objetivos abordados, y en la INTRODUCCIÓN se ha incluido un apartado dedicado específicamente a la evaluación de las diferentes metodologías disponibles para el estudio de comunidades microbianas, por ello en este

apartado sólo se presenta de forma resumida un listado de los procedimientos utilizados.

2.1. Extracción de ADN y métodos moleculares

Cada tipo de muestra puede requerir un tratamiento distinto para obtener un buen rendimiento de ADN. Para las secreciones de abubilla, de naturaleza viscosa, empleamos un kit comercial basado en columnas de intercambio iónico (FavorPrep Genomic DNA extraction kit, Favorgen Biotech) combinado con un tratamiento de lisozima para asegurar la rotura de las paredes celulares bacterianas. Las muestras de la superficie de la cáscara de los huevos, dada la forma de muestreo, mostraron mejor resultado empleando una técnica denominada extracción por Chelex (Martín-Platero *et al.*, 2010).

El ADN total de las muestras, una vez extraído, se sometió a amplificaciones por PCR, con cebadores y condiciones específicas según el objetivo perseguido. Para la obtención de huellas genéticas (*fingerprinting*) se emplearon cebadores específicos para amplificar regiones conservadas de los ADNs ribosomales, que por su naturaleza pueden dar información estructural de una muestra compleja, con distintos tipos de bacterias. Tanto si los patrones se establecen gracias a la variabilidad en longitud de una región como en el caso del RISA (Gürtler y Stanisich, 1996), como si las diferencias se basan en la composición de bases de una secuencia concreta, que pueden hacerse patentes empleando un gradiente de temperatura como en el TTGE (Ogier *et al.*, 2002), el resultado puede hacerse visible mediante una electroforesis. En el caso de emplear algún tipo de automatización, por ejemplo el ARISA, tras marcar las muestras usando un cebador con un fluorocromo, se emplearían un

sistema capilar y un detector de fluorescencia para ir midiendo la longitud de los fragmentos marcados y obtener el perfil genético deseado.

Otra opción empleada fue clonar directamente los fragmentos amplificados, para poder secuenciarlos individualmente tras aislarlos gracias al uso de plásmidos con resistencia a antibióticos y cepas bacterianas sensibles. No obstante, esta metodología está hoy en día en desuso, puesto que es posible secuenciar directamente el ADN amplificado de una muestra a gran escala empleando pirosecuenciación (tecnología 454) (Margulies *et al.*, 2005) o secuenciación por síntesis (tecnología Illumina) (Bentley *et al.*, 2008). Cada una de estas secuenciaciones ofrece en principio distintas ventajas, ya que la primera suele dar secuencias más largas, preferibles cuando se desea obtener información taxonómica más fiable, mientras que la segunda proporciona un mayor número de secuencias, más adecuado para estudios en los que es necesario comparar la composición microbiana de distintas muestras.

2.2. Cuantificación de poblaciones bacterianas: FISH y qPCR

El uso de sondas de ADN con marcaje fluorescente que hibridan con el ARN ribosómico (FISH) permite detectar bacterias activas en una muestra, localizarlas, y contarlas con ayuda de las herramientas adecuadas como TMARKER (Schüffler *et al.*, 2013). Esas sondas pueden ser generales, como la Eub338 (Amann *et al.*, 1991) empleada en esta tesis para detectar bacterias totales en la secreción, o específicas, como la Enc221 (Wellinghausen *et al.*, 2007), que permiten distinguir un grupo específico de bacterias, en este caso los enterococos. Además, especialmente cuando se usan sondas específicas,

puede usarse algún tipo de marcaje fluorescente del ADN como control positivo (en nuestro caso Hoescht).

Por su parte, la PCR en tiempo real permite acoplar a la reacción de amplificación clásica una medida de fluorescencia, de forma que en cada ciclo es posible cuantificar las copias generadas por la presencia de un fluorocromo que sólo emite al unirse al ADN de cadena doble. A partir de estas medidas es posible calcular el número de secuencias originales si se dispone de un patrón molecular que se amplifica a la vez que la muestra. Este patrón consiste generalmente en una dilución seriada de un vector de tamaño conocido en el que se clona el amplicón de interés, de forma que es posible aproximar el número de copias empleado midiendo la concentración de la muestra mediante una serie de cálculos. En nuestro caso, esta aproximación nos permitió calcular el número de bacterias de diferentes muestras de secreción uropigial, usando cebadores universales (Klammer *et al.*, 2008; Muyzer *et al.*, 1993).

3. Análisis de datos

El tratamiento necesario para los distintos tipos de datos obtenidos con las diferentes metodologías descritas, tiene que ser necesariamente diferente.

Para el análisis de los perfiles genéticos de la comunidad, el programa Fingerprinting II Informatix (Bio-Rad) permitió obtener patrones y compararlos entre si. BLAST (Altschul *et al.*, 1990) fue la herramienta de elección para identificar las secuencias procedentes tanto de clones individuales como de secuenciación masiva. No obstante distintas aproximaciones fueron necesarias para aplicar ese algoritmo dada la diferencia de escala entre ambas

estrategias. En la secuenciación masiva, primero hay que realizar cierto procesamiento, como separar las secuencias por muestra y posteriormente agruparlas por similitud en OTUs, lo que reduce considerablemente el volumen de los análisis posteriores. Con el avance de las tecnologías de secuenciación, se han desarrollado paralelamente numerosas herramientas informáticas adecuadas para el tratamiento de los datos resultantes, y entre ellas en esta tesis se han usado QIIME (Caporaso *et al.*, 2010), Mothur (Schloss *et al.*, 2009), USEARCH y UCHIME (Edgar, 2010; Edgar *et al.*, 2011) y el *Distribution Based Clustering* (Preheim *et al.*, 2013), siguiendo el esquema básico de procesamiento descrito en la INTRODUCCIÓN. Otra forma de emplear las secuencias es obtener filogenias para comparar la similitud y la composición de las muestras sin necesidad de obtener identificaciones, que se puede, obtener por encuadre taxonómico con secuencias ya caracterizadas. Este enfoque se empleó con las librerías de clones usando los programas Clustal (Larkin *et al.*, 2007) y Phylip (Felsenstein, 2005). Para la secuenciación masiva, aunque la filogenia no fue empleada como tal, se usaron medidas filogenéticas basadas en la generación de árboles mediante FastTree (Price *et al.*, 2009). La comparación entre comunidades en estos casos se hizo mediante la generación de matrices de distancias con UniFrac (Lozupone y Knight, 2005). Otras herramientas de análisis adicionales se obtuvieron a partir del *pipeline* establecido en QIIME (Caporaso *et al.*, 2010), uno de los más completos que existen actualmente.

4. Análisis estadísticos

En su mayoría estos análisis se llevaron a cabo empleando el programa estadístico R (R Development Core Team, 2014), que gracias a la adición de distintos paquetes que añaden funcionalidades de todo tipo, tiene un gran potencial. Entre los test más empleados en esta tesis se encuentran análisis discriminantes paramétricos (ANOVA) y no paramétricos (Mann-Whitney U y Kruskal Wallis). También se han empleado análisis multivariantes como Adonis (Oksanen *et al.*, 2013) y regresiones múltiples con matrices o MRM (Goslee y Urban, 2007). Ciertos análisis como el BEST para elegir los taxones que mejor explican un patrón o los cálculos de distancias geográficas se llevaron a cabo en programas como Primer 7 (Primer-E) y Quantum GIS (Quantum GIS Development Team, Open Source Geospatial Foundation Project, 2015).

Las figuras se obtuvieron principalmente usando el programa estadístico R (R Development Core Team, 2014) en combinación con el paquete básico Graphs (gráficos de cajas y de puntos), y los paquetes Ggplot2 (gráficos de interacción) (Wickham, 2009) y Made4 (*heatmaps*) (Culhane *et al.*, 2005).

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CAPÍTULOS

CAPÍTULO I:

The hoopoe's uropygial gland hosts a bacterial community influenced by the living conditions of the bird

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ABSTRACT

Molecular methods have revealed that symbiotic systems involving bacteria are mostly based on whole bacterial communities. Bacterial diversity in hoopoe uropygial gland secretion is known to be mainly composed of certain strains of enterococci, but this conclusion is based solely on culture-dependent techniques. This study, by using culture-independent techniques (based on the 16S rDNA and the ribosomal intergenic spacer region) shows that the bacterial community in the uropygial gland secretion is more complex than previously thought and its composition is affected by the living conditions of the bird. Besides the known enterococci, the uropygial gland hosts other facultative anaerobic species and several obligated anaerobic species (mostly clostridia). The bacterial assemblage of this community was largely invariable among study individuals, although differences were detected between captive and wild female hoopoes, with some strains showing significantly higher prevalence in wild birds. These results alter previous views on the hoopoe-bacteria symbiosis and open a new window to further explore this system, delving into the possible sources of symbiotic bacteria (e.g. nest environments, digestive tract, winter quarters) or the possible functions of different bacterial groups in different contexts of parasitism or predation of their hoopoe host.

INTRODUCTION

Bacteria are ubiquitous key players in a vast number of ecological and evolutionary processes (Moran, 2002; Pace, 1997). In particular, they are frequent counterparts of symbiotic interactions, where they have been an important force shaping the evolution of a wide range of living beings, ranging from protists to all multicellular groups including plants and animals (reviewed in Moran, 2006; Wernegreen, 2012). These symbiotic interactions have traditionally been classified according to their effects on host fitness as parasitism, mutualism, or commensalism. Nevertheless, distinguishing between them is not simple as they frequently have similar origins and involve similar mechanisms (Ochman and Moran, 2001), and can change from one status to another depending on circumstances (Pérez-Brocal *et al.*, 2013). In fact, it has been demonstrated that commensal (apparently neutral) bacteria can be key in the maintenance of microbiota homeostasis and thus of host health, resembling the effects of mutualistic (clearly beneficial) symbionts (Cabreiro and Gems, 2013; Hamdi *et al.*, 2011; Shin *et al.*, 2011; de Steenhuijsen Piters *et al.*, 2015). Although parasitism draws more research efforts due to its clinical relevance (see Barber *et al.*, 2013; Dando *et al.*, 2014; Giogha *et al.*, 2014 for some reviews), studies on mutualistic systems and their various known benefits for the hosts have been discovered to be a fruitful area of research. Mutualistic benefits of bacteria include processes related to nutrient uptake and assimilation, detoxification, tolerance to environmental factors and access to new ecological niches, prevention of infections and pathogen establishment, predator avoidance, signaling, and immunity development (Leser and Mølbak, 2009; Moran, 2006; Rajilić-Stojanović, 2013).

Systems that involve a single bacterial strain are particularly interesting for exploring coevolutionary relationships between host and bacterial symbionts because the bacterial specificity usually implies intimate coevolutionary association. These associations have been accurately described in some systems (Baumann, 2005; Dunlap *et al.*, 2007; Ehinger *et al.*, 2014; Moran *et al.*, 2008), but the generality of these so-called two-partner symbioses has been questioned, mainly because of the detection of secondary symbionts in several of these systems (Baumann, 2005), which implies a more relaxed coevolutionary process (Dunlap *et al.*, 2007). New data suggests that most symbiotic relationships (particularly commensalism and mutualism) involve several microorganisms, including complex microbial communities interacting with a single host, which offers a new picture of the interactions between hosts and bacteria and the multi-directional benefits involved (Hussa and Goodrich-Blair, 2013).

The hoopoe (*Upupa epops*), a hole-nesting bird, has a mutualistic association with bacteria residing in its uropygial gland (Martín-Vivaldi *et al.*, 2009; Soler *et al.*, 2008). Symbiotic bacteria can produce antimicrobial substances that can benefit their host, contributing to defense against parasites and pathogens, as described in salamanders, ants and plants for example (Banning *et al.*, 2008; Currie *et al.*, 1999; Saikkonen *et al.*, 1998), and also in birds (Rajchard, 2010; Soler *et al.*, 2010). In the case of strains inhabiting hoopoe uropygial glands, several studies have shown that they provide the bird with antimicrobial substances such as bacteriocins (Martín-Platero *et al.*, 2006) and volatile metabolites (Martín-Vivaldi *et al.*, 2010) that aid in protecting feathers against keratinolytic bacteria (Ruiz-Rodríguez *et al.*, 2009) and eggs against pathogens (Martín-Vivaldi *et al.*, 2014; Soler *et al.*, 2008). Until now, the hoopoe-bacteria symbiosis had been studied using culture-dependent techniques showing an association with *Enterococcus faecalis* and (less frequently)

with other *Enterococcus* species (Ruiz-Rodríguez *et al.*, 2013). However, it is well known that most microorganisms are unable to grow in laboratory conditions (Rappé and Giovannoni, 2003), and several lines of evidence suggest the presence of other groups of bacteria in the uropygial gland of hoopoes. For example, the elimination of bacteria from glands by means of antibiotics affected the presence of several metabolites not produced by enterococci (Martín-Vivaldi *et al.*, 2010), and the fingerprints of hoopoe secretions revealed several unidentified bands (Martínez-García *et al.*, 2015). Additionally, microscopic studies of the glands have revealed rod-shaped bacteria and spirochetes (Martín-Vivaldi *et al.*, in preparation). These evidences suggest that the microbiome hosted in hoopoe glands is a mix of strains belonging to different taxonomic groups. Therefore, the community may result from the incorporation of bacteria from different sources, such as the environment and the parental microbiota (Chaston and Goodrich-Blair, 2010; Mandel, 2010). Indeed, we already know from cross-fostering experiments that there is an environmental influence on the enterococci strains established in nestling hoopoe glands (Ruiz-Rodríguez *et al.*, 2014). Furthermore, the special properties of hoopoe uropygial secretions caused by bacteria in females are maintained only while incubating (Martín-Vivaldi *et al.*, 2009), and enterococci were not detected in the white secretions produced outside this period (Soler *et al.*, 2008), so the symbionts (or part of them) may be acquired each breeding season. Thus, the environmental influence mediating differences in samples taken from individuals living and breeding in different conditions (i.e. captivity *vs.* wild) may be of interest to clarify the origin of the uropygial bacterial symbionts.

It has been well established that molecular techniques uncover many unculturable microbes in complex microbial samples (Fisher and Triplett, 1999; Muyzer *et al.*, 1993; Smalla *et al.*, 2007). Therefore, the use of molecular

techniques is necessary to fully understand the relationships between hoopoes and bacteria living in their uropygial gland and the impact of the bird living conditions on its composition. This work aims to fill this gap, making a first approach to the molecular analysis and identification of the diversity of the bacterial community living in the hoopoes uropygial gland and its secretion. In addition, this study focuses on the effects of the living conditions on the community of bacteria established in the female uropygial gland, comparing wild and captive environments. The first identification of bacteria other than enterococci within the hoopoe uropygial gland secretion and the influence of bird living conditions on the composition of its bacterial community are described and discussed here.

MATERIALS AND METHODS

1. Ethics statement

Research was performed in accordance with national (Real Decreto 1201/2005 de 10 de Octubre) and regional guidelines. All necessary permits to perform this research were provided by the Consejería de Medio Ambiente of the Junta de Andalucía (Spain). The study was approved by the Ethics Committee of the University of Granada (Comité de Ética en Experimentación Animal, CEEA, Ref.: 785). Suffering and stress in the birds was carefully minimized. No bird died as a consequence of its manipulation for sampling during this study.

2. Study area, study species, and sampling procedures

Samples were collected during the 2006 and 2010 breeding seasons in both a wild and a captive population of hoopoes. The wild population is located in the Hoya de Guadix (southern Spain), where hoopoes breed within nest boxes placed in trees and buildings (for a more detailed description of the study area see Martín-Vivaldi *et al.*, 1999, 2006). The captive population was maintained at three different locations: Hoya de Guadix (Granada), the gardens of the Faculty of Science (Granada, University of Granada), and La Hoya Experimental Farm (Almería, Experimental Station for Arid Zones, CSIC). Breeding pairs were housed in independent cages (at least 3 m x 2 m x 2 m), with access to soil and provided with live food (crickets and fly larvae) and meat (beef heart) *ad libitum*. The females sampled in 2010 had been maintained in captivity at least since the previous breeding season.

During the breeding season, from mid-February to the end of July, nest boxes and cages were visited twice per week to collect uropygial gland secretion samples and record breeding parameters. All adults were ringed with numbered (Spanish Ministry of the Environment) and colored rings to aid identification. Sterile latex gloves were worn to extract uropygial gland secretions from breeding females and nestlings using a micropipette with a sterile tip introduced within the gland papilla, where secretion accumulates, after lightly washing the exterior of the gland with 96% ethanol. Samples were collected into sterile 1.5 ml microfuge tubes at 4ºC and then stored at -20ºC within 24 hours until processed. The extraction kit Realpure Spin Kit (Durviz S.L., Valencia, Spain) was used with 5–20 µL of each secretion sample for total nucleic acid extraction. The negative control showed no amplification for all the PCR conditions used in subsequent analyses.

3. Molecular characterization of symbiotic bacterial diversity

Two different approaches were used to estimate uropygial gland bacterial diversity and to identify the most common species: (a) Ribosomal Intergenic Spacer Analysis (RISA) (Borneman and Triplett, 1997), with 36 uropygial secretions of females (23 from wild individuals and 13 from captive individuals) sampled in 2010; (b) Sequencing of the 16S rDNA and the ribosomal intergenic spacer between the rDNA 16S and rDNA 23S genes. Partial 16S sequences were obtained from a Temporal Temperature Gradient Electrophoresis (TTGE) and ribosomal intergenic spacers from RISA profiles. The 11 most prevalent bands from TTGEs and the five most prevalent bands from RISA fingerprints were selected for sequencing. In addition, we built clone libraries for the 16S rDNA and for the ribosomal intergenic spacer region. Clone libraries of the 16S rDNA were obtained from three samples (two from 2010 and one from 2006) and clone libraries of the ribosomal intergenic spacer from nine samples (all from 2010).

4. Fingerprinting of bacterial communities

The RISA PCR from genomic DNA was performed according to (Gürtler and Stanisich, 1996) using the oligonucleotide primers 72f (5'-TGC GGC TGG ATC TCC TT-3') and 38r (5'-CCG GGT TTC CCC ATT CGG-3') (Gürtler and Stanisich, 1996; Martin-Laurent *et al.*, 2001). TTGE samples were prepared by nested PCR using the oligonucleotide primers W01 (5′-AGA GTT TGA TC[AC] TGG CTC-3′) and W012 (5′-TAC GCA TTT CAC C[GT]C TACA-3′) in the first PCR, and HDA1-GC (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC

TCC TAC GGG AGG CAG CAGT-3') and HDA2 (5'- GTA TTA CCG CGG CTG CTG GCA-3') in the second PCR. TTGE PCR and electrophoresis were performed following (Ogier *et al.*, 2002). The 50 µL PCR reactions for both RISA and TTGE were carried out in a Mastercycler thermocycler (Eppendorf, Hamburg, Germany) using a MBL-*Taq* DNA polymerase (Dominion-MBL, Cordoba, Spain). Gels were stained in ethidium bromide and photographed under UV transillumination using a UVP ImageStore 5000 system (Ultraviolet Products, Cambridge, UK).

After running the polyacrylamide gels to separate the bands produced by the amplification of the ribosomal intergenic spacer, the resulting images were processed with the Fingerprinting II Informatix Software (Bio-Rad, Hercules, California, USA) to standardize them and make them comparable. A dendrogram was then constructed using the Unweighted Pair Group Method with Arithmetic averages (UPGMA) algorithm based on the Jaccard similarity coefficients using the same software. We also compared groups of samples determined based on their living conditions (captive *vs.* wild), using a non-parametric permutation MANOVA test (Adonis function) included in the Community Ecology Package Vegan (Oksanen *et al.*, 2013) for the R statistical software (R Development Core Team, 2014). The richness was considered as the number of observed OTUs (Operational Taxonomic Units) recognized as different bands in our fingerprints. Beta diversity analyses to compare community composition between samples (i.e. Principal Coordinate Analysis (PCoA) based on the Jaccard similarity matrix) were performed using scripts from the Quantitative Insights Into Microbial Ecology (QIIME, version 1.8) pipeline (Caporaso *et al.*, 2010) and the EMPeror software for graphic representations (Vázquez-Baeza *et al.*, 2013). All other statistical analyses (including Mann-Whitney U-tests and Kruskal-Wallis tests for diversity comparisons and the two-sample test for equality of proportions with continuity correction to

check the relative abundance of OTUs within different groups) were performed using the respective functions within the R Stats Package included in the R statistical software (R Development Core Team, 2014). A Kruskal-Wallis test was performed to ensure that the three populations maintained in captivity could be used as one captive population in subsequent analyses (Kruskal-Wallis tests, N = 13; χ^2 = 0.94; P = 0.62; Almería, N = 8, samples mean (SD) = 15.0 (3.1); Faculty of Science, N = 4, samples mean (SD) = 16.3 (0.8); Guadix, N = 1, samples mean (SD) = 15.0 (0.0)). The taxonomic assignment of specific bands in our samples based on the sequencing of the five most prevalent bands in the fingerprint patterns was performed using the band-matching option in the Fingerprinting II Informatix Software (Bio-Rad, Hercules, California, USA), with 1% position tolerance and 0% optimization. The 36 analyzed samples from 2010 females were divided into two groups based on their living conditions (maintained in captivity, N = 13, and wild, N = 23). Both groups contained a single sample per individual to avoid pseudoreplication. The consistency of the groups regarding sample origin was analyzed using resampling based on the Jackknife average method (Cameron and Trivedi, 2005).

5. Clone libraries, sequencing, and OTU identification

A fragment of 700 base pairs (bp) from the 16S rDNA (including V1-V4 regions) from three samples and the variable ribosomal intergenic spacer from bacteria in the secretions of nine samples was amplified as described above. Then

the PCR products for each region were pooled. The mixed PCR products were cloned on pGEM-T Easy Vector according to the manufacturer's instructions (Promega Corporation, Madison, Wisconsin, USA) to generate clone libraries for the 16S rDNA and the ribosomal intergenic spacer.

The 29 selected clones for the 16S rDNA and the 21 for the ribosomal intergenic spacer libraries were characterized by TTGE and RISA respectively to group them by similarity in order to sequence only one per group. The result was 12 different groups for the 16S rDNA and 13 groups for the ribosomal intergenic spacer. One clone from each group was then used for sequencing from plasmid DNA preparations using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Life Technologies, Madrid, Spain). Additionally, the fragments obtained from the bands isolated from TTGE and RISA fingerprints were also sequenced. The bands selected for sequencing were purified from the polyacrylamide gels using passive diffusion in sterilized water (12 h at 4ºC), and were then re-amplified using the same primer and reaction conditions as before to be sequenced.

Before identification, the sequences obtained for the same marker (either 16S rDNA or ribosomal intergenic spacer) from clones and fingerprint bands were compared by aligning them with Clustal 2.1 (Larkin *et al.*, 2007). Sequences with 100% identity were considered the same, and only one (the longest) was used for the systematic study. A total of 18 different sequences have been deposited in the NCBI GenBank database with accession numbers KR076707 to KR076724.

The MegaBLAST module of the BLAST (Basic Local Alignment Search Tool) Sequence Analysis Tool BLASTN 2.2.30+ (available online at the National Center for Biotechnology Information (NCBI) website (Bethesda, USA, http://www.ncbi.nlm.nih.gov/)) was used to search for the closest phylogenetic neighbors using local alignments of our sequences with those in the GenBank

Nucleotide collection (nr/nt) database. The overall phylogenetic similarity was evaluated using an expected threshold of 10 and all the default parameters for MegaBLAST. For each OTU sequence (i.e. the different sequences retained), we selected the five different known strains with the highest Max Score, and also included all known species in the list whose Query Cover or Identity percentage were within the range of those of the five selected species. By following these criteria, we ensured that all the most probable candidates closely related to our OTUs were used for phylogenetic analyses. Whenever possible, two different strains per candidate species were included for each OTU. After this step, we detected that our ribosomal intergenic spacer candidates (due to the high variability in this region) did not have homology with the database sequences throughout all their length, but only in two clearly differentiated subregions (presumably associated to two tRNA genes, for alanine and isoleucine, that have been found in the ribosomal intergenic spacer amplified in other studies (Boyer *et al.*, 2001; Hayashi *et al.*, 2012; Nakagawa *et al.*, 1992)). Since these two subregions were not present simultaneously (nor were either of them present in all the sequences), they were used separately to avoid errors in the grouping of candidates with different subregions. Moreover, eight of the candidate sequences for the ribosomal intergenic spacer did not correctly align with the subregions used, and were therefore excluded from further analyses.

Sequences from these species were included together with those of our OTUs to build three different consensus trees (one for the 16S rDNA and another two for the two homology subregions within the ribosomal intergenic spacer), where our OTUs could be taxonomically situated. The sequences were aligned with Clustal 2.1 (Larkin *et al.*, 2007) using the default parameters, including one outgroup sequence for the pertinent region in each case from the algae *Caulerpa taxifolia*. Then a

standard bootstrap was performed with the Seqboot tool included in Phylip 3.695 (Felsenstein, 2005) and 100 trees were built corresponding to each subsample with FastTree 2.1.3 (Price *et al.*, 2010, 2009). Finally, a consensus tree was built using the Consense tool included in Phylip 3.695 (Felsenstein, 2005). The bootstrapping values were incorporated with the Compare-To-Bootstrap script from the MicrobesOnline tree comparison tools (available at http://www.microbesonline.org/fasttree/treecmp.html). Image editing of the trees was performed using FigTree 1.4.1 (Rambaut, 2006).

RESULTS

6. Bacterial diversity in hoopoe uropygial glands

The analyses of RISA and TTGE fingerprints revealed that the bacterial community inhabiting hoopoe uropygial glands is more complex than previously thought, showing 11 to 21 different bands per individual (Fig. 1). Nevertheless, the community was rather uniform among hoopoes, with 24% of the bands detected in RISA gels (N = 46) present in at least 50% of individuals (Fig. 1A). The fingerprint profiles of nestlings looked very similar to those of females (Fig. 1B). However, given the low number of nestlings sampled they were not used in our statistical comparison.

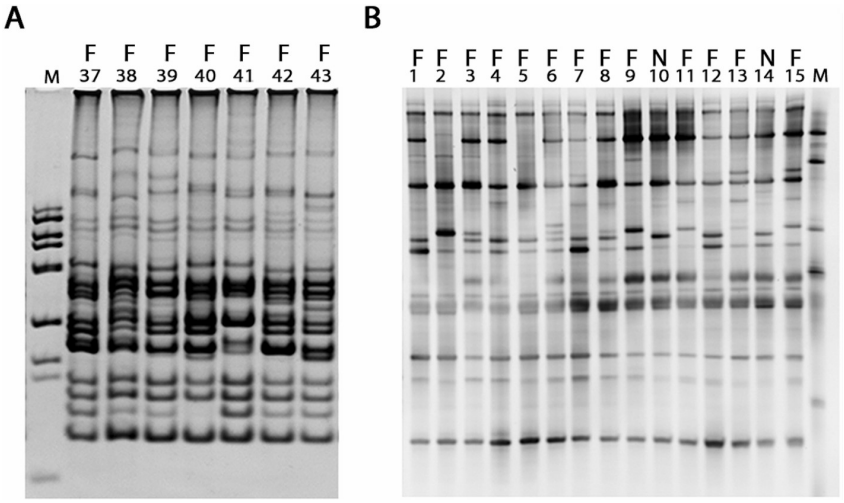

Figure 1. Uropygial bacterial community fingerprints. (A) Example of RISA gel with the band profiles of uropygial secretions of seven different wild hoopoe females used in this study. Lane M contained a 100-bp DNA ladder (Biotools, B&M Labs, Madrid, Spain). (B) TTGE gel showing the characteristic band profiles of the uropygial secretions of 13 wild hoopoe females (F), and also of two wild nestlings (N) to show their profile resemblance with those of females; lane M contained a band profile of bacterial strains from the laboratory collection.

7. Differences between wild and captive hoopoes

We compared the bacterial communities reflected in RISA gels among populations, and found that the richness (i.e. number of bands) in wild samples was non-significantly higher than in captive samples (Mann-Whitney U-test, N = 36; U = 93.5; P = 0.062; wild samples mean (SD) = 17.0 (2.2), captive samples mean (SD) = 15.4 (2.6)). When taking into account the identity of the OTUs in the communities, bacterial communities from females maintained in captivity differed significantly from those from wild females (Adonis, $F_{1,35}$ = 16.89; P < 0.001; Fig. 2). The consistency of the groups based on sample origin showed a high percentage (above 84%) of correct assignments for both captive and wild samples to their corresponding group when resampling our dataset. In addition, the Principal Coordinate Analysis (PCoA) confirmed our results, as both wild and captive female samples clustered separately. According to the PCoA, the difference between these populations

explained 15% of the variation among bacterial communities, with both principal components (PC) 1 and 2 accumulating 23% of total variation in our samples.

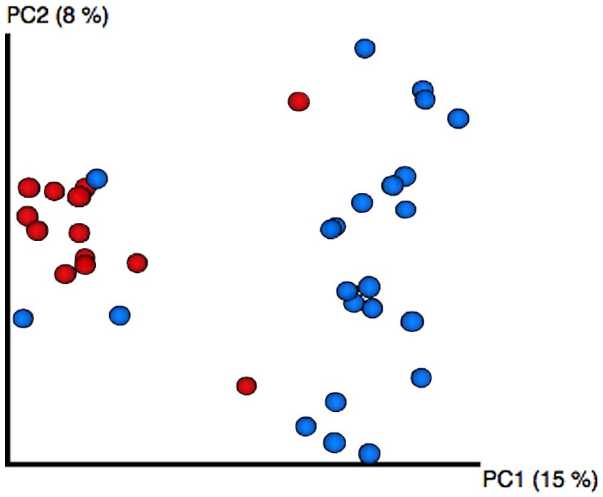

Figure 2. PCoA analysis of captive and wild hoopoe female samples. The PCoA shows sample grouping by similarities in OTU composition. Samples from captive (red) and wild (blue) female hoopoes cluster separately. The percentage of variation explained by the plotted principal components (PC) is indicated in parentheses.

8. Species composition

The 16S sequences revealed 14 different OTUs (i.e. the different sequences identified; Fig. 3), most (10 out of 14) grouped within clades of the phylum *Firmicutes*, two within the phylum *Actinobacteria* (family *Coriobacteriaceae*), and the remaining two within the phylum *Bacteroidetes* (genus *Porphyromonas*) (Fig. 3, Table 1). Among the *Firmicutes*, OTU 1 was an *Enterococcus* species and probably belongs to *E. faecium* or *E. faecalis* (Fig. 3). We found two different *Peptoniphilus* species (OTU 5 and 6), and within the same family (*Clostridiales Incertae Sedis XI*), two strains close to the genera *Murdochiella* and *Kallipyga* (OTUs 8 and 9). OTU 10 grouped with the genus *Clostridium* but did not match any of the candidate species. The three remaining OTUs grouped in the order *Clostridiales* were an

Eubacteriaceae (OTU 11) close to several species of the genus *Eubacterium* and two species (OTUs 2 and 12) linked to a clade of *Ruminococcaceae* (but with low consistency; Fig. 3). In four cases (8 OTUs), two of our OTUs were paired in the tree, with different bootstrap probabilities. In two instances (OTUs 3+4 and 8+9), that probability was 100% (Fig. 3). These OTUs were therefore considered as strains of the same species since, in all the other cases of 100% probability in the grouping of two sequences, the taxa were two strains of the same species (Fig. 3, 10 cases).

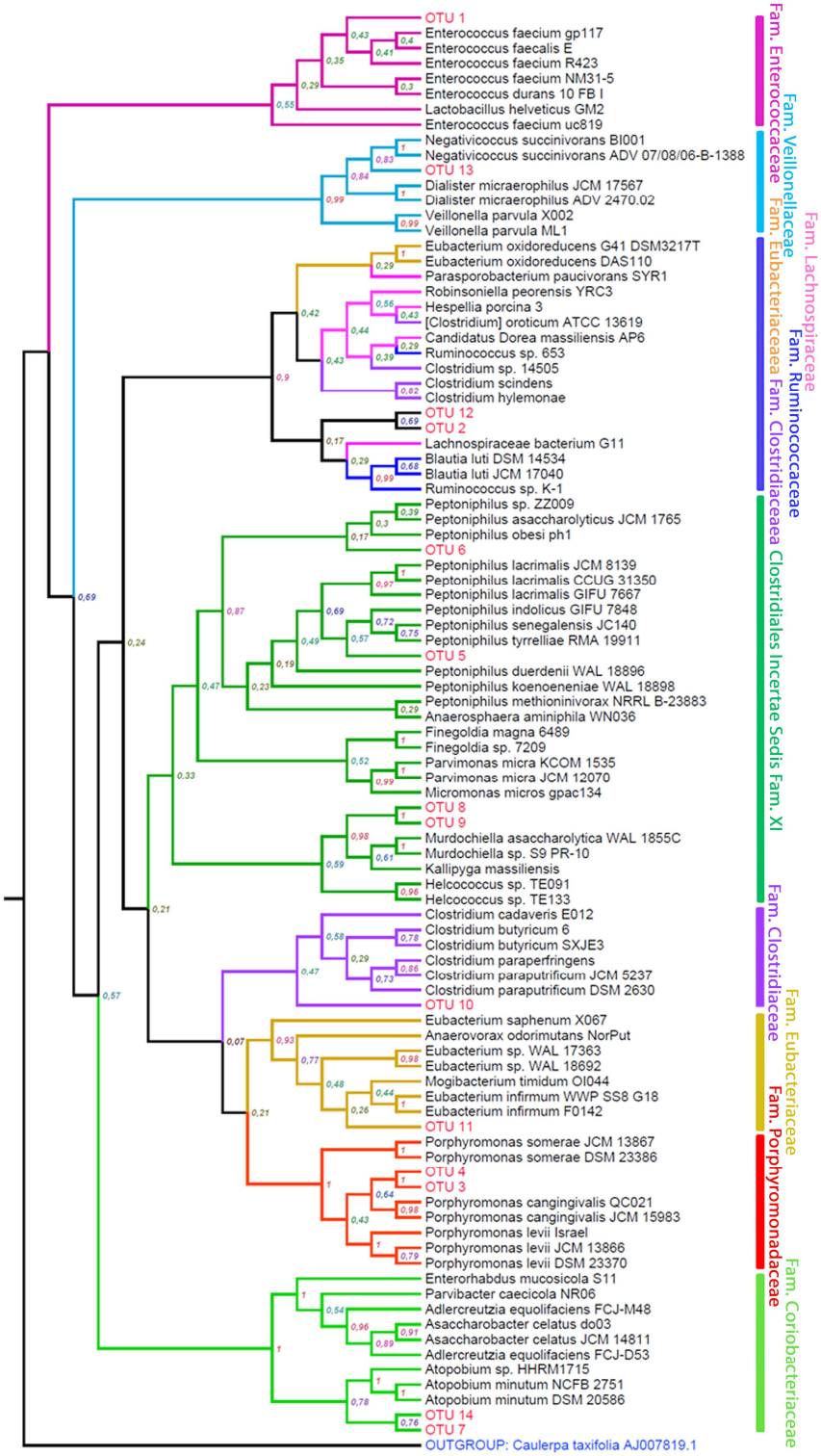

Figure 3. Consensus tree with the taxonomic position of the different bacterial OTUs detected in hoopoe uropygial secretions by sequencing the 16S rDNA. Labels in nodes indicate the bootstrap mean probability for each clade after 100 repetitions.

The analysis of ribosomal intergenic spacer sequences revealed eight different OTUs (in this case called ITS; Fig. 4), most included within the phylum *Firmicutes* (7 out of 8), and only one (ITS 7) grouped with the phylum *Actinobacteria* (order *Actinomycetales*) (Table 1). In the *Firmicutes*, two OTUs could be clearly assigned to the genus level. ITS 2 grouped with *Enterococcus* species and ITS 6 grouped with *Clostridium* species. These two OTUs, together with ITS 8 (grouped within *Clostridiales Incertae Sedis XI* in the genus *Peptoniphilus*), coincided with taxa detected using the 16S marker (Table 1). However, the ribosomal intergenic spacer marker provided the identification of five additional OTUs in groups not detected among the 16S sequences. ITS 1 was included in a clade within the family *Thermoanaerobacteriaceae* (close to the *Thermoanaerobacterium* species), ITS 4 formed a clade with a *Megasphaera* species (family *Veillonellaceae*), ITS 5 was grouped with a *Coprococcus* species (family *Lachnospiraceae*), and ITS 3 was grouped close to the family *Paenibacillaceae*. The ribosomal intergenic spacer approach, as the use of 16S (Table 1), identified an OTU within the *Actinobacteria* phylum (ITS 7), although in this case it was not a *Coriobacteriaceae* but was related to the families *Nocardioidaceae* and *Micrococcaceae*.

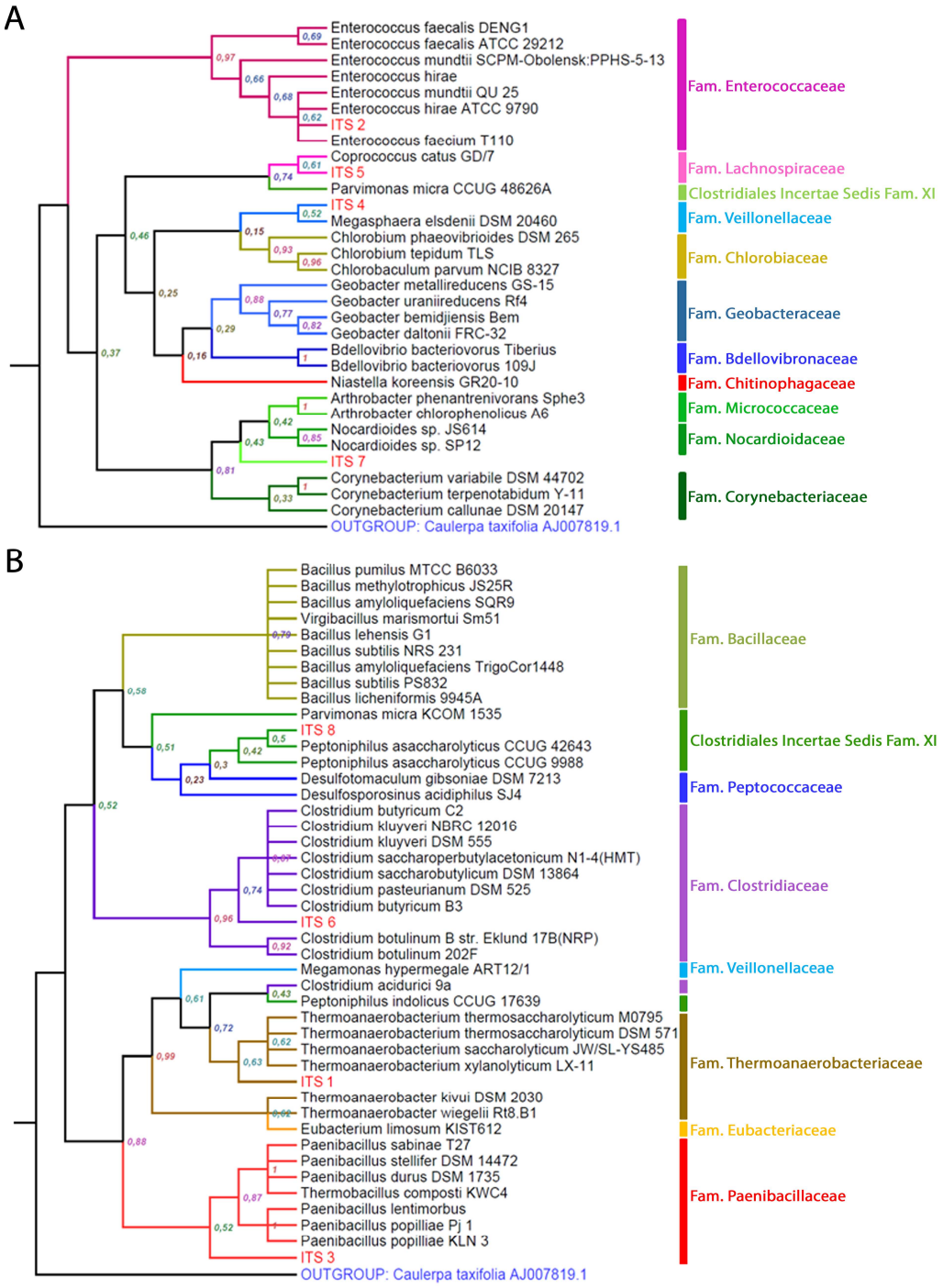

Fig. 4. Consensus trees with the taxonomic position of the different bacterial OTUs (ITS) detected in hoopoe uropygial secretions by sequencing the ribosomal intergenic spacer. Panels A and B include OTUs grouped according to each of the two subregions found within the ribosomal intergenic spacer. Labels in nodes indicate the bootstrap mean probability for each clade after 100 repetitions.

In no case did one of our OTUs (either from 16S or from the ribosomal intergenic spacer) get paired with a known species with a very high probability (more than 90%), so at most we could assign them to particular genera or families according to their position in the consensus trees (Table 1). OTUs were assigned to the family of the known species (from the GenBank Nucleotide collection database) they were more closely related to in the consensus trees. OTUs were assigned to a particular genus only if their sequence resulted clearly included within a clade of different species of the same genus. When an OTU was not included in a particular genus but grouped with one, it was considered to be related to that genus (in parentheses in Table 1).

Table 1. Taxonomic position of the OTUs detected in the uropygial secretion of female and nestling hoopoes.

TAXONOMY						OTUs	
Phylum	**Class**	**Order (Suborder)**	**Family**	**Genus**	**Species**	**16S**	**ITS**
Firmicutes	*Clostridia*	*Clostridiales*	*Incertae Sedis XI*	*Peptoniphilus*	*Peptoniphilus* sp1	OTU 5	
					Peptoniphilus sp2	OTU 6	
					Peptoniphilus sp3		ITS 8
				(Murdochiella/ Kallipyga)	Sp1 strain 1	OTU 8	
					Sp1 strain 2	OTU 9	
			Eubacteriaceae	*Eubacterium*	*Eubacterium* sp	OTU 11	
			Lachnospiraceae	*(Coprococcus)*	Sp		ITS 5
			Clostridiaceae	*(Clostridium)*	Sp	OTU 10	
				Clostridium	*Clostridium* sp		ITS 6
			Unknown	Unknown	Sp1	OTU 2	
					Sp2	OTU 12	
		Thermoanaerobacteriales	*Thermoanaerobacteriaceae*	*(Thermoanaerobacterium)*	Sp		ITS 1
	Bacilli	*Lactobacillales*	*Enterococcaceae*	*Enterococcus*	*Enterococcus* sp	OTU 1	ITS 2
		Bacillales	*Paenibacillaceae*	*(Paenibacillus)*	Sp		ITS 3
	Negativicutes	*Selenomonadales*	*Veillonellaceae*	*(Negativicoccus)*	Sp	OTU 13	
				Unknown	Sp		ITS 4
Actinobacteria	*Actinobacteria*	*Actinomycetales*	Unknown	Unknown	Sp		ITS 7
	Coriobacteria	*Coriobacteriales* (*Coriobacterineae*)	*Coriobacteriaceae*	*(Atopobium)*	Sp1	OTU 7	
					Sp2	OTU 14	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Porphyromonas*	*Porphyromonas* sp1 strain 1	OTU 3	
					Porphyromonas sp1 strain 2	OTU 4	

Taxonomic position inferred for the OTUs detected in the uropygial secretion of female and nestling hoopoes by several different molecular methods. When the sequences were not clearly included within a genus in the trees, the genus most closely related to the sequence is given in parentheses (see Figs. 3-4).

1. Composition of wild and captive hoopoes uropygial microbiomes

The comparison between captive and wild hoopoes in the frequency of appearance of the five most prevalent bands in RISA fingerprints revealed some differences (Table 2). There were two OTUs that appeared in most samples of both populations, while in the other three cases prevalence differed between them. ITS 8

(*Peptoniphilus* species) was present in 100% of the analyzed samples (N = 36). ITS 7 (an *Actinobacteria* species close to *Nocardioidaceae* and *Micrococcaceae*) also appeared in a high percentage of samples regardless of their origin; in fact, it occurred in a similar percentage in both sample groups, as confirmed by a two-sample test for equality of proportions with continuity correction (N = 36; χ^2 = 0.11; P = 0.736). In the other three cases, prevalence was higher in samples from wild females than in samples from captive females (Table 2), although differences were significant only for ITS 4 (probably a *Veillonellaceae* species, N = 36; χ^2 = 15.36; P < 0.001) and ITS 6 (a *Clostridium* species, N= 36; χ^2 = 6.40; P = 0. 011), whereas ITS 5 (probably a *Lachnospiraceae*) was more common in wild females but not significantly so (N = 36; χ^2 = 1.97; P = 0.161).

Table 2. Frequency of appearance of ITS OTUs in wild and captive hoopoe females.

Phylum	Class	Order	Family	Genus	OTU	% wild females (N=23)	% captive females (N=13)
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	-	ITS 5	34.8	7.7
			Clostridiaceae	*Clostridium*	ITS 6	65.2	15.4
			Incertae Sedis XI	*Peptoniphilus*	ITS 8	100.0	100.0
	Negativicutes	*Selemonadales*	*Veillonellaceae*	-	ITS 4	73.9	0.0
Actinobacteria	*Actinobacteria*	*Actinomycetales*	-	-	ITS 7	91.3	100.0

Comparison of the frequency of appearance of the five most prevalent bands (sequenced from RISA gels) between the uropygial secretions of wild hoopoe females and females maintained in captivity.

DISCUSSION

Molecular analysis of the bacterial community in hoopoe uropygial secretions revealed that it is more complex than previously found by culture-dependent methods. This study shows that, apart from *Enterococcus* species (the predominant bacteria found in hoopoe glands to date (Martín-Vivaldi *et al.*, 2009; Ruiz-Rodríguez *et al.*, 2012)), the dark secretions of breeding females and nestlings host at least 20 different OTUs from three different bacterial phyla (*Firmicutes*, *Actinobacteria*, and *Bacteroidetes*). The communities were very similar among individuals, indicating a specific, largely invariable assemblage of bacterial species residing in this particular bird organ, although a deeper sampling (for example using high-throughput sequencing) may be needed to confirm this finding. Interestingly, the bacterial OTUs in hoopoe glands have rarely resulted in the identification of known species, suggesting that this environment has a high potential to uncover strains with interesting properties.

This is the first time that bacteria other than enterococci are found to be abundant within the uropygial gland of birds. Multipartite mutualistic symbioses of animals with microorganisms are the norm rather than the exception (reviewed in Hussa and Goodrich-Blair, 2013). The stability of this kind of associations requires that different symbionts provide independent benefits to hosts or that, if producing redundant benefits, counterbalance their relative abundances or coevolve to shared pathways of resource consumption and production (Hussa and Goodrich-Blair, 2013). It is worth noting that the bacteria in hoopoe glands belong to a wide taxonomic range (at least 18 different species corresponding to 16 different genera in 13 families). We have previously shown that, within the *Enterococcus* genus, species and strains with higher antimicrobial potential (mediated by bacteriocin

production) prevail in hoopoe uropygial secretions (Ruiz-Rodríguez *et al.*, 2013). Although hoopoes may regulate the gland colonization by bacteria to keep an adequate assembly, these results suggest that the competition ability of different strains colonizing the uropygial gland of hoopoes determines the final bacterial communities (Scheuring and Yu, 2012). The coexistence in the gland of several unrelated species from different families opens the possibility of unrelated bacterial strains providing complementary benefits to hosts. The main benefit demonstrated for enterococci inhabiting the gland is the production of bacteriocins defending hoopoes from feather-degrading bacteria (Ruiz-Rodríguez *et al.*, 2009) and eggs from trans-shell infection of embryos (Martín-Vivaldi *et al.*, 2014; Soler *et al.*, 2008). Other species of the bacterial community in the uropygial secretion may be responsible for certain chemicals detected in the secretion that increase its antimicrobial potential. Indeed, we have shown that the experimental eradication of bacteria from glands by antibiotics eliminates several volatile compounds with antimicrobial activity, some of them typical metabolites from various of the bacterial groups detected in this study (such as 4-methyl pentanoic and 3-phenyl propanoic acids produced by clostridia) (Martín-Vivaldi *et al.*, 2010). It is well known that *Actinobacteria* are especially able to produce protective substances and are involved in mutualisms with animals that benefit from their antifungal capabilities (Kaltenpoth, 2009). One *Actinobacteria* genus related to the *Actinomycetales* found in hoopoe glands (*Nocardioides*) has been confirmed as an antibiotic-producing mutualist in fungus-growing ants (Barke *et al.*, 2010). *Atopobium*, a group close to *Coriobacterium* (Rainey *et al.*, 1994), is involved in nutritional endosymbiosis with *Pyrrhocorix* bugs (Kaltenpoth *et al.*, 2009). Therefore, these bacteria are good candidates to act as beneficial strains in hoopoes as well. Additionally, some of the bacteria detected in the gland of hoopoes are likely responsible for the stench of the

secretion. This may confer protection against predators (Cramp, 1998), as occurs for the closely related green woodhoopoe (Burger *et al.*, 2004). Future studies should examine the specific role in secretion functionality of the different bacterium species integrating the bacterial community.

All the genera discovered within the bacterial community of the hoopoe uropygial gland are obligated or facultative anaerobes. This result was expected since the gland ampulla where the secretion accumulates is near a sphincter that isolates the cavity from the surrounding air, and the viscosity of the secretion itself may prevent oxygen diffusion. Although hosts would benefit from antimicrobial compounds produced within the uropygial gland, only aerotolerant antibiotic-producing bacteria such as enterococci would be able to grow directly on host tissues (feathers and eggshells). However, strictly anaerobic bacteria may be able to grow in the numerous crypts of hoopoe eggshells, which rapidly fill with bacteria-rich secretion during incubation, thereby providing the anaerobic environment required by some of the bacteria detected (Martín-Vivaldi *et al.*, 2014). Further research is needed on the composition of communities established on eggshells to confirm these hypotheses.

Our results emphasize the importance of molecular methods to characterize the symbiotic community of the hoopoe uropygial gland. Nevertheless, even though molecular methods are powerful tools for the study of bacterial communities (Weng *et al.*, 2006), results may vary depending on the method employed. In this study, we used two different approaches (sequencing of bands from fingerprints and clone libraries) for two different markers (16S rDNA and ribosomal intergenic spacer), which produced complementary results. Remarkably, enterococci (the taxonomic group whose presence and importance in this system has been repeatedly confirmed by culture-dependent methods (Martín-Vivaldi *et al.*, 2014; Ruiz-

Rodríguez *et al.*, 2009, 2012, 2013; Soler *et al.*, 2008)) have only been detected by sequencing of bands from fingerprints and not in clone libraries. This disparity is probably because this group is in low abundance in comparison to other bacteria groups within the uropygial secretion. The combination of methods used here suggests that we have detected the most prevalent (selected in the fingerprints) and abundant (cloned in libraries) strains residing in hoopoe uropygial gland secretions. Nevertheless, in future research on this topic other approaches such as high-throughput sequencing may be needed to describe the whole uropygial bacterial diversity, as well as the relative abundances of the different strains.

The diversity of bacterial communities in terms of composition differed for captive and wild hoopoes, suggesting environmental influences on the uropygial bacterial community assemblage. Although the variation among our bacterial populations explained by the first two axes of the PCoA constituted only 23% of the total, this is a common result in diversity studies (see for example Aagaard *et al.*, 2012; Alekseyenko *et al.*, 2013; Suchodolski *et al.*, 2012). Most of the variation among bacterial communities comes from rare strains that are less relevant than predominant ones when describing such communities, and whose influence is usually not included within the first PCoA axes (Pedrós-Alió, 2006). Environmental influences have previously been described for the enterococci population (Ruiz-Rodríguez *et al.*, 2014), and our own results now extend this conclusion to the entire bacterial community. Wild and captive hoopoes breed in nest boxes installed using the same materials, but several environmental differences (including diet) may be the cause of the detected differences. In fact, many bacteria closely related to the OTUs found in hoopoe secretions inhabit the digestive tract of animals (Attwood *et al.*, 2006; Hugon *et al.*, 2013; Mishra *et al.*, 2013; Vesth *et al.*, 2013), including birds (Benskin *et al.*, 2009; Berger *et al.*, 2003; Hupton *et al.*, 2003; Janiga

et al., 2007; Klomp *et al.*, 2008; Kühn *et al.*, 2003; Lu *et al.*, 2003, 2008; Moreno *et al.*, 2003; Ruiz-de-Castañeda *et al.*, 2011). *Atopobium* has appeared in the faeces of two different passerine species (Benskin *et al.*, 2010, 2015), whereas *Eubacterium* and *Ruminococcus* occur in chickens and gulls (Lu *et al.*, 2003, 2008). Finally, several species of *Clostridium* have been detected in some of these systems (Benskin *et al.*, 2009; Lu *et al.*, 2003, 2008). Therefore, if bacteria in the uropygial gland originate from the digestive tract of hoopoes, diet differences among captive and wild individuals may explain the detected differences in bacterial communities. Another possible explanation for the detected differences between wild and captive populations is the influence of the migratory behavior of hoopoes. While hoopoes maintained in captivity are kept in a similar environment the whole year, part of the wild hoopoe population breeding in our study area migrates to African winter quarters (Reichlin *et al.*, 2012). Therefore, migrating individuals are exposed to quite different environments from which they may acquire new bacteria. Winter quarter characteristics affect plumage microbiota of migratory birds (Bisson *et al.*, 2009) and, thus, may also explain the higher diversity of the bacterial community of wild hoopoes. All these possibilities deserve further research to untangle the bacterial colonization process of the hoopoe uropygial gland and the possible sources of variation affecting the composition of its symbiotic community (especially in the wild).

In conclusion, our results indicate that the bacterial community residing in the uropygial gland of female and nestling hoopoes is much more complex than previously reported. It includes at least 18 species belonging to 16 different genera and 13 families. Composition is largely invariable across individuals, although partially influenced by the environment as suggested by the differences found

between wild and captive hoopoes. These results open interesting new avenues of research in the understanding of the relationship between hoopoes and bacteria. Particularly intriguing is the possibility of different bacterial groups functioning additively (e.g. antimicrobial characteristics) or in different contexts (e.g. antipredatory, antiparasitic, or immunological). Moreover, the nature of interactions among the different taxa sharing the gland may be key in the ecological processes determining the final community composition, which can include bacteria acquired from different sources.

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CAPÍTULO II:

Seasonal and sexual differences in the microbiome of hoopoe uropygial secretions

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ABSTRACT

Communities of microbial symbionts have drastic consequences on host phenotype and survival, but factors affecting symbiotic microbiomes including host characteristics have been scarcely explored. Here we characterized the microbiomes of uropygial secretion of hoopoes (*Upupa epops*) collected at different phases (reproductive or not) and from different sexes and ages (chicks *vs* adults) and estimated differences among them. Secretion of nestlings and breeding females differed from those of males and non-breeding females in color, odor and antimicrobial properties. Although we found consistent patterns of bacterial assemblage, we detected variations associated to environmental conditions (i.e. captivity), sex, and reproductive phase and, therefore, with color and antimicrobial properties of secretion. Dark (i.e. with antimicrobial activity) secretions from breeding females harbored 10 fold more bacteria than white secretions from males or non-breeding females. These results imply the existence of a reservoir of bacterial symbionts in glands of non-breeding hoopoes, and suggest a host-regulating proliferation of symbiotic bacteria in phases of high risk of pathogenic infections (i.e. reproduction). We also discuss the importance of the detected bacterial genus that, due to known antibacterial properties, may play a central role defending their hosts against pathogenic infections.

INTRODUCTION

Ecological and evolutionary studies, that have been traditionally focused on explaining individual variation and factors determining associated fitness, can nowadays benefit from new perspectives of separately considering the phenotypic characteristics with direct link to the individual genome (i.e. organs) and those more related to the microbiome (i.e., holobionts perspective, Bordenstein and Theis, 2015). This is because microbial symbionts and factors affecting their stability and homeostasis may have drastic consequences on host phenotype and survival (Flórez *et al.*, 2015; Peterson *et al.*, 2015). The necessary first steps to understand these effects are to describe the composition and variation of the microbial communities and explore environmental factors that affect their stability, as well as identifying key microbial members associated to essential functions (Rajilić-Stojanović, 2013). In addition, exploring variation among symbiotic bacterial communities in relation to host characteristics and reproductive cycle would help to understand microbiome functioning within the hosts as well as consequences for the coevolutionary process (Moran, 2006; McFall-Ngai, 2006; Scheuring and Yu, 2012).

Living beings from all taxonomic groups host mutualistic microorganisms that are essential for survival (Leser and Mølbak, 2009; Moran, 2006; Rajilić-Stojanović, 2013). Among functions of microbial symbionts, those related to nutrient acquisition, immunity and physiological regulation of gut microbiome have been broadly studied, especially in mammals (Belkaid and Hand, 2014; Hooper and Gordon, 2001). Some other important functions of microbial communities, as those related to antiparasitic defenses, have been mainly explored in marine invertebrates and insects, but rarely

in vertebrate hosts (Flórez *et al.*, 2015) where variation of communities in relation to host characteristics are still poorly understood and deserve further investigations. Among the functions for symbiotic bacteria described in birds, such as nutrient absorption, immunity development, and defense against pathogens (Kohl, 2012; Ruiz-Rodríguez *et al.*, 2009a; Soler *et al.*, 2010), it has recently been discovered that a couple of species harbor complex bacterial communities with anti-predatory and/or anti-parasitic functions in their uropygial gland (Law-Brown and Meyers, 2003; Soler *et al.*, 2008). The uropygial gland secretion is used onto the plumage (Jacob and Ziswiler, 1982) and even to smear the eggs (Martín-Vivaldi *et al.*, 2014; Soler *et al.*, 2014) and its antimicrobial activity in the hoopoe (*Upupa epops*) depend on the action of symbiotic bacteria living within the gland (Martín-Platero *et al.*, 2006; Martín-Vivaldi *et al.*, 2010, 2014; Soler *et al.*, 2008). The uropygium is the only exocrine gland of birds which content is isolated from external environmental conditions. Therefore, the uropygial gland of hoopoes, which harbours bacterial communities, is a suitable space to study dynamics of bacterial communities in relation to the environmental conditions experienced by bird hosts. Indirect evidences of variation in the bacterial community of hoopoe's uropygial gland come from associations between density of bacteria and characteristics (color and odor) of secretion on the one hand and associations between color of secretion and sex, reproductive activity (nesting period of females) and age (nestlings *vs* adults) (Martín-Vivaldi *et al.*, 2009; Soler *et al.*, 2008). Interestingly, antimicrobial activity of secretions is also related to their color (Soler *et al.*, 2014) and the presence of symbionts (Martín-Vivaldi *et al.* 2010). That suggests that the dynamics of the bacterial communities should explain variation in functionality for hosts.

Within the bacterial community of hoopoes, enterococci are likely a key component. Previous studies on this system with culture dependent methods

pointed out their ability to produce antimicrobial compounds like bacteriocins (Martín-Platero *et al.*, 2006; Ruiz-Rodríguez *et al.*, 2012) and its relationship with antimicrobial functions (Martín-Vivaldi *et al.*, 2014; Ruiz-Rodríguez *et al.*, 2009b; Soler *et al.*, 2014). Thus, we paid particular attention to the dynamics (i.e. variations due to reproductive phase, sex, or age) of enterococci within the whole microbiome of the uropygial secretion of hoopoes.

With the aim of understanding variation in hoopoes phenotypes due to the dynamics and functionality of their microbial symbionts, here we characterized microbial communities of males, breeding and non-breeding females, and nestling hoopoes. To do so, qPCR, fluorescence *in situ* hybridization (FISH) and pyrosequencing approaches were used.

MATERIALS AND METHODS

1. Study area, study species and sampling procedures

The study was performed in 2009, 2010, 2011 and 2012 including a wild and a captive population of hoopoes maintained in Granada (Faculty of Science, Granada, University of Granada), Guadix, and Almería (La Hoya Experimental Farm, Almería, Experimental Station for Arid Zones, CSIC) (southern Spain). Breeding pairs were housed in independent cages of at least 3 m x 2 m x 2 m, with access to soil and provided with live food (crickets and fly larvae) and meat (beef heart) *ad libitum*. The wild population was studied in the Hoya de Guadix where hoopoes breed within nest-boxes placed in trees and buildings.

Prior to pairing, in winter, captive individuals were sampled once per month to collect white secretion samples. After pairing, nest-boxes and cages were visited twice per week until the end of the breeding season to record laying dates and collect uropygial gland secretion samples in both the wild and the captive populations. For individual recognition, all adults were marked with numbered (Spanish Ministry of Environment) and colored rings. The uropygial gland secretion samples were obtained as described in (Martínez-García *et al.*, 2015).

2. Fluorescence *In Situ* Hybridization

A small amount of the uropygial secretion extracted from the glands of some individuals (20 breeding females: 16 captive and 4 wild; 20 nestlings: 12 from 5 captivity nests and 8 from 6 wild nests; and 23 captive individuals in winter: 10 males and 13 females) was used to study abundance of metabolic active bacteria by means of Fluorescence *In Situ* Hybridization (FISH) of smears of uropygial secretions. Briefly, after fixing 5 µL of secretion on Polysine glass slides (Thermo Scientific), cells were hybridized with Eub338 (Amann *et al.*, 1991) labeled with Cy3 (Thermo Scientific) as a universal probe for Eubacteria and with Enc221 (Wellinghausen *et al.*, 2007) labeled with FITC as a probe for all enterococci. As positive control for the presence of bacteria we stained slides with Hoescht (Sigma-Aldricht). For a detailed explanation of sampling fixation and the hybridization process followed see Appendix 1. Using the 100x objective and an Olympus DP72 camera, two pictures of each of the three microscopic fields analyzed per hybridized slide were taken through an Olympus BX51 fluorescence microscope with DAPI, FITC and TRITC filters. Since amount of hybridized bacteria estimated with the

TMARKER v1.20163 software (Schüffler *et al.*, 2013) was repeatable within individual samples ($F_{1,78}$ = 7.66, P < 0.001, R = 0.69 for the 39 individuals with three available microscopic fields of the same sample), the average values of bacterial density was used in the analyses. See Appendix 2 for detailed information of estimations of bacterial densities.

3. DNA extraction and purification

Total DNA from uropygial secretion samples (2–20 µL) was extracted using the FavorPrep Genomic DNA extraction kit (Favorgen Biotech) according to manufacturer's instructions, adding a lysozyme treatment (10 mg/ml, at 37 °C for 30 min). PCR product purifications were performed using the GenElute PCR clean-up kit (Sigma-Aldrich) according to manufacturer's instructions. All final elutions were made in ultrapure sterile water.

4. Quantification by qPCR

A qPCR assay was used to estimate the amount of bacteria present in 2 µL aliquots of all kinds of collected secretion samples. Universal bacterial primers 338f (5'-ACTCCTACGGGAGGCAGCAG-3') and 518r (5'-ATTACCGCGGCTGCTGG-3') (Klammer *et al.*, 2008; Muyzer *et al.*, 1993) were used to amplify the samples along with a standard reference. The standard reference was a 197 base pair amplicon produced with the 338f and 518r primer pair cloned in the TOPO-TA vector (Life Technologies), and diluted in tenfold steps to obtain a standard curve covering concentrations from 10^{10} copies to 10^{1} copies after quantification by

spectrophotometry in a Nanodrop (Thermo Scientific). Each qPCR reaction contained a 20 µl mixture including 1x SensiFAST SYBR & Fluorescein Kit mastermix (Bioline), 0.3 µM of each primer and 2 µL of template DNA. The qPCR was carried out in a CFX96 Real-Time PCR System (Bio-Rad) with the following conditions: initial denaturalization at 95 ºC for 3 minutes, 40 cycles of 95 ºC for 5 seconds, 55 ºC for 10 seconds and 72 ºC for 10 seconds with plate read each cycle end, and a final melt curve from 55 ºC to 95 ºC with a temperature increment of 0.5 ºC each 5 seconds. All quantifications were performed in duplicate and the mean values were used to approximate the number of 16S rDNA copies per microliter of the original samples with the CFX Manager Software (Bio-Rad). As pyrosequencing let us know the bacterial composition of the samples, the 16S rDNA number of copies was transformed into approximate number of bacteria in our samples using the mean copies per genome of the most abundant group found (Větrovský and Baldrian, 2013).

5. Amplicon libraries construction and pyrosequencing

DNA samples for pyrosequencing were obtained from different individuals during the breeding season: 50 breeding females (34 wild and 16 captive) at day 12–15 of incubation and 33 nestlings from 33 different broods (15 wild and 18 captive) at day 15–20 after hatching. Additionally, DNA of secretions from non-nesting individuals also sampled in the middle of the breeding season (April–June) was pyrosequenced. The non-nesting individuals included 6 breeding males (2 wild and 4 captive) and 2 non-paired, captive females (one never paired and the other one previously paired in the same season).

The V1–V3 region for the 16S rRNA gene was amplified with the universal eubacterial primers 27F (5'-TCA GAG TTT GAT CMT GGC TCAG-3') and 533R (5'-GCT TAC CGC GGC KGC TGG CACG-3') (modified from Fierer *et al.*, 2008; Huse *et al.*, 2008). Those primers were modified to include an 8 base pair barcode (specific for each sample) for multiplexing, and the pyrosequencing adaptors A and B. The PCR was performed at an annealing temperature of 52 °C, with 25 cycles of amplification, using a Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific). Then the PCR products were purified, quantified by spectrophotometry using a Nanodrop (Thermo Scientific), and pooled in equimolar concentrations. After this step, amplicons suitable to be pyrosequenced with the 454 GS FLX Titanium system (Roche) using the Lib-L emulsion PCR method were obtained. Pyrosequencing was performed at the Genomics and Health Unit of the Center for Public Health Research (CSISP) in Valencia, Spain.

6. Data analysis and taxonomic identification

The preprocessing was carried out using scripts from Mothur v.1.33.3 (Schloss *et al.*, 2009) and QIIME 1.8 (Caporaso *et al.*, 2010) for sequence formatting and quality filtering and demultiplexing, respectively. The Distribution Based Clustering Method with a value for abundance cutoff of 10 (Preheim *et al.*, 2013) and the October, 2012 Greengenes database trimmed to adapt it to the amplified region in our 454 dataset (McDonald *et al.*, 2012) were used for OTU picking. Chimeric sequences were checked and filtered out using UCHIME v4.2.40 (Edgar *et al.*, 2011). Singletons and OTUs with prevalence lower than 10% were excluded from subsequent analyses. Samples were classified according to the sex and

reproductive phase, and filtered out when they had less than 1000 sequences for females (both breeding and non-paired) and nestlings, and less than 300 sequences for males. Rarefaction curves showed a quite good coverage of diversity for females (both breeding and non-paired) and nestlings, whereas more sequencing effort would be required to reach a good coverage for males. In order to obtain subsets of data within which all samples were comparable, a multiple rarefaction at 75 % of the minimum number of sequences per sample for each group was performed, and then the average OTU composition was calculated. When it was needed to compare different groups of samples, they were all filtered and rarefied using the lower parameters (i.e. those used for males), but keeping only the samples that previously passed each group filtering. After rarefaction, diversity indexes and core microbiomes were calculated using QIIME 1.8 (Caporaso *et al.*, 2010). The taxonomical classification was obtained using BLAST 2.2.22 (Altschul *et al.*, 1990) against the October, 2012 Greengenes database (McDonald *et al.*, 2012). Two samples from captive nestlings were thought to belong to individuals suffering an infection due to their altered profile (they showed above 50 % of Bacilli), so they were discarded from statistical analyses.

7. Statistical analyses

The values of bacterial density estimated from qPCR were used to test differences between sexes and among different reproductive phases using a two-way ANOVA with sex and phase as the independent factors, and Tukey multiple comparisons test for post-hoc comparisons. Variance within group of samples was homogenneous (Levene's Test; sex: $F = 1.32$, $P = 0.288$; reproductive phase: $F =$

1.74, P = 0.181), which validates the use of the ANOVA (McDonald, 2014). That was not the case for pyrosequencing values, and non-parametric Mann-Whitney U-test and the Kruskal-Wallis test were used instead. All the statistical analyses and plots were obtained with the R statistical software (R Development Core Team, 2014), using the appropriate functions within the basic R packages Stats (one-way and two-way ANOVA, U-test, Kruskal-Wallis test) and Graphics (boxplots). Some additional functions from the Mulcomp (Hothorn *et al.*, 2008) and Ggplot2 (Wickham, 2009) packages were needed to perform the Tukey multiple comparisons post-hoc test and the interaction plots respectively. Kruskal-Wallis test with Bonferroni correction for multiple comparisons was used to compare specific taxonomic compositions among groups, using the group_significance.py script from QIIME 1.8 (Caporaso *et al.*, 2010).

RESULTS

8. Bacterial counts in hoopoe uropygial secretions

Total bacterial counts showed significant differences between females and males (two-way ANOVA; F = 4.71, df = 2, P = 0.044) and along the different reproductive phases studied (two-way ANOVA; F = 4.22, df = 2, P = 0.032) (see Fig. 1). The interaction of both sex and reproductive phase was also significant (two-way ANOVA; F = 4.46, df = 2, P = 0.027) (see Fig. 1). The post-hoc comparisons revealed that those effects were due to sex differences during the breeding phase (Tukey; T = 3.68, P = 0.017) and phase differences for female

samples (Tukey; T = 3.83, P = 0.013). Consequently, samples of pre-breeding males and breeding females did also differ (Tukey; T = 3.53, P = 0.024).

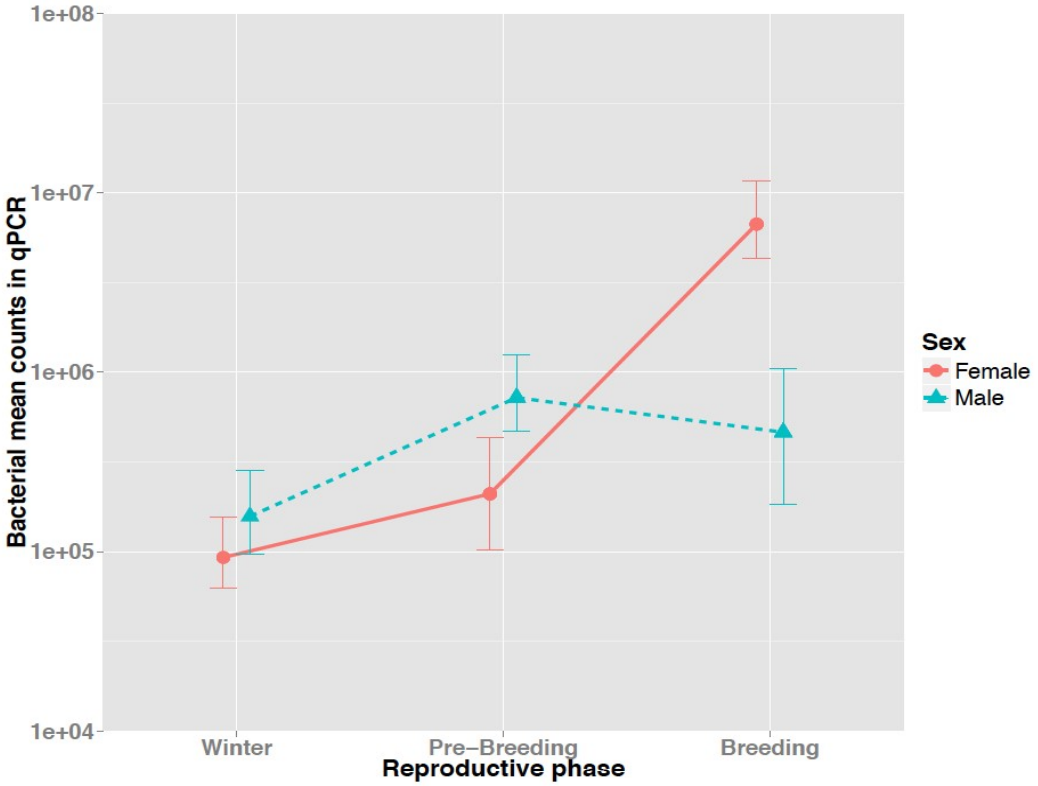


Figure 1. Interaction plot showing qPCR results for bacterial counts per microliter of uropygial secretion for females and males in different reproductive phases.

In addition, qPCR total bacteria counts did not significantly differ between samples from nesting females and chicks (Mann-Whitney U-test; U = 26.0, P = 0.052; breeding females samples: N = 5, mean (SD) = 6.70E+06 (5.61E+06); nestlings samples: N = 6, mean (SD) = 1.90E+06 (2.39E+06)). Equally, when comparing qPCR total bacteria counts of female secretions during the breeding season, no significant differences where found ((Mann-Whitney U-test; U = 13.0, P = 0.143; breeding females samples: N = 5, mean (SD) = 6.70E+06 (5.61E+06); non-paired females samples: N = 3, mean (SD) = 2.09E+06 (1.88E+06)).

White and dark secretions showed very different aspects when observed at fluorescence microscopy looking for bacteria (Fig. 2). While all smears of dark secretions from nesting individuals were full of bacteria stained with both Hoescht and universal probe (an example in Fig. 2a-c), bacteria were only rarely detected with FISH in winter secretions from either females (one out of 13 individuals) or males (none of 10 individuals) (Fig. 2d-i). Secretions of breeding females and nestlings did not differ in the amount of bacteria per microscope field stained with Hoescht (one-way ANOVA, $F_{1, 38} = 1.301$, $P = 0.261$; females: N = 20, mean (SD) = 17418.8 (8270.4); nestlings: N = 20, mean (SD) = 21051.8 (11600.3)). In both cases the amount of those bacteria showing also RNA staining with the universal probe was less than 100 % (one-way ANOVA, $F_{1, 38} = 3.602$; $P = 0.808$; females: N = 20, mean (SD) = 84.1 % (33.9); nestlings: N = 20, mean (SD) = 61.1 % (42.3)).

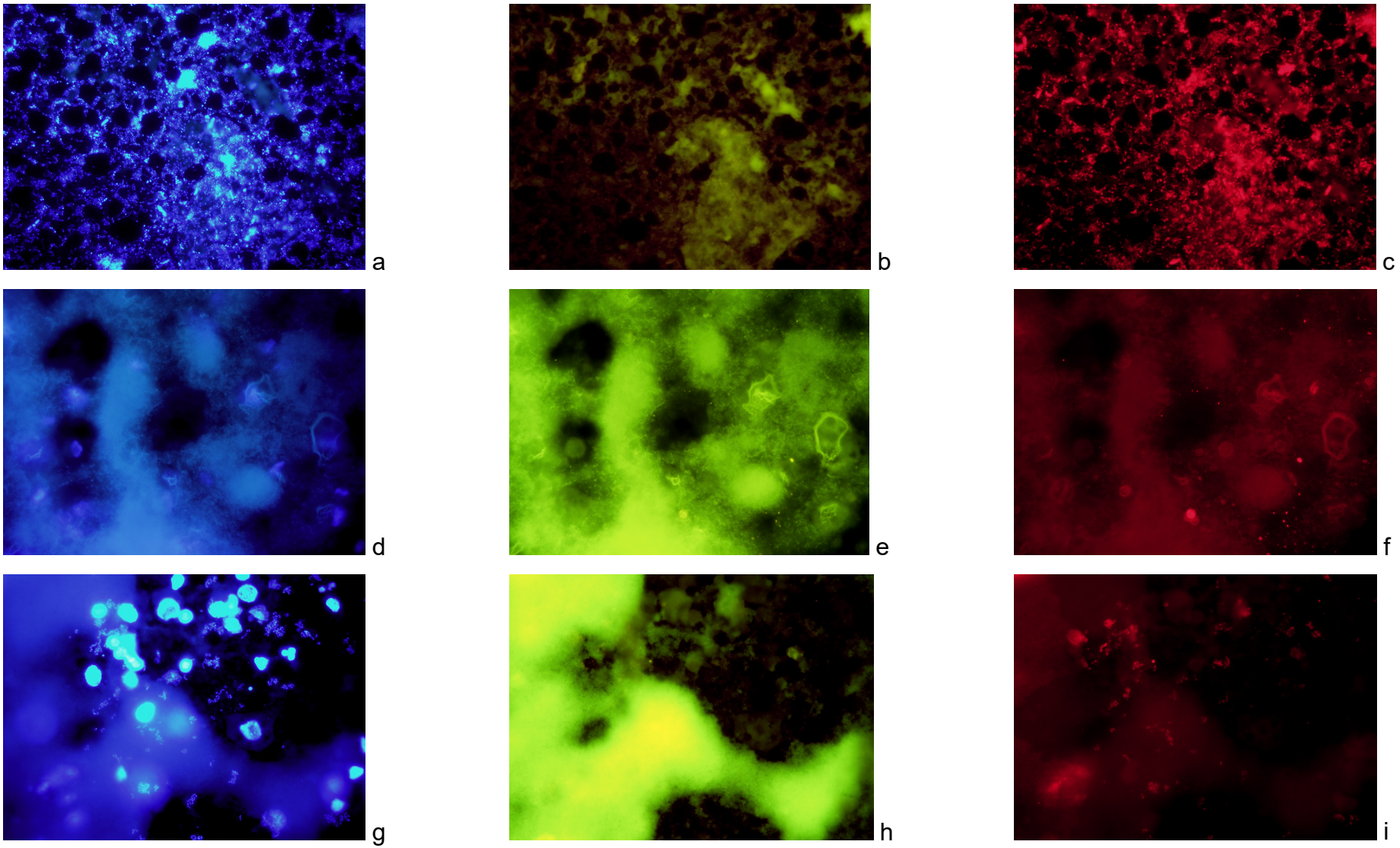


Figure 2. Fluorescence microphotographs of the dark uropygial secretion of a hoopoe breeding female (a-c), and two white secretions of females in winter (d-f and g-i) hybridized with the universal probe Eub338 marked with Cy3 to stain bacterial RNA, and with Hoescht to stain DNA. The three images in each line are of the same microscope field, but taken with the DAPI (blue), FICT (green-yellow) and TRITC (red) filters. In the first and third samples, bacteria are stained in blue (a and g) and red (c and i). The images taken with the green FICT filter are included to show that a fine grain texture usually found in white secretions (example in d-f) are not stained nucleic acids since show the same aspect in the three filters..

9. Bacterial diversity in uropygial secretions during the breeding season

The richness estimated by means of the chao1 index indicated that the bacterial communities, on average, included more than 30 OTUs in all kinds of samples (breeding females: N = 40, 35.3 ± 6.3; nestlings: N = 19, 33.0 ± 4.8; breeding males: N = 3, 36.2 ± 5.8; non-paired females: N = 2, 40.0 ± 2.8). Additionally, the PD whole tree index was calculated to quantify the phylogenetic diversity for each group (breeding females: N = 40, 2.07 ± 0.33; nestlings: N = 19, 2.05 ± 0.32; breeding males: N = 3, 2.32 ± 0.22; non-paired females: N = 2, 2.53 ± 0.32).

Wild samples showed slightly higher diversity values for the PD whole tree index than captive samples for both breeding females and nestlings (see Fig. 3). However, significant differences between samples coming from wild and captive populations were not detected either for breeding females (Mann-Whitney U-test; U = 115, P = 0.08; wild samples: N = 27, mean (SD) = 2.14 (0.24); captive samples: N = 13, mean (SD) = 1.92 (0.44)) nor for nestlings (Mann-Whitney U-test; U = 62, P = 0.15; wild samples: N = 11, mean (SD) = 2.15 (0.24), captive samples: N = 8, mean (SD) = 1.90 (0.36)). Additionally, no significant differences were found when comparing phylogenetic diversity between breeding females and nestlings (Mann-Whitney U-test; U = 397, P = 0.79; female samples: N = 40, mean (SD) = 2.02 (0.33); nestling samples: N = 19, mean (SD) = 2.05 (0.36)).

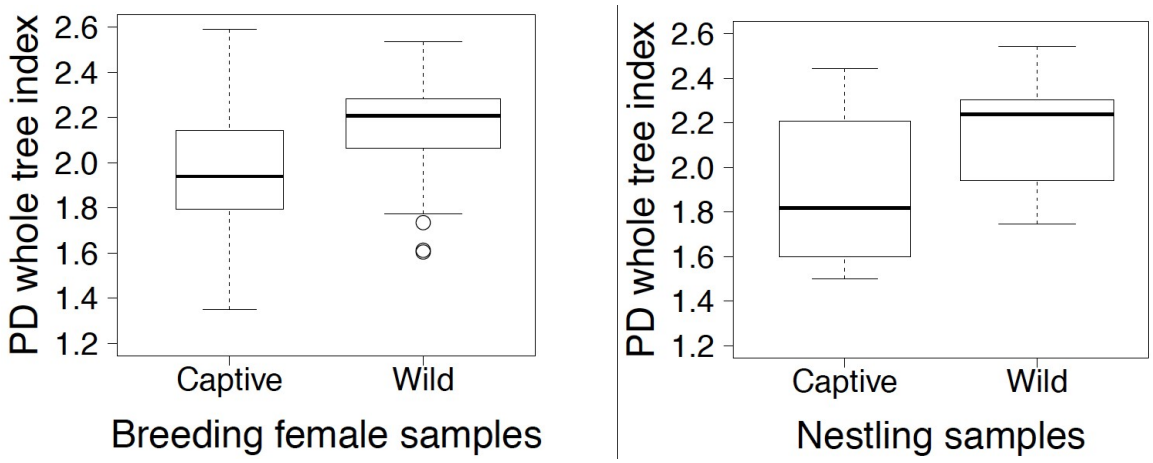


Figure 3. Box plots showing phylogenetic diversity represented by the PD whole tree index for wild and captive breeding females (A), and wild and captive nestlings (B).

10. Hoopoe uropygial microbiome

Firmicutes constituted over 90 % of the sequences amplified from our samples. *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* were also found in uropygial secretions but in lower proportions (see Table 1). In general, the bacterial communities from all individuals were similar, with 11 out of 28 genera present in 50 % of the samples.

Table 1. Mean composition at class level (in proportion of sequences) of hoopoe uropygial microbiome for breeding females, nestlings, males and non-paired females. Mean for total sampled hoopoe population is given at phylum level.

phylum; class	breeding females	nestlings	males	non-paired females	total
Actinobacteria; Actinobacteria	0.001	0.002	0.004	0.016	0.002
Bacteroidetes; Bacteroidia	0.053	0.032	0.049	0.002	0.045
Firmicutes; Bacilli	0.004	0.071	0.001	0.063	**0.925**
Firmicutes; Clostridia	**0.920**	**0.859**	**0.921**	**0.841**	
Proteobacteria; Alphaproteobacteria	0.004	0.001	0.000	0.073	0.028
Proteobacteria; Betaproteobacteria	0.000	0.000	0.002	0.000	
Proteobacteria; Epsilonproteobacteria	0.017	0.009	0.014	0.003	
Proteobacteria; Gammaproteobacteria	0.001	0.027	0.009	0.001	

The core microbiome (considered as taxa at genus level found in more than 75 % of the samples within each group) included 10 genera out of 27 (four present in all samples) for breeding females and 8 genera out of 28 (five present in all samples) for nestlings. Seven of the genera were common in both breeding female and nestling core microbiomes. For males and non-paired females, the core microbiome was 11 genera out of 23 and 19 genera out of 20 respectively. It is worthy to note that six genera (*Clostridium*, *Peptoniphilus*, *Coprococcus*, *Ruminococcus*, and another two unidentified genera of clostridia) were common in the core microbiomes of all groups of samples. Furthermore, there were two genera that were found only in dark secretions (*Vagococcus* and *Parascardovia*), and another one found only in nestlings (*Pseudomonas*), although with low prevalence (26 %) and very low abundances (< 1 %). On the contrary, no genus was found only in male and non-paired female secretions. For a summary of the number of OTUs included within each genus, see Table 2.

Table 2. Number of OTUs (Operational Taxonomic Units) included within the different genera found in this work.

Genus	OTUs	Genus	OTUs
Parascardovia	2	*Anaerotruncus*	1
Intrasporangiaceae unknown genus	1	*Ruminococcus*	7
Propionibacteriaceae unknown genus	1	*Veillonellaceae* unknown genus	3
Porphyromonas	5	*Veillonella*	1
Vagococcus	1	*Clostridiales* unknown genus	7
Bacilli unknown genus	3	*Coriobacteriaceae* unknown genus	2
Clostridiaceae unknown genus	33	*Clostridia* unknown genus	2
Anaerococcus	7	*Sphingomonadales* unknown genus	1
Clostridium	7	*Acidovorax*	1
Helcococcus	3	*Campylobacter*	5
Peptoniphilus	12	*Enterobacteriaceae* unknown genus	1
Lachnospiraceae unknown genus	2	*Proteus*	1
Coprococcus	8	*Pseudomonas*	1
Ruminococcaceae unknown genus	6	*Vibrionaceae* unknown genus	1

Pyrosequencing methods failed to detect the enterococci that otherwise were apparent when applying FISH to smears of secretions (Fig. 4). They were detected in 55 % of samples (N = 20) of breeding females, and in 35 % in the case of nestlings (N = 20). In any case, enterococci constituted a very low proportion of the bacterial cells stained with the universal probe (females: mean (SD) = 0.0012 % (0.0025); N = 20; nestlings: 0.0011 % (0.0032); N = 20), even when considering samples with detected enterococci (females: mean (SD) = 0.0021 % (0.0031); N = 11; nestlings: 0.0031 % (0.00350); N = 7).

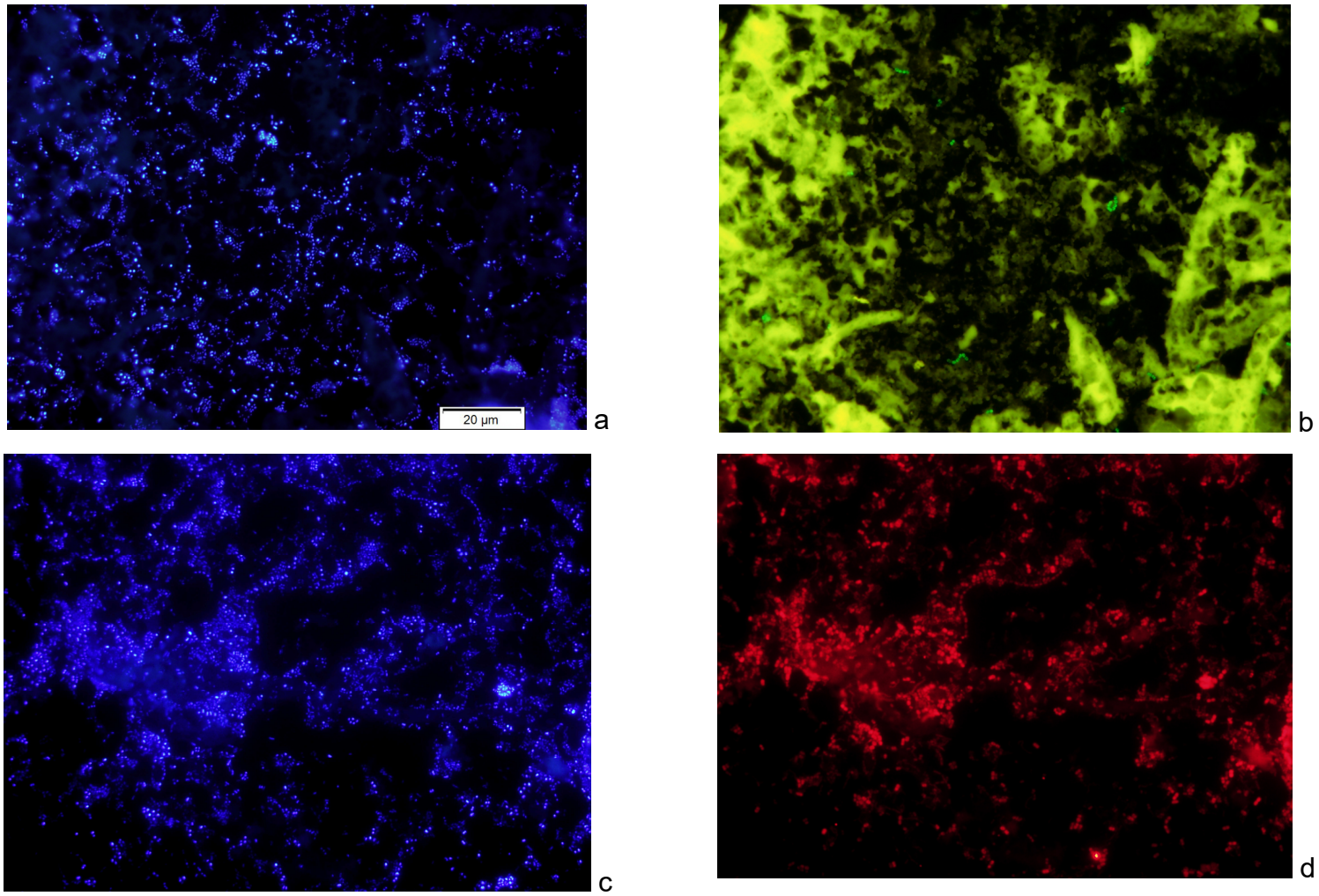
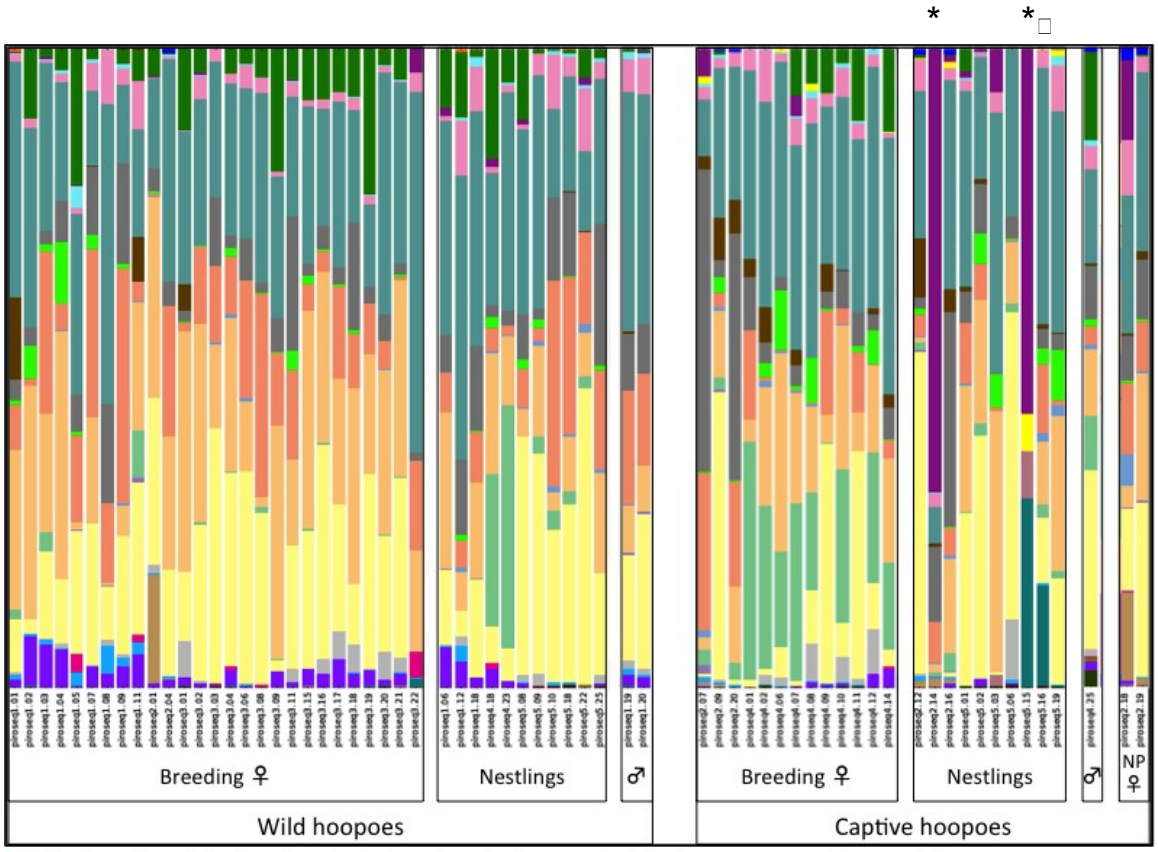


Figure 4. Fluorescence microphotographs of the uropygial secretion of a female hoopoe hybridized with the probe specific for RNA of the genus *Enterococcus* Enc-221 labeled with FITC (b) and the universal probe Eub-338 labeled with Cy3 (d). Images a and c are from the same microscopic fields than b and d respectively, taken through the DAPI filter to observe the staining of bacterial DNA with Hoescht.

Wild and captive hoopoe uropygial secretions slightly differ in their bacterial community composition (Fig. 5). For females, an unidentified genus of *Ruminococcaceae* and the genera *Anaerococcus* and *Vagococcus* were significantly more frequent in captive than in wild populations (Kruskal-Wallis test with Bonferroni correction for multiple comparisons; *Ruminococcaceae* genus, χ^2 = 15.98, P < 0.01; Anaerococcus, χ^2 = 12.68, P = 0.01; *Vagococcus*, χ^2 = 11.51, P = 0.01), while the genus *Campylobacter* was significantly more frequent in wild than in captive populations (Kruskal-Wallis test with Bonferroni correction for multiple comparisons; *Campylobacter*, χ^2 = 10.33, P = 0.04). samples from wild and captive nestlings only differ at the order level, whith *Bacteroidales* being more frequent in wild than in captive nestlings (Kruskal-Wallis test with Bonferroni correction for multiple comparisons; Bacteroidales, χ^2 = 8.99, P = 0.04).



k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae;g__Parascardovia
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Intrasporangiaceae;g__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Porphyromonas
k__Bacteria;p__Firmicutes;c__Bacilli;o__;f__;g__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Vagococcus
k__Bacteria;p__Firmicutes;c__Clostridia;o__;f__;g__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Anaerococcus
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Helcococcus
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Peptoniphilus
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Anaerotruncus
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella
k__Bacteria;p__Firmicutes;c__Clostridia;o__Coriobacteriales;f__Coriobacteriaceae;g__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__;g__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Acidovorax
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae;g__Campylobacter
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Proteus
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__

NP=non-paired

*=nestlings showing signs of infection

Figure 5. Relative abundance of genera in the hoopoe uropygial microbiome during the breeding season. Samples are organized by groups according to living conditions, and sex and breeding phase. Each group of samples shown was rarefied at 300 (males) or 1000 (females and nestlings) sequences per sample.

DISCUSSION

The present work describes for the first time the taxonomic composition of the whole microbiome of the special hoopoe uropygial gland secretion (125 OTUs distributed within 28 genera). This may be a conservative description given the sequencing depth of the 454 platform and the filtering parameters used, but still provides a good approach to the main taxa richness found in uropygial secretions (Pedrós-Alió, 2012). More importantly, we have described sexual differences and the dynamic variation of the microbial communities in terms of abundances and diversity of microorganisms. Independently of the method used, white secretions of males of all reproductive phases and winter and non-breeding females host bacteria at lower densities than those of breeding females (and non-paired females along the breeding season) and nestlings. However, the composition of the microbiome is quite invariable along the breeding season independently of the sex and age of the individual sampled.

The uropygial microbiome of hoopoes is dominated by clostridia, a group that is abundant in the gut microbiome (Keenan *et al.*, 2013; Roggenbuck *et al.*, 2014; Sankar *et al.*, 2015; Wienemann *et al.*, 2011). Clostridia are known to be essential maintaining the stability of gut microbiota (Lopetuso *et al.*, 2013) and may have the same role in the uropygial gland of hoopoes. Clostridia produce butanoic acid that is one of the more abundant volatiles of dark secretion of hoopoes (Martín-Vivaldi *et al.*, 2010). This compound is able to inhibit cell growth, but can also work as a trophic factor (at least for human cells) depending on its concentration (Lopetuso *et al.*, 2013). Butanoic acid may be thus related to the exaggerate size of glands in breeding females and nestlings (Martín-Vivaldi *et al.*, 2009). This compound was

not detected in white secretions suggesting either that clostridium are at a lower density in these secretion or that clostridia do not produce butanoic acid there.

The importance of rare taxa in microbiome functioning for hosts has been previously described (Dohrmann *et al.*, 2013; Pedrós-Alió, 2012). However, scarce bacteria are difficult to detect by pyrosequencing and other more traditional methodologies may be more indicated to evaluate their function. This is likely the case of enterococci within hoopoe uropygial secretions. Enterococci was the most abundant bacteria in traditional culture media, but were only found at low density in less than 50% of samples using an *Enterococcus* specific probe with the FISH approach. However, the contribution of enterococci to the antimicrobial properties of the secretion has been previously reported (Martín-Platero *et al.*, 2006; Ruiz-Rodríguez *et al.*, 2012), and their abundance within the secretion of females and on eggshells impregnated with female secretion positively affect hatching success (Martín-Vivaldi *et al.*, 2014). Some other candidates as key bacterial components of the secretion due to their theoretical antimicrobial activity are *Vagococcus* and *Parascardovia*. Bacteria in the genus *Parascardovia* (related to the genus *Bifidobacterium*) are acetic acid and lactic acid producers (Jian and Dong, 2002). The genus *Vagococcus* is phylogenetically related to the genera *Enterococcus* and *Carnobacterium*, and it is widely distributed in nature. Some strains of *Vagococcus* have been found to produce bacteriocins active against *Listeria*, *Staphylococcus* and *Hafnia* (Batt, 1999). All these bacteria are more abundant and prevalent in secretions collected during the breeding season, particularly in females and nestlings, which suggest that hosts enhance specific bacterial growth during the phase of higher risk of pathogenic infections. Similarly to what has been recently found to influence the microbiome in women and men (Dominianni *et al.*, 2015), we speculate with the possibility that sex hormones were the physiological causes

driving detected variations in hoopoes microbiome. Our finding of non-paired female secretions being more similar in bacterial counts to those of breeding females than to those of females sampled out of the breeding season may be interpreted in accordance with this possibility.

Previous work with traditional growth methodologies failed to detect bacteria in secretions of males and of non-breeding females, which suggested that symbiotic bacteria should colonize the uropygial gland of females every year. Our results here demonstrate that most bacterial taxa were found in white secretions at much lower density. Thus, a baseline reservoir of bacteria is maintained within the gland out of the breeding season. We however cannot reject the possibility that hoopoes collected bacteria from other environments to colonize their gland. It is well known that the gastrointestinal tract of animals is inhabited by complex communities in which *Firmicutes* (along with *Bacteroidetes*) are abundant (Young and Schmidt, 2008). *Firmicutes* colonization of the gut in birds has been described for several species, although it never seems to be over 75 % of total diversity (reviewed in Kohl, 2012). However, the gut microbiome is exposed to conditions directly related with food intake that may not affect the uropygial gland (David *et al.*, 2014). The differences in relative abundances with gut microbiome that would produce a predominant population of *Firmicutes* (mostly clostridia) against *Bacteroidetes* in the gland could be related to a more restrictive nutrient availability.

Interestingly, and similarly to previous work (Martínez-García *et al.*, 2015; Rodríguez-Ruano *et al.*, 2015), we have also detected differences in microbiome of the uropygial gland of captive and wild hoopoes. These differences posit the importance of environmental conditions determining microbial communities that may be related to diet. Diet is known to affect gut microbiome of animals (David *et al.*, 2014) including birds (Wienemann *et al.*, 2011). Given that many of the taxa

detected in the hoopoe secretion are also important in gut microbiomes (i.e. *Bacteroidetes*, *Clostridia*, etc.), it is possible that the growth of some bacteria collected from the gut within the uropygial secretion was the cause of the detected differences between captive and wild hoopoes. Another non-exclusive possibility is that nest materials from previous years functioned as reservoirs of symbiotic bacteria. Further research in this sense is needed to clarify the origin of the uropygial symbiotic bacteria in hoopoes as well as the factors driving the changes observed in the secretions of nesting individuals.

Summarizing, the low variability detected among the uropygial microbiome of individual hoopoes suggest a characteristic but dynamic bacterial assemblage. Most variations were due to bacterial abundance and appeared in association with special secretion characteristics (i.e. color) related with the breeding phase, which opens the opportunity of host-regulating proliferation of symbiotic bacteria in phases of high risk of pathogenic infections (i.e. reuse of hole-nests for breeding). We also highlight the importance of possible key bacterial genus due to their known antibacterial properties. This would include enterococci, a group of symbiotic bacteria for which beneficial effects in hoopoes had been previously detected although, due to their low density, are difficult to detect by pyrosequencing. Finally, we find evidence supporting environmental influences on the microbiome of the uropygial secretion that may be essential for understanding the variation in functionality for hosts. The detected dynamic variation may suggest mechanisms for hosts to control symbiotic microbiomes that should be further explored.

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APPENDIX 1

Method for Hybridization

A small amount of the uropygial secretion extracted from the glands of some individuals (20 breeding females: 16 captive and 4 wild; 20 nestlings: 12 from 5 captivity nests and 8 from 6 wild nests; and 23 captive individuals in winter: 10 males and 13 females) was used to study abundance of metabolic active bacteria by means of Fluorescence *In Situ* Hybridization (FISH). In the case of dark secretions (breeding females and nestlings) two smears of 5 µL for each individual were prepared in two different Polysine glass slides (Thermo Scientific) by extending the drop to form a layer as thin as possible with the help of a second clean slide rubbed over the secretion. One of the slides was used to estimate the total amount of bacteria and the other to estimate the amount of enterococci. In the case of white secretions (winter individuals), all the available volume of secretion for each individual was used to prepare an only smear to study total amount of bacteria in the same way than with dark secretions. Smears were immediately fixed in 4 % paraformaldehyde, washed in sterile distilled water, dehydrated in successive 50º, 80º y 96º ethanol baths, air-dried and stored until hybridization.

The fixed smears stored were hybridized several months after collection. In the moment of hybridization, to permeate cell walls,10 µL of lisozime solution (100 mM TRIS-HCl; 50 mM EDTA; pH 8; 2 mg/mL lisozime, USB Corporation) were added to each smear and incubated at 37 ºC during 60 minutes in a wet atmosphere. Then, slides were washed with filtered distillated water, air-dried and dehydrated in successive 50º, 80º y 96º ethanol baths.

Pre-hybridization was performed by dropping 5 µL of Dig Easy Hib detergent (Roche) at 45 ºC during 2 hours in a wet atmosphere. Then, immersions in pre-

hybridization buffer (20 mM TRIS-HCl; 0.9 M NaCl; SDS 0.01%; pH 7.2-7.4) and in filtered distilled water were used to wash the detergent, and slides were dried at 28 ºC.

The Eub338 (5'-GCTGCCTCCCGTAGGAGT-3', Amann *et al.*, 1990) labeled with Cy3 (Biomers.net) as universal probe for Eubacteria and Enc 221 (5'-CACCGCGGGTCCATCCATCA-3', Wellinghausen *et al.*, 2007) labeled with FITC for all enterococci (Biomers.net) were used. Hybridization was performed by adding 30 µL of the probe - hybridation buffer (1 µL probe, 20 mM TRIS-HCl; 0.9 M NaCl; SDS 0.1%; formamide 25%, Sigma-Aldricht; pH 7.2-7.4) to smears and incubating at 45 ºC overnight in a wet atmosphere. Then, temperature was increased to 48 ºC during 20 minutes in a wet atmosphere. A 15 minutes wash was performed in hybridization buffer followed by a second wash in filtered distilled water during 2 minutes. Slides were dried at room temperature. Hybridization buffers were pre-heated to ensure that SDS was dissolved in such high NaCl concentrations. Afterwards, buffers were kept at room temperature.

After hybridization, 10 µL Hoescht (10 µg/mL, Sigma-Aldricht) were added to smears and the mixture was maintained for 30 minutes in a wet atmosphere to stain DNA as positive control for the presence of bacteria. Then, slides were washed with PBS for 2 minutes and dried at room temperature.

Slides were mounted applying a drop of antifading solution (Vectashield Mounting Medium H-1000) and a coverslip, and then sealed with varnish.

The whole process was performed maintaining slides in the dark to avoid fading of the fluorescence probes and pigments, and they were stored in dark boxes at 4 ºC until observation at fluorescence microscope.

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APPENDIX 2

Slides were observed through an Olympus BX51 fluorescence microscope with DAPI, FITC and TRITC filters. Three different fields per slide with a homogeneous distribution of secretion were photographed. For each of these fields, two pictures were performed: one with the DAPI filter (for Hoescht stain) and another one with the adequate filter for each probe (Enc221 was detected with FITC filter and Eub338 with TRITC filter). Pictures were captured using the 100x objective and an Olympus DP72 camera, and processed by the cellSens 1.4.1 software (Olympus). The amount of bacteria (see quantification method below) detected with Hoescht was repeatable within individuals ($F_{1,78}$ = 7.66, P << 0.001, R = 0.69 for the 39 individuals with three available microscopic fields of the same sample). Therefore we used the mean of the number of bacteria obtained for the three pictures with each filter as the count value of each individual for that filter.

The amount of bacteria stained with Hoescht and Eub338 was estimated with the TMARKER v1.20163 software (Schüffler *et al.*, 2013). For this we trained the program to recognize positive and negative superpixels with the semiautomatic labeling application using at least 150 positives and 150 negatives of variable intensity manually selected on a typical picture of a sample. Superpixels were created by the program based on the color histogram of images, with our specification of the number of superpixels, and with the maximum smoothness value. The number of superpixels to create was decided by examining which of several values best resulted in a grid with the size of the stained cells. This was 70000 for the blue stained DNA of cells in Hoescht pictures, and 45000 for the red stained RNA of cells in Eub338 pictures. The classifier trained for each probe was subsequently applied to the remaining images to calculate the number of positive superpixels in each picture, and that number was adopted as an estimation of the number of stained cells.

In the case of enterococci, they were very scarce (Fig. 4b) so the number of green cells visible in the images was counted manually on a computer screen. To calculate the proportion of all active cells that were enterococci in each sample, we referred the number of stained enterococci in the Enc221 slide to the amount of Hoescht stained cells in that slide that were expected to be marked with the universal probe. This was given by the percentage of Hoescht stained bacteria in the Eub338 slide that were also detected by the universal probe.

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CAPÍTULO III:

Spatiotemporal variation in the hoopoe uropygial microbiome: A role for seasonal and geographical factors?

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ABSTRACT

Microbial communities in nature are exposed to their environment and may be affected by different physico-chemical factors, either unique, cyclic or in gradients. This environmental influence may cause shifts in the bacterial communities that are easily detectable due to the high growth rates of microorganisms. The dynamics of free-living communities have been studied for different systems, but symbiotic communities may show environmental influenced patterns as well, either in a temporal or a spatial manner. For symbiotic communities, the mode of acquisition may affect the way that the environment can affect their structure. Usually, vertically transmitted communities are less diverse than those with at least some environmental influence, as the external conditions may determine the availability of certain bacteria or the host predisposition to maintain certain groups over others. In the uropygial microbiome of hoopoes, it has been shown that the acquisition of symbionts during its development can have an environmental component subject to horizontal transmission. Here we test whether the meteorological or other environmental factors varying with geographic location, type of habitat or the advance of the breeding season affect the composition of uropygial communities. A largely invariable composition of the uropygial microbiome was found across the different spatiotemporal factors studied here. Only the year showed a significant effect on the natural uropygial microbiome variation, with a particular group of OTUs being more frequent in 2012 in comparison with 2010 and 2011, which were not different between them. Almost half of the sequences present in all the samples correspond to a few OTUs, including six members of class *Clostridia*, two members of class *Actinobacteria* and one member of class *Bacteroidia*. This shows that

the community inhabiting the uropygial glands of female hoopoes is rather stable across the environmental range covered by the study.

INTRODUCTION

Microbial communities in nature are exposed to their environment and can be affected by physicochemical factors such as pH, salinity, light, temperature, humidity, and nutrient concentration (Bachar *et al.*, 2010; Su *et al.*, 2014). Those factors may vary along gradients in depth (i.e. aquatic systems) or altitude (i.e. mountain systems), or depend on geographical location. Sometimes they show a seasonal pattern that may cause cyclic changes in bacterial communities along time (Andersson *et al.*, 2009; Kim *et al.*, 2014). Given the high growth rates of microorganisms their changes can be easily detectable in comparison to other ecological measurements (Prosser *et al.*, 2007). Several studies have focused on those dynamics, and the results show that the environment may play a key role not only in determining free-living communities, but also in shaping symbiotic assemblages of organisms (Bisson *et al.*, 2009; Filteau *et al.*, 2010). The biogeographic patterns of free microbial communities have also been studied (Fierer and Jackson, 2006). In this case symbiotic communities show different spatial scales in which variations can be detected, including different habitats (Pantos *et al.*, 2015) but also locations within the same host individual (The Human Microbiome Project Consortium, 2012).

Mutualism occurs when two or more species interact in nature and the result is an increase in the actual or potential genetic fitness of each participant (Janzen, 1985). Many of these mutualistic interactions involve bacteria that may constitute whole complex symbiotic communities inside the host (Hussa and Goodrich-Blair, 2013). Those symbiotic communities are maintained due to their multiple benefits to the host regarding metabolism, immunity or defense (reviewed in Flórez *et al.*,

2015; Leser and Mølbak, 2009). Their acquisition also has different possible mechanisms. Symbiotic bacteria may be acquired vertically (from mothers to offspring) or horizontally (between individuals or directly from the environment) (Moran, 2006). Usually, vertically transmitted communities are less diverse than those with at least some environmental influence (Zeng *et al.*, 2015). If symbionts are acquired from the environment, their availability may be dependent on environmental conditions and thus the final community assemblage inside the host may be shaped by external factors (Fierer and Jackson, 2006). Additionally, changes in environmental conditions may affect the host metabolism and physiology, by means of food availability or pathogenic risk for example (Davenport *et al.*, 2014; Jacob *et al.*, 2014). This way, environmental factors could also determine different host disposition to maintain certain kinds of bacteria over others, indirectly influencing the final symbiotic community structure (Costello *et al.*, 2012). On the other hand, hosts may also develop strategies that can counteract the effect of the environment when a regulation of bacterial populations (either symbiotic or pathogenic) is important for fitness (Ruiz-De-Castañeda *et al.*, 2012). Some microclimatic aspects related with the stay within the nest during incubation are known to affect the eggshell bacterial loads (Ruiz-De-Castañeda *et al.*, 2011a), and it has been demonstrated that incubation behavior can modify some of the factors affecting eggshell bacterial loads (i.e. humidity) (Ruiz-De-Castañeda *et al.*, 2011b).

The hoopoe (*Upupa epops*) hosts bacterial symbionts in its uropygial gland. The uropigial secretion carries bacteria (and their products) out of the gland and thus mediate the bacterial positive effects on the host by means of preening (Martínez-García *et al.*, 2015). The uropigial microbiome of the uropigial secretion of hoopoes shows a quite complex bacterial community dominated by clostridia (Rodríguez-Ruano *et al.*, unpublished data). Some approaches to bacterial origin

and acquisition in the uropigial gland of this species have shown both environmental and genetic influences (Ruiz-Rodríguez *et al.* 2014; Martínez-García 2015). However, the role of the environment in shaping the uropigial microbiome is still not well understood.

The hoopoe is a hole-nesting bird in which only nesting individuals present a characteristic dark uropygial secretion with antimicrobial properties apparently mediated by bacteria (Martín-Vivaldi *et al.*, 2009). It is known that the hoopoe breeding season occurs from mid February to the end of July in southern Spain (Martín-Vivaldi *et al.*, 2009). Each female starts the laying at different dates along the breeding season, so the stay inside the hole-nest may occur either in late winter, spring or summer for each female. The hypothesis proposed here is that, if the composition of the microbiome of the gland and its secretion in females is mediated at least partially by environmental factors (i.e. because some bacteria are acquired from the surroundings of the hole-nest), there should be variations in the acquired populations of bacteria appearing in the dark secretions of breeding females in relation to those factors. Those changes could be motivated either by the general weather conditions at the time when each female starts the breeding attempt, or even by the specific environment determined by the location of the hole-nest. In the first case, a seasonal shift in the uropigial microbiome of hoopoe females should appear when studying females breeding at different dates during the breeding season, along different years. In the second case, geographical differences would be detected when analyzing females nesting in variable landscapes and from different study populations. The direct influence of the bacterial composition of the hole-nest material in the uropigial microbiome has been previously discarded (Martínez-García, 2015). However, the seasonal variations of uropygial microbiome due to weather conditions such as temperature or humidity

remain unexplored, and so remains the influence of location of the hole-nest in bacterial acquisition.

Here we intend to explore the possible effect of environmental (i.e. temperature and humidity) and biogeographic (i.e. hole-nest location) factors in shaping the uropigial microbiome of hoopoe females. To do that, we survey the seasonal and spatial changes that bacterial communities in the uropigial secretion of breeding females experience along the breeding season across different years and between different locations, as good as the particular environmental variables that could affect such changes.

MATERIALS AND METHODS

1. Study area and sampling procedures

The study was performed during the breeding seasons of 2010, 2011 and 2012 with a wild population of hoopoes located at the Hoya de Guadix (Granada, Spain). Next boxes and cages were visited twice per week from mid February to the end of July to detect the laying date of clutches. Samples of uropygial secretions of the breeding hoopoe females were collected at day 15 of incubation. That day, they were also ringed with numbered (Spanish Ministry of Environment) and colored rings for individual recognition. The uropygial gland secretion was extracted directly from the gland papilla using latex gloves cleaned with 96 % ethanol and a micropipette with a sterile tip, after lightly washing the surface of the gland with 96

% ethanol. Samples were stored in sterile 1.5 ml microfuge tubes at 4 ºC and then frozen at -20 ºC within 24 hours until processed. A total of 51 first clutches of wild females sampled along the three breeding seasons (2010, 2011 and 2012) were used for the analyses after discarding clutches of repeated individuals to avoid pseudoreplication.

2. DNA extraction and purification

Extraction of total DNA from uropygial secretion samples was performed using the FavorPrep Genomic DNA extraction kit (Favorgen Biotech) according to manufacturer's instructions, adding a lysozyme pretreatment (10 mg/ml, at 37 °C for 30 min). For PCR product purification a Solid Phase Reversible Immobilization (SPRI) using AMPure XP magnetic beads (Agencourt) was performed. Final elutions were made in ultrapure sterile water for total DNA and in EB buffer for PCR products.

3. Amplicon libraries, sequencing and sequences processing

DNA amplification was normalized using qPCR and diluting the samples to ensure they achieved the exponential phase at the same number of cycles. Then a first PCR step to amplify the V4 region of the 16S rDNA was performed. The universal primers 515F and 806R (Nikkari *et al.*, 2002; Wang and Qian, 2009) were modified by Polz *et al.* to include part of the Illumina adapters (and a 5 bp barcode in the forward primer for multiplexing) as follows: U515F (5'-ACACGACGCTCTTCCGATCT-NNNNN-GTGCCAGCMGCCGCGGTAA-3') and

E806R (5'-CGGCATTCCTGCTGAACCGCTCTTCCGATCT-GGACTACHVGGGTWTCTAAT-3'). PCR was carried out using a Q5 High-fidelity DNA polymerase (New England BioLabs) at an annealing temperature of 52 ºC and terminated at exponential phase as determined by the initial qPCR. Then a second PCR step of 9 cycles was performed to generate the final construction adding the complete Illumina adapters (including a 9 bp barcode in the reverse primer for multiplexing). The purified PCR products were normalized by qPCR and mixed accordingly. The library pool was sequenced in an Illumina MiSeq sequencer at the MIT BioMicro Center, Cambridge, Massachusetts, USA.

The preprocessing was carried out using scripts from USEARCH 8.0.1623 (Edgar, 2010) for paired-end sequences concatenation and from QIIME 1.8 (Caporaso *et al.*, 2010) for quality filtering and demultiplexing. Filtered sequences were aligned and trimmed before the Distribution Based Clustering Method (Preheim *et al.*, 2013) was used for OTU picking against the Greengenes_12_10 database (trimmed to the V4 region included in our dataset) (McDonald *et al.*, 2012). UCHIME (Edgar *et al.*, 2011) was used to remove chimeric sequences. The OTUs were taxonomically classified using BLAST 2.2.22 (Altschul *et al.*, 1990) against the Greengenes_12_10 database (McDonald *et al.*, 2012). Then, the OTUs identified as *Archaea* were discarded to keep only bacterial information. The OTU table was filtered to eliminate low abundance OTUs (with less than 0.0005 % of the reads) as recommended in (Bokulich *et al.*, 2013). Samples with less than 5000 sequences were discarded and then a multiple rarefaction at 75% of the minimum number of sequences per sample was performed to standardize the dataset using scripts from QIIME 1.8 (Caporaso *et al.*, 2010). The standard QIIME 1.8 pipeline was also used to calculate diversity indexes, and the core OTUs in the microbiome

(those present in 100 % of samples). Distance matrices for bacterial composition based on abundance of bacterial groups were obtained with UniFrac (Lozupone and Knight, 2005).

4. Environmental variables

Weather information for the Hoya de Guadix in the years 2010, 2011 and 2012 was obtained from the meteorological databases of the Consejería de Medio Ambiente of the Junta de Andalucía (http://www.juntadeandalucia.es/medioambiente/servtc5/sica/sima_av.jsp). Particularly, we used the available data for temperature, humidity and precipitation, calculating the mean for each female depending on its laying date (using values from one week before the beginning of laying to one week after laying, to include the period when the reproductive uropygial traits develop). The progress of the breeding season was measured considering day 1 as the first of April. Geographical distances among the sampled nest-boxes from the Hoya de Guadix were calculated using QGIS 2.8.3-Wien (Quantum GIS Development Team, Open Source Geospatial Foundation Project, 2015). Additionally, the habitat (holm oak meadows, dry plains and irrigated olive groves) and population (groups of nests placed in a continuum patch of a particular habitat separated several km from other groups of nests) were taken into account for the geographical approach.

5. Statistics

Statistical analyses were performed using the R statistical software (R Development Core Team, 2014), with the basic Stat and Graphics Packages. Multiple regression on distance matrices (generated for all the variables in the same software) was performed using the MRM function (an extension of the partial Mantel tests) of the ecodist Package (Goslee and Urban, 2007). We performed a stepwise backward approach with all the available variables for each model, progressively eliminating those without significant effects and the highest p-values until leaving only significant predictors. We tested the simultaneous influences of all types of variables considered (both involving geographical and seasonal effects) in a global model, as good as two separate models with each type of variables, in all cases including the year of sampling. Since both approaches produced the same results, we present only the two separate models. To identify the particular OTUs responsible of the grouping of samples we used heatmaps obtained using the heatplot function of the made4 Package developed for R (Culhane *et al.*, 2005).

RESULTS

1. Composition of the uropygial microbiome

Firmicutes were predominant in all the samples regardless the year and other variables considered here. A whole distribution of OTUs into different phyla, classes, orders and families can be consulted in Table 1. Among the 5403 OTUs

found, 17 were present in 100% of the samples (N = 50) and they accounted for between 21.5 % and 51.1 % of the postprocessing sequences per sample (mean relative abundance for the core OTUs (SD) = 45.8 % (9.8 %)). Moreover, 98.7 % of those sequences corresponded to only 9 of the core OTUs, which were identified as *Coprococcus*, *Peptoniphilus*, *Parabacteroides*, *Streptomyces,* and some unidentified genera of the families *Clostridiaceae*, *Coriobacteriaceae* and *Actinomycetaceae*. The remaining 8 core OTUs were identified as *Actinomyces*, *Streptomyces* and some genera of *Clostridiaceae.*

2. Seasonal variation

The model exploring seasonal and meteorological variables (year, date in the season, temperature, humidity and precipitation) did not include any significant effect except for year (MRM; global model with all variables: N = 50, R^2 = 0.033, F = 8.4, P = 0.247; final model with year: N = 50, R^2 = 0.020, F = 25.0, P = 0.001).

Table 1. Distribution of OTUs into different taxonomic levels from phylum to family. For brevity, some levels have been grouped as “Other” within the immediately higher level.

Phylum	Class	Order	Family	Number of OTUs
Acidobacteria	Unidentified			5
Actinobacteria	*Actinobacteria*	*Actinomycetales*		343
	Other			3
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	265
			Other	5
	Flavobacteriia	*Flavobacteriales*	*Flavobacteriaceae*	25
		Unidentified		1
	Sphingobacteriia	*Sphingobacteriales*		7
Chloroflexi	*Anaerolineae*	*DRC31*	Unidentified	1
Cyanobacteria	*Chloroplast*			3
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	3418
			Lachnospiraceae	496
			Veillonellaceae	163
			Other	19
		Coriobacteriales	*Coriobacteriaceae*	229
			Other	5
		Natranaerobiales	*ML1228J*	82
		Thermoanaerobacterales	*Caldicellulosiruptoraceae*	1
	Bacilli	*Lactobacillales*	*Streptococcaeae*	8
			Other	2
		Bacillales		3
		Gemellales	*Gemellaceae*	1
	Erysipelotrichi	*Erysipelotrichales*	*Erysipelotrichaceae*	2
Fusobateria	*Fusobacteria*	*Fusobacteriales*	*Fusobacteriaceae*	3
Lentisphaerae	*Lentisphaeria**	*Lentisphaerales*	*Lentisphaeraceae*	2
NPL-UPA2	Unidentified			1
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	194
			Helicobacteraceae	2
	Betaproteobacteria	*Burkholderiales*	*Comamonadaceae*	25
			Other	5
		Rhodocyclales	*Rhodocyclaceae*	7
		Unidentified		1
	Gammaproteobacteria			27
	Deltaproteobacteria	Unidentified		12
		Other		8
	Alphaproteobacteria			15
Verrucomicrobia	*Opitutae*			2
Other Bacteria	Unidentified			12

3. Geographical variation

A similar model was used to test geographical and habitat influences. In this case the variables habitat, population and distance between nests were used, although the year was also included to control its effects in the uropygial microbiome. Again, the model resulted in no significant effects of any of the geographical variables (MRM; global model with all variables: N = 50, R^2 = 0.022, F = 6.961, P = 0.193; the final model only included year as above).

4. Interannual variations in the uropygial microbiome

The effect of year on the composition of the uropygial microbiome of incubating hoopoe females was due to both 2010 and 2011 being significantly different to 2012 (MRM pairwise comparisons; 2010 and 2012: N = 31, R^2 = 0.119, F = 62.385, P = 0.001; 2011 and 2012: N = 36, R^2 = 0.014, F = 8.667, P = 0.002), but not between them (MRM; N = 33, R^2 = 0.001, F = 0.561, P = 0.435). These differences were evident in the grouping of most 2012 samples together in the same cluster in a heatmap reflecting the distribution of the 100 more abundant OTUs in our samples (Fig. 1). Some specific OTUs seem to be responsible for the detected clustering of the majority of the uropygial samples from 2012, mainly including representatives of the genera *Coprococcus*, *Parabacteroides*, *Campylobacter* and several unidentified *Clostridiaceae*. Most of the samples of 2010 and 2011 also clustered together, but showed less clear patterns of defining OTUs.

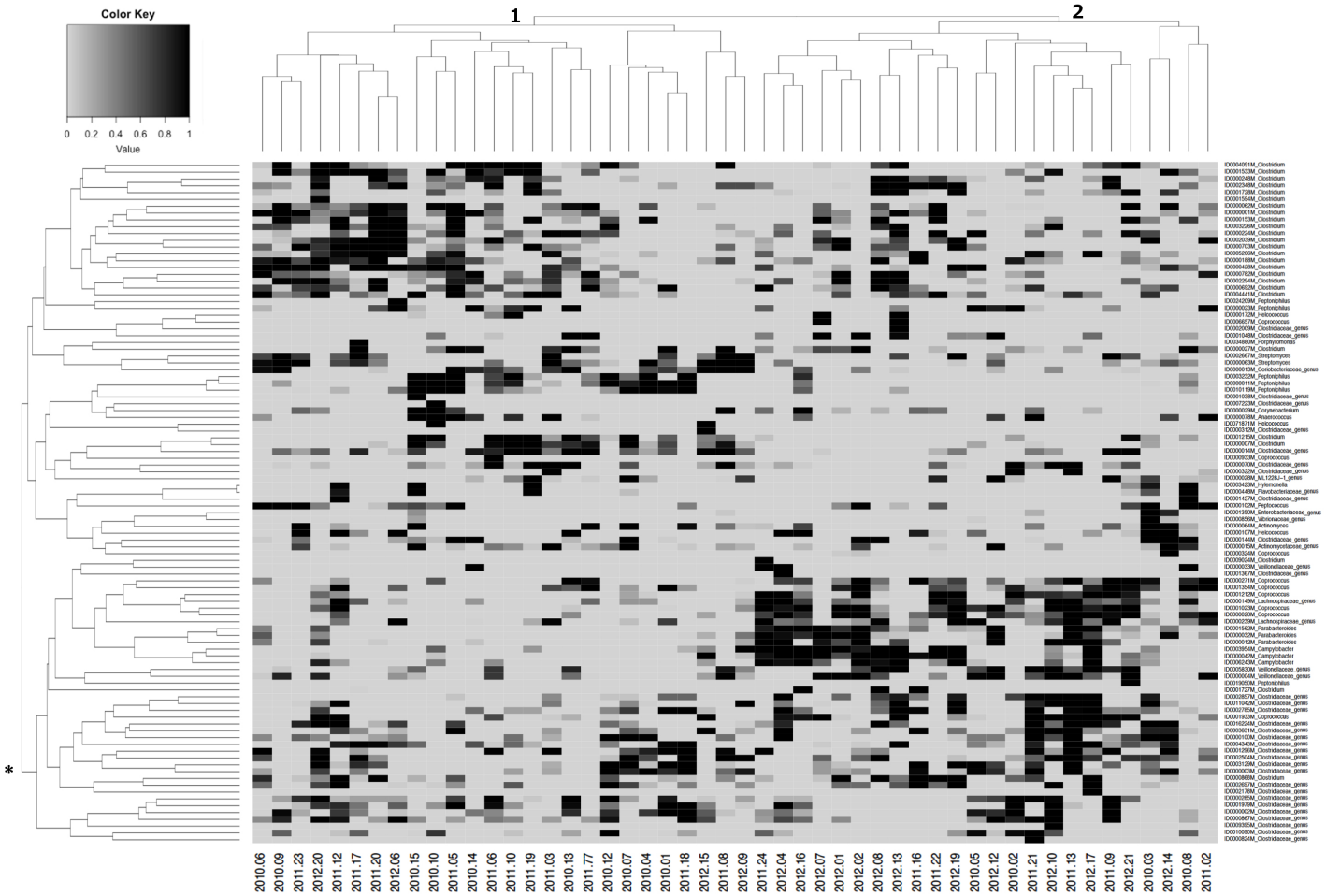

Figure 1. Heatmap representing the distribution of the 100 more abundant OTUs in our samples along the different years studied. Most samples from 2012 grouped together in cluster number 2, separated from most of the 2010 and 2011 samples (grouped together in cluster 1). An OTU cluster (marked with an asterisk) appears associated with the cluster 2 of samples.

DISCUSSION

We have found that, among the environmental factors analyzed, only the year of study affected the composition of the microbial community inhabiting the uropygial gland of female hoopoes. These results highlight the invariability of the uropygial microbiome of breeding females across different environmental factors (i.e.: season, weather conditions and location). Our current results also confirm the taxonomic composition of the uropygial microbiome previously obtained by 454 pyrosequencing (Rodríguez-Ruano *et al.*, 2015; this work: chapter II).

The composition of the uropygial microbiome of female hoopoes is clearly dominated by *Firmicutes* (mainly *Clostridia*) as previously described with pyrosequencing. However, the deeper sequencing obtained from the MiSeq Illumina technology allowed us to find much more OTUs compared to the 454 technology (a total of 5403 OTUs against 125 OTUs previously found) (Rodríguez-Ruano *et al.*, 2015; this work: chapter II). Most of them, however, represent rare groups in this microbiome, as only 9 OTUs (including six members of class *Clostridia*, two members of class *Actinobacteria* and one member of class *Bacteroidia*) constituted around 45 % of the total diversity found (in percentage of sequences). The rare taxa may include OTUs which only occasionally live in the gland or other present in low abundances and whose detection is quite stochastic for this reason (Pedrós-Alió, 2006). The existence of a main core of taxa and the invariability of their presence across different individuals and environmental conditions in the wild suggests that this bacterial assemblage is characteristic of the uropygial gland and quite stable.

Particularly, the season, temperature, humidity and precipitation did not have any detectable effect on the uropygial microbiome of hoopoe breeding females. Additionally, the habitat, the population and the distance between nests were also discarded as predictors of the uropygial microbiome variations. Spatiotemporal stability has been detected in other symbiotic systems. Particularly for sponges, it has been determined that the bacterial communities that ensure their ecological functions are maintained over seasons and years in several different host species, even when the environmental factors largely vary along time and the surrounding free-living bacteria suffer wide seasonal fluctuations (Erwin *et al.*, 2012). The invariability and stability of bacterial communities in symbiosis, along with the existence of core representatives (either abundant or rare) may be important for community adaptation to their environment (Campbell *et al.*, 2011). Moreover, phylogenetic redundancy in the core microbiome (if we assume similar functions for members of the same lineage) may imply a better community response to disturbance (Shade and Handelsman, 2012). In the uropygial microbiome of hoopoes a core bacterial assemblage was found in all breeding females. Some of the core components were phylogenetically related, and thus they may have an important homeostatic function in the uropygial symbiotic community. Additionally, the uropygial bacterial core was composed by both main and rare OTUs, in accordance with new insights into the possible activity and functional relevance of both high and low abundance taxa in the microbial assemblage and ecology (Campbell *et al.*, 2011).

In contrast to all the other seasonal, meteorological and geographical factors studied in this work, the year showed a significant influence in the bacterial communities of the female's uropigial secretion. The interannual variations detected were due to a differentiation of 2012 from 2010 and 2011. Although 2012 was

climatologically characterized by lower mean relative humidity percentages in the area of study, caused by a decrease in the mean seasonal precipitations for both spring and summer that year, we did not detect any direct influence of the humidity and precipitation experienced by the different females before laying and during incubation on their uropygial microbiome. These results suggest that the interannual variation may be due to other meteorological factors not included within the present study or that the climatic differences in a wider temporal scale affect (directly or indirectly) the communities of bacteria in the habitats of hoopoes. It is known that certain seasonal-scale adaptation occurs in some symbiotic systems either due to direct weather effects (i.e. precipitation) (Torres-Cortés *et al.*, 2012) or to indirect seasonal changes in food availability for example (Davenport *et al.*, 2014). In the case of the pea aphid (*Acyrthosiphon pisum*) defensive symbionts, both weather conditions and enemy-mediated pressures seem to influence the seasonal patterns found (Smith *et al.*, 2015). Another possible explanation for the variations detected in 2012 may be related to indirect influences of climate if the migratory behavior of the study population changes among years depending on climatology or other reasons, given that our wild population is a mix of sedentary and migrant individuals (Reichlin *et al.*, 2012; Van-Wijk *et al.*, pers. com.). Birds' microbiota can be affected by winter quarter characteristics in migratory species (Bisson *et al.*, 2009), and therefore the uropygial microbiome in hoopoes may vary accordingly, depending of the migratory behavior of the individuals of the population each year.

In conclusion, the overall invariable structure of the uropygial community of the gland of hoopoe females over seasonal and spatial factors has been demonstrated, thus suggesting a specific adaptation of the uropygial microbiota to its environment and an intimate symbiotic relationship between bacteria and hoopoes at least for

females while nesting. The presence of a core microbiome including related taxa may also imply a better adaptation to disturbance, allowing some cushioning against changes that otherwise would imply a disruption of the symbiotic homeostasis. The only factor explaining some of the variation detected here was the year, although none of the meteorological factors studied explained it. More research in this direction may reveal the environmental sources of uropygial bacterial variation, letting a better understanding of the way this particular microbiome is acquired and maintained.

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CAPÍTULO IV:

Changes in the uropygial microbiome of hoopoe females along different reproductive phases

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ABSTRACT

Microorganisms play a key role in multiple functions when they establish symbioses with hosts. From gastrointestinal tract to airways, and reproductive tract, symbiotic bacteria may be related to normal body function and health, although their role may vary among physiological stages of the host. In the hoopoe, a hole-nesting bird, a relevant function for symbiotic bacteria has been described for breeding. The presence of certain bacteria in female uropygial gland causes some special characteristics in its secretion (i.e. antimicrobial activity) that provide an increase in the reproductive success of the host. In this work, hoopoe females were sampled at different stages from the days previous to breeding to the end of their breeding attempt, and the changes in the bacterial community of their uropygial secretion were tracked using high throughput sequencing. A relative increase in the *Firmicutes* populations (mainly class *Clostridia*) was found during breeding phase, when bacteria load is the highest. In the pre-breeding and post-breeding samples, when bacteria in the secretion are scarce, *Proteobacteria* and *Bacteroidetes* dominated. Moreover, the changes detected may involve certain turnover of the strains present in the secretion, suggesting that part of the breeding assemblage may be acquired each breeding season. The selective pressures that have driven to the apparition of this cyclic pattern closely linked to female stay within the nest-hole, and the mechanisms that cause the changes each breeding season remain uncertain, although some hypotheses are proposed as avenues for future research.

INTRODUCTION

Microorganisms are key in the dynamics of ecosystems, including their main role in biogeochemical cycles and nutrient recycling (Nazaries *et al.*, 2013). Moreover, they affect the functioning of other organisms, directly determining their resistance to pathogens or toxins, their ability to avoid predators or use determined compounds as nutrients, and even the overall host health status (reviewed in Flórez *et al.*, 2015; Hacquard *et al.*, 2015; Moran, 2006). When microorganisms interact in that way with macro-organisms, they obtain some benefits from their host too, establishing mutualisms that can evolve (in more or less degree) to very specific and close relationships (Moran, 2006). Hosts frequently maintain symbiotic relationships with different microbiomes at different body locations (Spor *et al.*, 2011), and those microbiomes can be interrelated, sharing strains among them and acting as sources for each other (Edmonds-Wilson *et al.*, 2015; Martínez-García *et al.*, 2015).

Seasonal changes in the presence or composition of microbiomes hosted by animals are usually related to reproductive cycles, with enhancement of beneficial microbes useful for protecting embryos or improving offspring growth during the breeding phase (Ceh *et al.*, 2013; Reid *et al.*, 2015; Therrien *et al.*, 2015). It is the case for example of changes in the composition of the microbiome during pregnancy in women vagina which turns into a stable state in which bacteria related with immunity enhancement and defense against pathogens are predominant (Braundmeier *et al.*, 2015). Bacteria present in both the vagina and the placenta may be important not only in protection during gestation, but also in fetus colonization and therefore in microbiome functionality in the newborn. However, that

colonization also involves bacteria from other body locations (i.e. intestinal) and changes in the woman gut microbiome during pregnancy are also related with the innate immunity boost of the fetus (Konstantinov *et al.*, 2013). In any case, the bacterial community is affected by hormonal changes. Indeed, the vaginal microbiome in women changes along the menstrual cycle, as the hormonal status varies (Braundmeier *et al.*, 2015). This example shows the complex influence of seasonal physiological changes of the host on its relationship with symbionts maintained throughout its body. Thus, the neuroendocrine system, the key driver for seasonal physiological changes in response to environmental changes (Wood and Loudon, 2014), probably regulates the release of particular nutrients, chemical signals or immune molecules favoring the presence of particular bacterial strains in the periods they are most important for host success. These seasonal changes may suggest the existence of a cost in the regulation of microbiomes hosted within the body, only paid seasonally, or variation in the optimal community among phases of the life cycle.

A remarkable case of seasonal dynamics in an animal organ hosting symbiotic bacteria is the uropygial gland of the European hoopoe (*Upupa epops*). The females and nestlings of this species experience drastic changes in gland size and secretion properties (color, odor and chemical composition) that are related to the presence of bacterial symbionts during their stay within the hole-nest in the breeding season (Martín-Vivaldi *et al.*, 2009, 2010; Soler *et al.*, 2008). These bacteria (enterococci) produce bacteriocins with antimicrobial activity against feather degrading and pathogenic bacteria (Martín-Platero *et al.*, 2006; Ruiz-Rodríguez *et al.*, 2009, 2012, 2013), and their presence in the secretion (used by females to impregnate eggs) increases hatching success (Martín-Vivaldi *et al.*, 2014; Soler *et al.*, 2008).

Recent analyses using molecular methods to detect non-cultivable bacteria have shown that the bacterial community hosted in the uropygial gland of female and nestling hoopoes during the nesting phase is much more complex than shown by cultivation, including not only enterococci but a variety of strains of several bacteria phyla (Rodríguez-Ruano *et al.*, 2015). In addition, some of the strains found in female secretions seem to be transmitted from the gland to the beak, brood patch and also eggshells through preening using the secretion (Martínez-García *et al.*, 2015). Moreover, it has been previously shown in cross-fostering experiments that the composition of the community inside nestling glands is subject to environmental effects, incorporating strains from other hoopoes or a common environmental source (Martínez-García, 2015; Ruiz-Rodríguez *et al.*, 2014). Despite all these results suggest that the microbiome inside glands is a dynamic system, no study has analyzed the changes experienced by the composition of the communities inside female glands along the breeding season, among phases involving different host interests and environmental influences. A related question is how these communities are established within the gland of females each season. The secretions of males throughout the year, and those of females out of the breeding season, lack the particular properties shown in females while breeding (Martín-Vivaldi *et al.*, 2009), when female secretions are full of cultivable bacteria (Soler *et al.*, 2008). Additionally, several characteristics of breeding females secretion such as color or chemical composition are directly caused by bacteria (Martín-Vivaldi *et al.*, 2009, 2010). These evidences suggest that the gland may be colonized seasonally by bacteria available out of the gland. However, until now there have not been clear evidences of the origin of the bacteria that each breeding season proliferate in the glands of females, and studies designed to test two probable sources (nest material and female gastrointestinal microbiomes) have not

found clear support for such origin (Martínez-García, 2015). Some evidence suggests that glands may retain the whole year a reservoir of bacteria able to grow and replenish the entire community periodically, each breeding season (Rodríguez-Ruano *et al.*, unpublished data). In order to test that hypothesis and describe the changes in the composition of the uropygial gland microbiome of female hoopoes along the breeding attempt, here we compare the bacterial communities found in the same individuals in seven different phases of the cycle using high-throughput sequencing.

MATERIALS AND METHODS

1. Study area, study species and sampling procedures

The study was performed in 2011 using a captive breeding population of hoopoes maintained in three different installations at Hoya de Guadix (Granada), the gardens of the Faculty of Science (Granada, University of Granada), and La Hoya Experimental Farm (Almería, Experimental Station for Arid Zones, CSIC). Females were kept together in a common cage in Granada isolated from males until pairing. Starting in March, each female was housed with an unrelated male in an independent cage of at least 3 m x 2 m x 2 m, with a nest box, access to soil and provided with live food (crickets and fly larvae) and meat (beef heart) *ad libitum*. After pairing, cages were visited daily up to the start of laying to record laying dates. Afterwards, visits were performed weekly to record clutch information and hatching dates, and collect uropygial gland secretion samples.

The hoopoe is a hole-nesting bird with asynchronous hatching that usually lays clutches of 6-8 eggs. Incubation starts with the first egg (day 1), last 17 days, and is carried out solely by the female, which stays within the nest brooding the nestlings until the first nestling hatched is about 8 days old (day 25). Afterwards, nestlings are fed by both parents within the nest until fledging (day 41-47) (Cramp, 1998). Hoopoes breed one or two clutches each breeding season, and when a second clutch occurs, it usually starts by the end of the fledging phase of the first one (Cramp, 1998). With this information we established seven different sampling periods along the breeding cycle: pre-breeding (between 98 and 8 days before day 1, named using negative numbers), laying (day 1), incubation (day 7), end of incubation (day 14), nestlings (days 20-27), fledging phase (days 35-42) and post-breeding (days 49-56). Sometimes it was possible to sample a second or replacement clutch, which let to compare the microbiome of the same phases in different breeding attempts for the same female.

All females were identified with numbered (Spanish Ministry of Environment) and color rings. The uropygial gland secretion was extracted using latex gloves clean with ethanol 96º C and a micropipette with a sterile tip introduced within the gland papilla, after lightly washing the opening and tuft of the gland with 96% ethanol. Samples were stored in sterile 1.5 ml microfuge tubes at 4ºC and then frozen at -20ºC within 24 hours until processed. A total of 134 samples were obtained from 13 captive females sampled at the different phases of the breeding attempt. The complete cycle (at least one sampling point for each phase) could be recorded only for two females, while seven were sampled in all phases except post-breeding (three of them had two lays, and one lacked one sampling point in the middle of nesting). Three more females were sampled from laying to fledging phase and another one from pre-breeding to nestlings phase. As the pre-breeding samples

were taken periodically for all females before knowing their laying date, the number available for each individual varied from one to five.

2. DNA extraction and purification, amplicon libraries and sequencing

Extraction of total DNA from uropygial secretion samples was performed using the FavorPrep Genomic DNA extraction kit (Favorgen Biotech) according to manufacturer's instructions, adding a lysozyme pretreatment (10 mg/ml, at 37 °C for 30 min). PCR product purification was performed using AMPure XP magnetic beads (Agencourt). Final elutions were made in ultrapure sterile water for total DNA and in EB buffer for PCR products.

DNA amplification was normalized diluting the samples after a qPCR, to ensure they achieved the exponential phase at the same number of cycles. Then the V4 region of the 16S rDNA was amplified in a first PCR step. The universal primers 515F and 806R (Nikkari *et al.*, 2002; Wang and Qian, 2009) were modified by Polz *et al.* to include part of the Illumina adapters (and a 5 bp barcode in the forward primer for multiplexing) as follows: U515F (5'-ACACGACGCTCTTCCGATCT - NNNNN - GTGCCAGCMGCCGCGGTAA-3') and E806R (5'-CGGCATTCCTGCTGAACCGCTCTTCCGATCT - GGACTACHVGGGTWTCTAAT-3'). PCR was carried out using a Q5 High-fidelity DNA polymerase (New England BioLabs) at an annealing temperature of 52 ºC and finished at exponential phase according to the initial qPCR. Then a second PCR step of 9 cycles was performed to generate the final construction with the complete Illumina adapters (including a 9 bp barcode in the reverse primer for multiplexing). The purified PCR products were normalized again by qPCR and mixed accordingly.

Sequencing was carried out in an Illumina MiSeq sequencer at the MIT BioMicro Center, Cambridge, Massachusetts, USA.

3. Data analysis and taxonomic identification

The preprocessing was carried out using scripts from USEARCH 8.0.1623 (Edgar, 2010) for paired-end sequences concatenation and from QIIME 1.8 (Caporaso *et al.*, 2010) for quality filtering and demultiplexing. Filtered sequences were aligned and trimmed before running the Distribution Based Clustering Method (Preheim *et al.*, 2013) for OTU picking against the Greengenes_12_10 database (trimmed to keep the V4 region included in our dataset) (McDonald *et al.*, 2012). UCHIME (Edgar *et al.*, 2011) was used to find and remove chimeric sequences. Taxonomical classification of the OTUs was performed using BLAST 2.2.22 (Altschul *et al.*, 1990) against the Greengenes_12_10 database (McDonald *et al.*, 2012). Then, the OTUs identified as *Archaea* were discarded and the OTU table was filtered to eliminate low abundance OTUs (with less than 0.0005 % of the reads) as recommended in (Bokulich *et al.*, 2013). The dataset was standardized with a multiple rarefaction at 75% of the minimum number of sequences per sample before the standard QIIME 1.8 pipeline (Caporaso *et al.*, 2010) was used to calculate diversity indexes and perform beta diversity analyses based on the distance matrices obtained with the weighted option of UniFrac (Lozupone and Knight, 2005). PCoA representations were implemented using EMPeror (Vázquez-Baeza *et al.*, 2013). Statistical analyses (including Kruskal-Wallis tests, Mann-Whitney U-tests with Bonferroni correction, and Permutational Multivariate Analysis of Variance) and heatmap plots were performed using the R statistical software (R

Development Core Team, 2014). Apart from the basic functions available in the standard R Stats and Graphics Packages, the functions adonis from the vegan Package (Oksanen *et al.*, 2013) and heatplot from the MADE4 Package (Culhane *et al.*, 2005) were used for those purposes.

RESULTS

The phylogenetic diversity of the microbiome hosted in the uropygial gland of females (calculated with the PD whole tree index) changed among reproductive stages (pre-breeding mean (SD) = 17.29 (5.23); laying mean (SD) = 6.29 (2.16); incubation mean (SD) = 6.19 (0.98); hatching mean (SD) = 5.91 (0.68); nestlings phase mean (SD) = 7.62 (3.31); fledging phase mean (SD) = 10.75 (3.99); post-breeding mean (SD) = 19.15 (2.34); Kruskal Wallis test $\chi^2 = 67.017$, $P < 0.001$). A post-hoc test using Mann-Whitney U-tests with Bonferroni correction revealed that the significant differences occurred between pre-breeding and all the other phases except post-breeding, between fledging phase and laying, incubation and hatching, and between nestlings phase and post-breeding.

When comparing the community structure of the different reproductive stages determined, significant differences were also found (Adonis, $F_{6\ 133} =$, $P < 0.001$). A pairwise comparison showed specific significant differences for pre-breeding and post-breeding against all phases from laying to fledgings (breeding phases), between fledgings and the other breeding phases except laying, and between laying and all the other breeding phases except fledgings. The differences are apparent

when representing the bacterial communities of the females in a PCoA plot (Fig. 1). Laying, incubation, hatching, nestling and fledging phases clustered more or less together and separated from pre-breeding and post-breeding along the PC1 axis, which explained 44 % of the variation found in our samples (Fig. 1A). Moreover, the uropygial microbiomes of females in the breeding phase seemed to vary along the PC2 and PC3 axes in an opposite way to those of the pre-breeding and post-breeding phases (Fig. 1B). The three first PCoA axes accounted for 61 % of the total variation found in our samples.

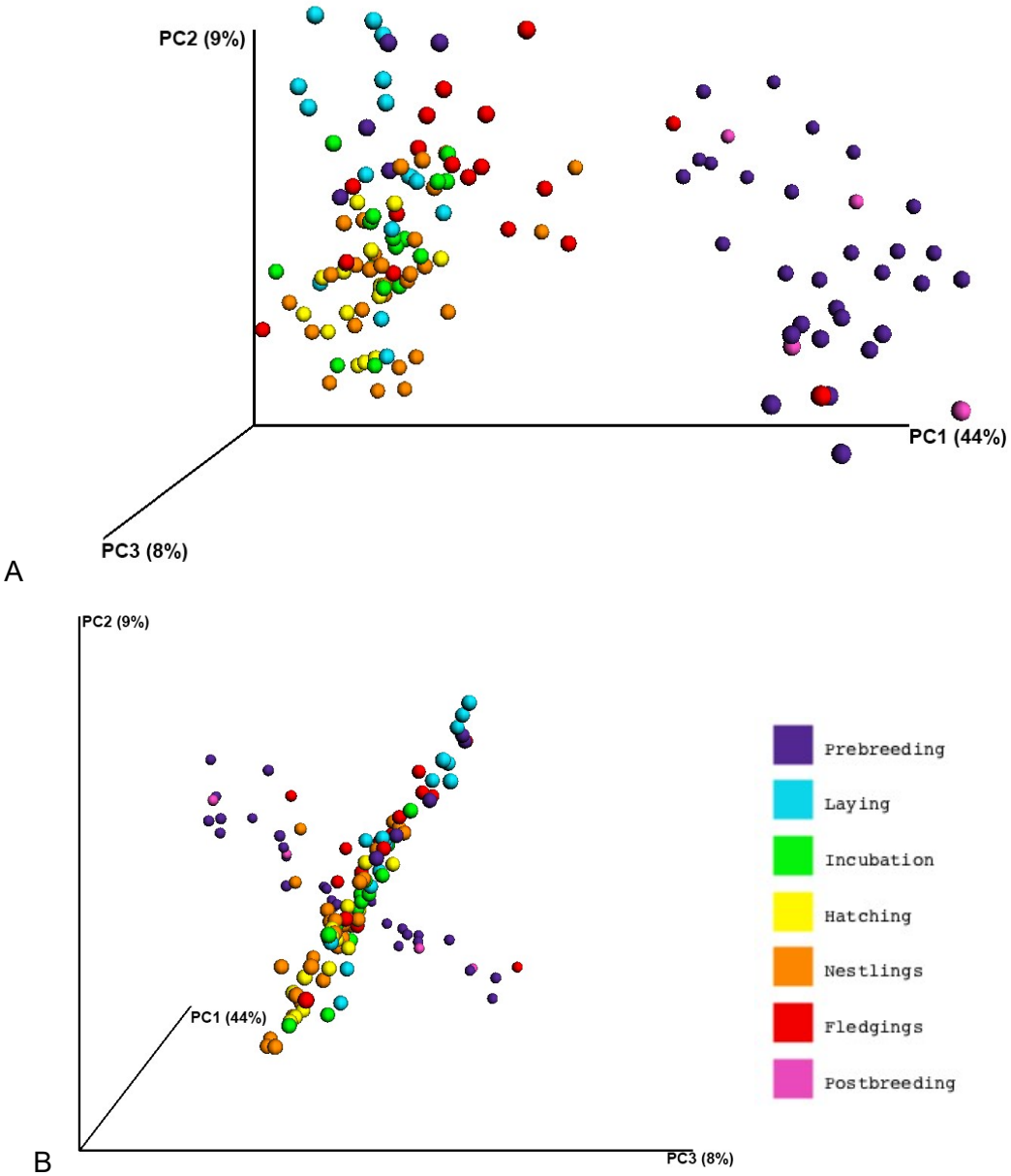


Figure 1. PCoA showing the different uropygial microbiomes of hoopoe females. All the phases sampled during breeding clustered together and separated from pre-breeding and post-breeding. The uropygial microbiomes of females in the breeding phase vary along PC 2 and PC 3 axes in an opposite way to the microbiomes out of breeding.

Four phyla accounted for 98.6 % of the OTUs found (N = 7558). Those phyla were *Firmicutes* (66.6 %), *Proteobacteria*, (17.2 %), *Actinobacteria* (8.3 %) and *Bacteroidetes* (6.6 %). One family within the phylum *Firmicutes* accounted for 46.4 % of the total OTUs (class *Clostridia*, order *Clostridiales*, family *Clostridiaceae*). The taxonomic composition of the samples was examined in order to find the specific

groups responsible for the structural differences among phases detected above. Samples from the breeding phases (from laying to fledgings) were dominated by *Firmicutes*, which represented between 69 % and 90 % of the sequences within each phase. Within *Firmicutes*, *Clostridia* was the predominant class for breeding samples. The predominant group for both pre-breeding and post-breeding samples was *Proteobacteria* (42 % and 54 % of the sequences respectively). In these phases, both *Proteobacteria* (mainly *Gammaproteobacteria* and *Betaproteobacteria*) and *Bacteroidetes* (mainly *Flavobacteria*) were relatively increased against *Firmicutes* compared to the breeding samples. But also within *Firmicutes* the relative abundances of *Bacilli* against *Clostridia* were higher, although *Clostridia* was still the predominant class of *Firmicutes* in the samples out of breeding.

When analyzing the variation in the taxonomic structure of our communities along the different reproductive phases considered, there was a progressive change from the *Proteobacteria* dominated samples before breeding to the *Firmicutes* dominated samples typically found in females dark secretions while nesting (Fig. 2). Towards the end of breeding and the post-breeding phase, some females started to show a kind of reversion to a bacterial community more similar to those of the pre-breeding phase (Fig. 2).

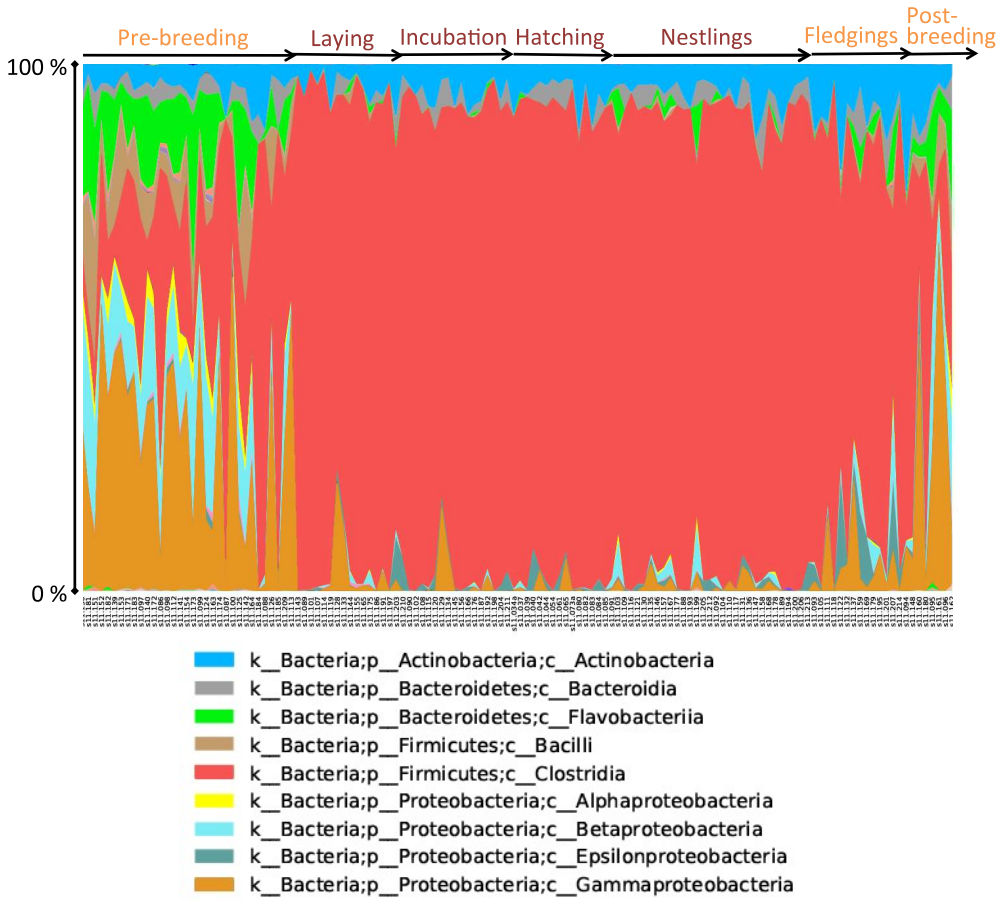


Figure 2. Evolution of the microbiome taxonomic composition in the uropygial secretion along the different reproductive phases established for females (each color represents the proportion of sequences for a different taxon). The central phases included in the breeding period do not show a differentiation in bacterial composition among them. Samples from before and after reproduction show a quite different bacterial assemblage from those of the samples from incubating and brooding females. For clarity, the legend has been adjusted to the main groups observed in the graphic.

A heatmap plot with the 100 most abundant OTUs in our samples showed a twofold clustering (Fig. 3). First, among samples, it was very clear that those of the pre-breeding and post-breeding phases (cluster 1) were very similar among them and very different to those of the phases in which females stayed in the nest (incubating or brooding) or had fledglings (cluster 2) (Fig. 3). Second, among OTUs, there was a group that was typical of the pre-breeding and post-breeding phases and very infrequent in the other phases. The samples of the different breeding

phases were pooled, including sampling days from laying to fledging phase, although the samples in fledging showed a tendency to cluster together more than with the others (cluster 2-A) (Fig 3). There was also a trend for the samples from the nestling period to group together more frequently than in the case of the remaining phases (laying to hatching). Most of the 29 samples of the nestling period of cluster 2-B2 (Fig. 3) resulted included in cluster 2-B2b (83 %), while the 38 samples from laying to hatching were evenly distributed between clusters 2-B2a and 2-B2b (53 % and 47 % respectively; Chi-square test, $\chi^2 = 6.71$, $P < 0.01$).

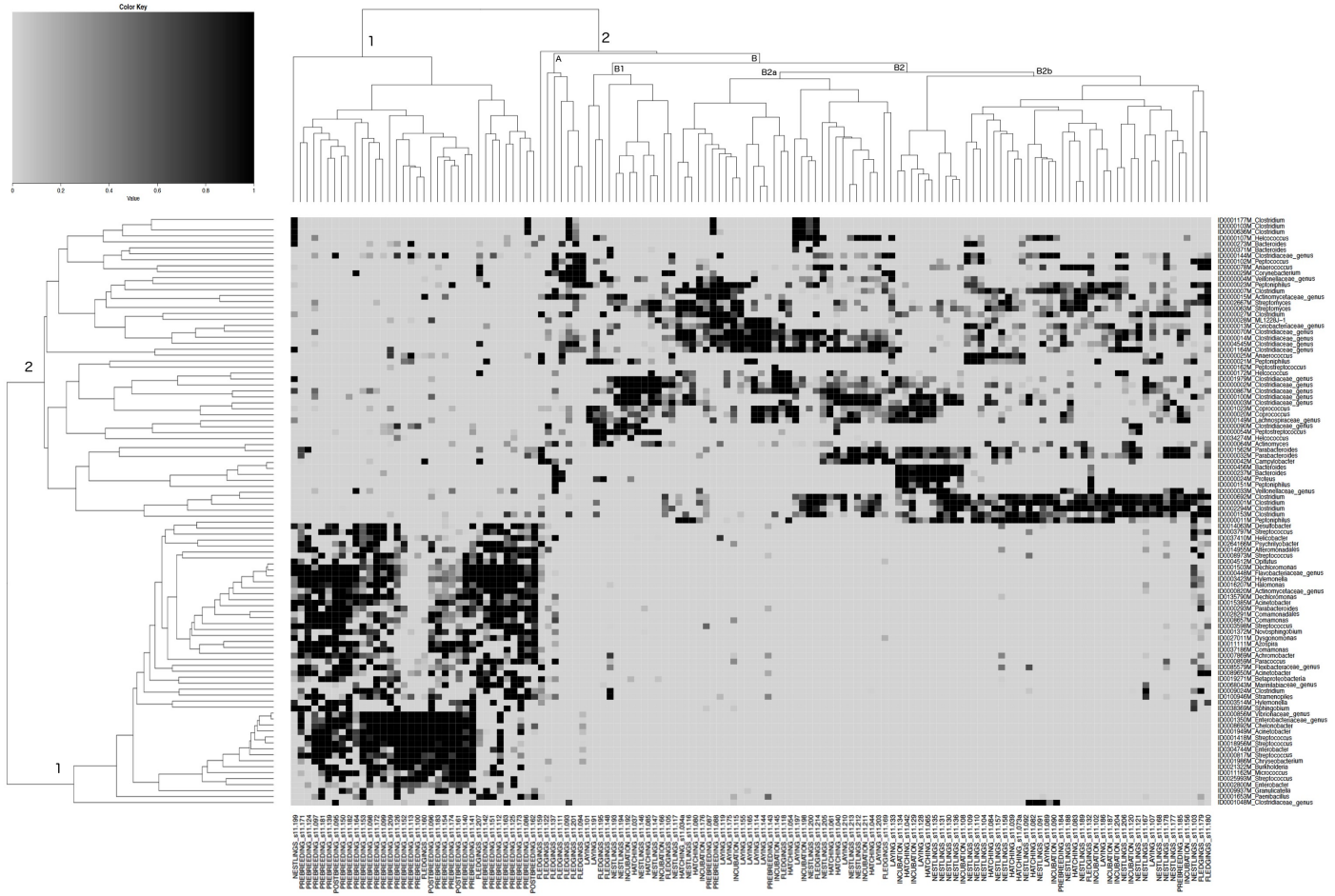
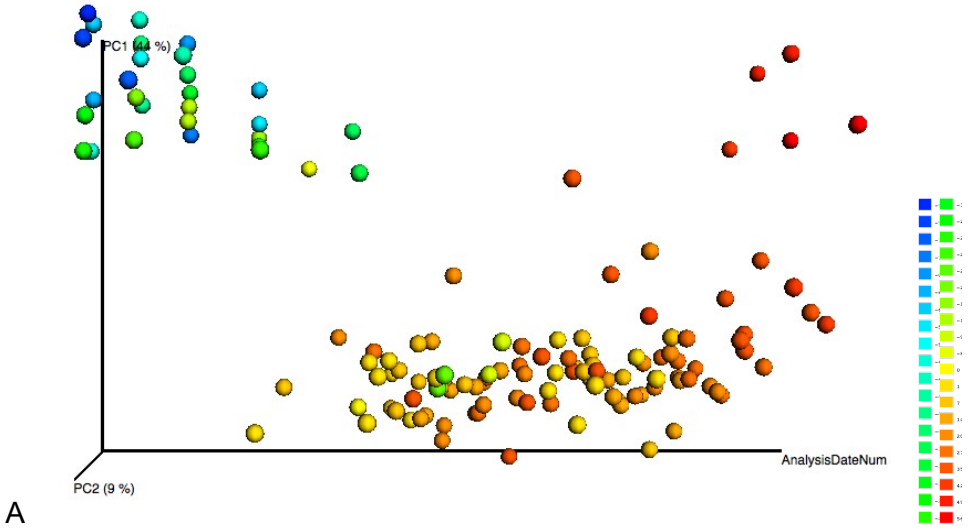
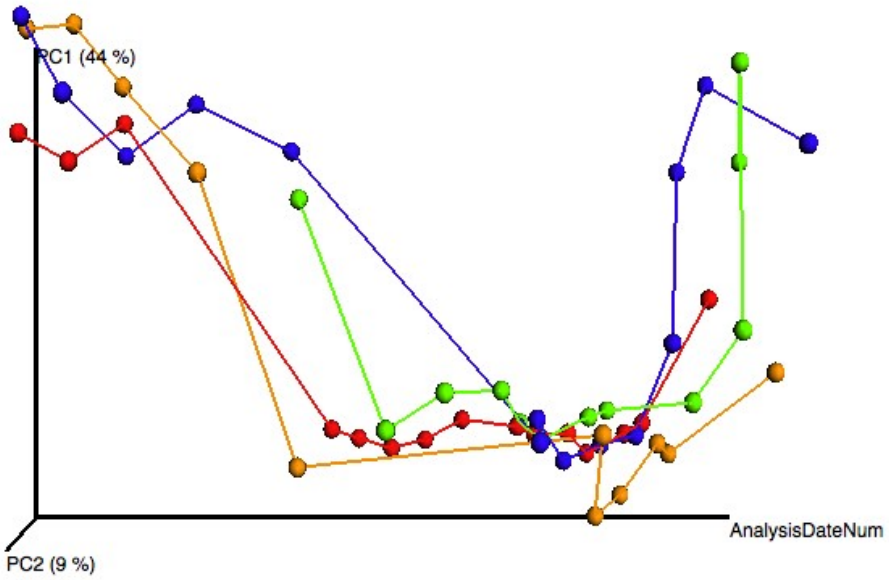


Figure 3. Heatmap showing the distribution of relative abundances (from light grey representing 0 to black representing 1) of the 100 most abundant OTUs in our samples across the different samples obtained from about 100 days before reproduction to about 60 days after laying for the 13 females tracked. A clear clustering separates the typical OTUs from the pre-breeding and post-breeding stages (cluster 1) from those of the central reproductive stages sampled, that are more or less mixed among them (cluster 2).

The change in composition can be also noticed if the PCoA is plotted against a fixed axis reflecting the progress of the breeding season (Fig. 4). In that case, the changes can be followed for all the samples together (Fig 4A), but they are easily tracked also for individual females (Fig. 4B).



A



B

Figure 4. Changes in bacterial communities of females along time. (A) All the samples colored by day of the reproductive cycle (from dark blue 100 days before laying to red 60 days after lay). (B) Example of the changes of the uropygial bacterial community experienced by four specific females sampled for this study (note that samples for all the possible time points for each female were not available).

DISCUSSION

In the present work we describe for the first time the variation of the uropygial bacterial assemblage of hoopoe females along the reproductive cycle. We have found that the microbiome present out of the breeding phase (pre- and post-breeding periods) differs substantially from that of the breeding phase (from laying to fledging) Within the breeding phases the microbiomes are more similar and hardly distinguishable among them. Indeed, there was not a drastic shift in the bacterial populations synchronous to the changes observed in the properties of the secretion along a breeding attempt. When the female leaves the nest after finishing brooding and starts to participate in looking for food out of the nest (day 25), the characteristics of its secretion start to change from the dark and pungent one to the whitish one that characterizes the non-breeding periods (Martín-Vivaldi *et al.*, 2009). However, the most evident change in the bacterial community detected in this study occurred later, once the nestlings left the nest (fledging period, day 41). In this phase the female community started to incorporate some OTUs more typical of the non-breeding period. Nevertheless, the study also detected a trend for samples of the nestling period differentiating from those of the egg phases (laying, incubation and hatching). All these results show a very dynamic structure of the microbiome present within female glands along the successive reproductive phases.

The difference in the composition of communities of the non-breeding and breeding phases is very interesting since it involves not just a change in the importance of groups, but also an important turnover of the particular OTUs present in high proportion within the community. One of the hypotheses proposed for the seasonal development of the typical microbiome found in female hoopoe glands is

that, given the presence of bacteria in the glands out of the breeding season, glands may be a reservoir of the main strains important for the bird (Rodríguez-Ruano et al 2015; this work: chapter II). However, the shift in the presence of some OTUs suggests that some strains responsible for the antimicrobial properties of the secretions that benefit the bird (Martín-Vivaldi *et al.*, 2010, 2014; Ruiz-Rodríguez *et al.*, 2009, 2012, 2013; Soler *et al.*, 2008) should be acquired each breeding season. Previous studies have shown that part of these symbionts can be acquired horizontally from the environment (Martínez-García, 2015; Ruiz-Rodríguez *et al.*, 2014), but the possible sources for bacterial acquisition by females (such as the nest materials and female gut) have not been confirmed (Martínez-García, 2015; Martínez-García *et al.*, 2015, but see Rodríguez-Ruano *et al.*, 2015). Therefore, the origin of those strains that periodically appear in female glands remains as one of the main questions to solve in the understanding of the system formed by the hoopoe and the symbionts of its uropygial gland.

Along breeding (from laying to fledging) the uropygial microbiome was dominated by *Firmicutes* of class *Clostridia*, in accordance with previous studies (Rodríguez-Ruano *et al.*, 2015; this work: chapters II and III). However, our results here show that, before breeding, the uropygial bacterial assemblage is dominated by *Proteobacteria* within the classes *Gammaproteobacteria* and *Betaproteobacteria, Bacteroidetes* of class *Flavobacteria*, and *Firmicutes* within the classes *Clostridia* and *Bacilli*. Towards the end of the breeding attempt, again, the microbiome of the uropygial secretion shows a composition typical of the pre-breeding phase. This high-level taxonomical shift in the predominance of bacterial populations (either due to a relative increase of some groups against another ones or to the relative decrease of the latter against the first) could reflect a deep change in the functions of the uropygial symbiotic assemblage (Langille *et al.*, 2013). For example,

Clostridia are known for their relevant homeostatic function in microbiomes, but also because they produce certain kind of substances (i.e. short chain fatty acids) with antimicrobial and regulatory functions in the host (Lopetuso *et al.*, 2013; Martín-Vivaldi *et al.*, 2010), while *Proteobacteria* are a group with a high metabolic versatility that allows its colonization of quite different environments, and their ability to produce antimicrobial peptides has been also described (Desriac *et al.*, 2013; Esposti, 2014). Given that several evidences (see above) suggest an antimicrobial function for the breeding microbiome, there are two possible explanations for the dynamic found in the uropygial microbiome. First, an alternative role of the non-breeding community may exist out of the nesting phase. Second, the reduction of the utility of the breeding assemblage when females are out of the nest may drive to a non-selective colonization of the gland in this moment. Since the abundance of bacteria in the secretion is reduced in great amounts out of the breeding season (Rodríguez-Ruano *et al.*, 2015; this work: chapter II), the second option seems more plausible, although more research is needed to distinguish between both possibilities.

The OTUs more commonly found in non-breeding secretions are quite diverse, and include representatives of the genera *Streptococcus*, *Enterobacter*, *Helicobacter*, *Parabacteroides*, and *Paracoccus* among others. During breeding, genera such as *Clostridium*, *Bacteroides*, *Parabacteroides*, *Coprococcus*, *Helcococcus*, *Peptoniphilus, Streptomyces* and several unidentified genera of *Clostridiaceae* are frequently found. All these species are obligated or facultatively anaerobic, as expected for the enclosed environment inside the uropygial gland. Some of them are also representatives of the gut microbiota, in accordance to previous work suggesting a gastrointestinal origin of part of the uropygial microbiota (Rodríguez-Ruano *et al.*, 2015).

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Other organisms depend on certain bacterial assemblages to improve their fitness during reproduction. It is the case for adult sponges (Ceh *et al.*, 2013) or female humans (Konstantinov *et al.*, 2013). Our results show that the microbiome in hoopoe glands drastically change towards a particular community for the period the female stays within the nest. These changes should be controlled at least partially by the host to favor a community that is the most profitable for its interests according to the "partner choice" (Bronstein, 2001; Bull and Rice, 1991; Douglas, 1998; Ferrière *et al.*, 2007; Sachs and Hollowell, 2012; Schwartzman *et al.*, 2015; Shapiro and Turner, 2014; Simonsen and Stinchcombe, 2014). Such control may be mediated by hormonal changes and involve the release of particular substances as nutrients, pH regulators or immune factors in the organ where the symbiosis is established (Chaston and Goodrich-Blair, 2010; Scheuring and Yu, 2012; Schwartzman *et al.*, 2015).

In the hoopoe, the properties of the secretion are partially determined by the bacteria living in the gland because chemical composition and color changed when bacteria were eliminated with antibiotics (Martín-Vivaldi *et al.*, 2009, 2010). However, in those experiments the secretions did not return to a stage similar to that of the non-breeding season (white) but remained different (Martín-Vivaldi *et al.*, 2009), suggesting that also the substances produced directly by the bird secretory cells are special during breeding. Probably, the secretion produced in this phase is in some way responsible for the changes experienced by the microbiome of the gland, exerting some kind of partner choice. Such a mechanism may have evolved to improve the beneficial effects of the community established in the gland favoring the taxa that provided nesting individuals with appropriate defensive characteristics

(as production of antimicrobial compounds) during nesting. A detailed analysis of the changes experienced by female glands and their secretions before the change in microbiome composition would help to unravel the key determinants of the dynamics of this bacterial community.

In conclusion, the mutualistic microbiome developed in the uropygial gland of hoopoes during breeding seems to be a particular combination of bacteria selected among the available stocks that is favored by the bird only during this stage. More research is needed to untangle the reasons why this symbiotic assemblage is not maintained for all the life stages of the bird, and the specific mechanisms that drive its changes. Further studies about the bacteria that are specifically related with benefits for the hoopoe, especially during breeding, may also help in the understanding of its highly dynamic system.

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CAPÍTULO V:

Influence of the uropigial microbiome on the eggshell bacterial community in hoopoes

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ABSTRACT

Symbiotic bacteria associated with hosts not only provide benefits regarding nutrition, immunity and development, but also contribute to a homeostatic environment that prevents invaders establishing, and therefore dysbiosis and even disease. However, changes in the microbiome are not always detrimental, and can lead to better-adapted symbiotic assemblages. In this sense, the environmental microbiota that affects hosts may be of interest, to better understand the ecological and evolutionary processes involved in symbiotic systems assemblages. Especially in birds, the nest environment may be an important source of bacteria for both acquisition of beneficial strains and infection by pathogens. In breeding hoopoes, a symbiotic relationship with bacteria in its uropygial gland has been previously studied. Some special adaptations in both the bird and the microbiome had been described that favor an increase of hatching success. Here we evaluate the specific effect of the uropygial bacteria on the eggshell microbiome by experimentally impeding female access to the uropygial gland both in normal and artificially increased pathogenic environments. The manipulation decreased the presence of uropygial bacteria in the eggshell microbiome. When pathogenic risk was experimentally increased in clutches, the presence of certain OTUs on the eggshell was affected by the manipulation of female access to the gland. There was a trend for the contribution of uropygial OTUs to the eggshell reducing the load of strains coming from the experimental contamination source. Moreover, several particular OTUs were associated with the hatching success of clutches including both positive and negative effects. Our results thus show that the microbiome present on the eggshell of hoopoes is affected by the arrival of secretion with symbiotic bacteria

from the female gland and that some eggshell components of this microbiome affect hatching success.

INTRODUCTION

The influence of bacteria on animal physiology and evolution is a key research field for life sciences in the twenty-first century (Bordenstein and Theis, 2015; McFall-Ngai *et al.*, 2013). Symbiotic bacterial assemblages are important for animal development, nutrition, immunity and defense (Flórez *et al.*, 2015; Hacquard *et al.*, 2015; Leser and Mølbak, 2009). Bacterial communities are also essential for host health, helping in maintaining the homeostasis and an overall good physiological function (Clemente *et al.*, 2012; Peterson *et al.*, 2015). Symbiotic bacteria can even determine social behaviors and communication, as it happens in hyenas (Theis *et al.*, 2013) or influence reproduction enhancing the protection against pathogens during gestation or favoring weight gain for both mother and newborn in humans (Konstantinov *et al.*, 2013). Today it is thus impossible to consider animal individuals as complete biological units without taking into account their microbial symbionts, and some authors propose the term holobiont to define that association (Bordenstein and Theis, 2015). In this sense, the study of symbiotic bacterial communities is essential to understand the evolutionary and ecological associations between animals and bacteria and their beneficial outcome.

When newly colonizing bacteria reach an established bacterial community, the result may depend on characteristics of both the original community and the colonizer. In general, bacterial communities that are well established tend to maintain their homeostasis and provide colonization resistance against other bacteria, that therfore have no ability to establish in the community and eventually disappear (Sassone-Corsi and Raffatellu, 2015). However, sometimes an invader can find a good colonizing opportunity if the original assemblage has been

disturbed, and then dysbiosis occurs (Peterson *et al.*, 2015). In some cases, the colonizer may have specific infective abilities that allow it to settle in the new habitat even though indigenous bacteria are present and the homeostasis of the community has not been previously altered. In this manner, some pathogens can evade the microbiome defense and cause infection even in healthy hosts (Curtis and Sperandio, 2011). But pathogens are not the only bacteria able to outcompete the indigenous microbiota. Some colonizers may demonstrate better (more useful or efficient) characteristics than previous native populations, and therefore may gain a foothold in the microbiome, replacing the original populations over time, as occurs in the gut microbiome with dietary changes (Voreades *et al.*, 2014).

Even if we consider only host-associated communities, some environments (i.e. body habitats) are more prone to be exposed to colonization than others. For example the airways keep a stable bacterial assemblage that is invaded by pathogens only when perturbation occurs (de Steenhuijsen Piters *et al.*, 2015). On the contrary, the gut microbiota is more exposed to changes that do not necessarily imply infection and disease. It is the case of the differences found in the gut microbiota of omnivorous versus vegetarian humans, that are just related with different nutrient availability and thus different metabolic activities necessary in that environment (Glick-Bauer and Yeh, 2014).

The fact that the environment can contribute to the host microbiota and shape its composition offers interesting insights for both ecology and evolutionary studies. Studying the way that the microbiota is acquired and how the different sources of bacteria contribute to the final symbiotic assemblage may help us understanding symbiotic relationships but also clarifying ecological and evolutionary processes involving the holobiont.

Bacteria have been detected in different body habitats in birds. These habitats include the plumage (Bisson *et al.*, 2009), the gastrointestinal tract (Waite and Taylor, 2015), the lower airways (Shabbir *et al.*, 2015), and the uropygial gland (Rodríguez-Ruano *et al.*, 2015), and each habitat hosts its own microbiota. The egg surface is also colonized by bacteria (Grizard *et al.*, 2014, 2015), but in most cases bacteria on the eggshell have been related to the probability of embryo infection more than to symbiotic associations (Ruiz-de-Castañeda *et al.*, 2011; Shawkey *et al.*, 2009; Soler *et al.*, 2011). It is known that several nest environment factors can influence the bacterial loads on eggshells (Ibáñez-Álamo *et al.*, 2014; Møller *et al.*, 2015; Peralta-Sánchez *et al.*, 2010), but few work has studied the transmission of bacteria from the nest environment to the eggs, and it mainly focuses on pathogenic bacteria (Brandl *et al.*, 2014). However, recently the same approach has been successfully used to determine the possible transmission of both beneficial and pathogenic bacteria to the eggshell in the nest environment (Martínez-García *et al.* unpublished results).

It is known that the uropygial gland of hoopoes (*Upupa epops*) hosts bacteria able to produce different antimicrobial compounds (Martín-Vivaldi *et al.*, 2010; Ruiz-Rodríguez *et al.*, 2012; Soler *et al.*, 2008). Hoopoes use the secretion of that gland to smear the eggs, and the eggshells present crypts that allow the accumulation of that secretion during incubation (Martín-Vivaldi *et al.*, 2014; Soler *et al.*, 2014). It has been recently demonstrated that some of the bacteria in the uropygial gland reach the eggshell from the uropygial secretion (Martínez-García *et al.*, 2015), and that the presence of a particular group of bacteria (enterococci) or their antimicrobial compounds enhances hatching success in this species (Martín-Vivaldi *et al.*, 2014; Soler *et al.*, 2008).

The hoopoe is a hole-nesting bird that nests in cavities that usually have been reused for breeding several times. Due to the accumulation of food, eggs and feces debris in the hole-nest, the growth of bacteria and therefore the possibility of transmission of bacteria to the eggs are high in this enclosed environment (Walls *et al.*, 2012), and in fact such transmission occurs in hoopoes (Martínez-García *et al.*, unpublished results). However, the special relationship that hoopoes have with their uropygial bacteria favors the presence and overgrowth of certain groups of bacteria during the nesting period (Rodríguez-Ruano *et al.*, unpublished results). Several bacteria found in the dark secretion typical of the nesting period (i.e. *Actinobacteria* and *Clostridia* representatives) are likely antimicrobial producers that may help in the defense against the possible pathogens that can reach the eggs from the nest environment (Rodríguez-Ruano *et al.*, 2015). In fact, both culture and molecular fingerprinting techniques have confirmed the presence of uropygial bacteria in eggshells (Martínez-García *et al.*, 2015; Martín-Vivaldi *et al.*, 2014), although the identification of these bacteria has been limited by the particular techniques used.

The aim of the present work is to determine the importance of bacterial colonization of the eggshells by strains present in the uropygial gland of the incubating female, considering the whole bacterial community in both locations, and its possible effect on the reproductive success of hoopoes. To achieve this, we tested the effect of experimentally manipulating female access to the gland and experimentally increasing the amount of potential pathogens present within the nest.

MATERIALS AND METHODS

1. Study population, experimental manipulation and sampling procedures

The field work was performed during the 2010 and 2012 breeding seasons in a captive population of hoopoes, maintained at Hoya de Guadix (Granada), the gardens of the Faculty of Science (Granada, University of Granada), and La Hoya Experimental Farm (Almería, Experimental Station for Arid Zones, CSIC). More details can be found in (Rodríguez-Ruano *et al.*, 2015). Pairs were maintained in independent cages and nest boxes checked twice a week in order to detect the initiation of breeding attempts. Afterwards each nest was visited at least weekly to register clutch size, the number of eggs hatched and number of fledglings produced. Hatching success was estimated as the proportion of eggs that successfully hatched in a clutch. To do that we counted the number of alive and dead nestlings found in the nest bottom as well as unhatched eggs staying in the nest several days after the hatching period.

In 2010 the available nests were assigned to one of three different experimental treatments: experimental, control I and control II. The manipulation consisted in preventing female access to the uropygial secretion by means of a sterile cat catheter (Buster, 1.0 mm width) inserted in the opening of the papilla of the gland and connected to a flexible silicone tube (70 mm length) for secretion collection. The structure was fastened with surgical glue (3M Vetbond) and adhesive bandage, and checked and renewed every two days. Control I individuals carried the same structure of the experimental group but without impeding the access to the gland opening to check the effect of the manipulation itself. Control II

individuals were visited and handled in a similar way as the previous groups, but no manipulation was performed. A whole description of the manipulation structure and experimental procedure can be found in (Martín-Vivaldi *et al.*, 2014). To increase the available number of clutches for the experiment, different breeding attempts of the same female were considered (different treatments were applied when possible, trying to keep a balanced number of experimental and control nests). Samples from both breeding female secretion and one eggshell in its clutch were obtained for 11control I nests, 7 control II nests and 12 experimental nests. There were not significant differences between both control groups neither in richness (Mann-Whitney U-test; N = 17, U = 31, P = 0.740; control I mean (SD) = 1201.2 (565.4), control II mean (SD) = 1222.4 (630.3)), phylogenetic diversity (Mann-Whitney U-test; N = 17, U = 39, P = 0.740; control I mean (SD) = 7.85 (4.03), control II mean (SD) = 7.36 (3.89)) or community composition (Adonis, $F_{1,16}$=0.509, P = 0.698). Therefore, both control groups were considered together as female control treatment.

In 2012 the same experimental procedure was repeated, but this time a second factor was added to all treatments. Some organic (without any treatment) hen eggs were exposed to nest environment contamination for two weeks in the field. After that, the egg content was homogenized and mixtures 1:1 of egg and sterile phosphate buffer were distributed into sterile microfuge tubes and incubated at 37 ºC overnight before they were applied in the field to clutches. This was performed smearing the whole surface of two eggs (the second and third in the laying sequence) from each clutch with a sterile cotton swab wetted with the solution of the rotten hen egg at days six and eight of incubation (the other eggs in the same clutch were not manipulated). With this manipulation we intended to

simulate an increased infection risk environment in the hole-nest to test the influence of the access to the uropygial secretion in the eggshell microbiota and hatching success in this challenging situation. Samples from both breeding female secretion and two eggshells (one from a smeared egg and another from an untreated egg) were obtained for 9 control I nests, 15 control II nests and 19 experimental nests on the eight day of incubation (before the second smearing with hen egg content). Again both control groups could be combined for subsequent analyses, as there were no significant differences between them for richness (Mann-Whitney U-test; N = 19, U = 52, P = 0.545; control I mean (SD) = 1914.8 (420.7), control II mean (SD) = 1846.5 (445.9)), phylogenetic diversity (Mann-Whitney U-test; N = 19, U = 58, P = 0.272; control I mean (SD) = 16.18 (5.21), control II mean (SD) = 13.31 (4.63)) and community composition (Adonis, $F_{1,18}$=1.160, P = 0.298).

Uropygial secretion samples were collected at day 15 of incubation using sterile latex gloves and a micropipette with a sterile tip introduced within the gland papilla, after lightly washing the exterior of the gland with 96% ethanol. Eggshell samples were collected at day 8 (combined experiment in 2012) or 15 (experiment of manipulated access to the secretion in 2010) of incubation by completely cleaning the surface of the eggs with a sterile swab slightly wet with sterile phosphate buffer. Then the samples were individually stored in sterile microfuge tubes either directly (secretion) or with 1.2 ml of buffer solution (eggshell swabs) and frozen at -20ºC within 24 hours until processed. A complete description of the sampling procedures is given in (Martínez-García *et al.*, 2015).

2. DNA extraction, amplicon libraries construction and sequencing

Total DNA from uropygial secretion samples was extracted using the FavorPrep Genomic DNA extraction kit (Favorgen Biotech) according to the manufacturer's instructions, adding a lysozyme treatment (10 mg/ml, at 37 °C for 30 min). All final elutions were made in 100 microliters of ultrapure sterile water.

For MiSeq Illumina sequencing, the 16S rRNA variable region V4 was amplified from the total genomic DNA by PCR using the bacterial primer set 515F/806R according to the Earth Microbiome Project (EMP) protocols at the BioFrontiers Institute at the University of Colorado (Boulder, Colorado, USA). All primers for amplification and sequencing are available at (http://www.earthmicrobiome.org/emp-standard-protocols/16s/). In the 2010 experiment, it was not possible to obtain amplification for three eggshell samples (two from experimental nests and one from a control I nest). In the 2012 experiment, it was not possible to obtain amplification for at least one of the eggshell samples in one control I nest, four control II nests and nine experimental nests. In these cases, the respective secretion and paired eggshell samples were also discarded from the analyses. In addition to the secretion and eggshell samples, 11 of the hen egg mixtures used in the experiment in 2012 were also sequenced to check the influence of its application in the eggshell microbiota and confirm the intended effect of the experiment. The PCR products were pooled, quantified, and sequenced on the Illumina MiSeq platform at the BioFrontiers Institute at the University of Colorado (Boulder, Colorado, USA).

3. Data analysis and taxonomic identification

Paired-end sequence concatenation was carried out with USEARCH 8.0.1623 (Edgar, 2010) and then QIIME 1.8 (Caporaso *et al.*, 2010) was used for preprocessing (quality filtering and demultiplexing).After preprocessing, the sequences were aligned and trimmed, and the Distribution Based Clustering Method with a value for abundance cutoff of 10 (Preheim *et al.*, 2013) was used for OTU picking against the Greengenes_12_10 database (also trimmed to the V4 region in our dataset) (McDonald *et al.*, 2012). Chimeric sequences were checked using UCHIME (Edgar *et al.*, 2011). Taxonomical classification was performed using BLAST 2.2.22 (Altschul *et al.*, 1990) against the Greengenes_12_10 database (McDonald *et al.*, 2012). After that, the OTUs not identified as *Bacteria* were checked, and those assigned to *Archaea* were discarded. Low abundance OTUs (including less than 0.0005 % of the reads) and samples with less than 2000 sequences were excluded from subsequent analyses using scripts from QIIME 1.8 (Caporaso *et al.*, 2010). Then a multiple rarefaction at 75% of the minimum number of sequences per sample was performed and the average OTU composition was calculated. After rarefaction, the standard QIIME 1.8 pipeline (Caporaso *et al.*, 2010) was used to construct a phylogenetic tree and calculate diversity indexes. Comparisons between groups (Mann-Whitney U-test, T-test, Wilcoxon signed rank test and ANOVA), and graphical representations (box-plots and scatter-plots) were performed using the respective functions within the R Stats and Graphics Packages included in the R statistical software (R Development Core Team, 2014) and the Ggplot2 Package (Wickham, 2009). Source Tracker 1.0 (Knights *et al.*, 2011) was used to determine the possible origin of the bacteria found in the eggshells under different experimental treatments. Before this analysis was performed, OTUs

present in less than 5% of the samples were filtered out (the standard 1% resulted in no filtering due to the small number of samples) and sequences were rarefied at 1000 per sample to reduce computing time. To test whether there is some effect on hatching success of the OTUs found on eggshells, we performed a stepwise regression between the similarity matrices of samples for the abundance of the 100 most frequent OTUs (Bray Curtis similarity) and hatching success (Euclidean distance) using the BEST tool available in software Primer.7. Afterwards, a multiple regression with the OTUs selected in the best model was used to analyze the particular effect of each OTU.

RESULTS

1. Effect of the access to the gland on the eggshell microbiome

Most of the OTUs detected on the eggshell were not assignable to the uropygial secretion of the female (Fig. 1). The majority of the control nests (82.4 %, N = 17) showed a small fraction of uropygial contribution in the eggshell microbiome, while only half of the experimental eggshells microbiome (50 %, N = 10) showed uropygial influence. Additionally, the proportion of the eggshell microbiome coming from the uropygial secretion was significantly different between experimental and control nests (T-test; N = 27, T = 2.36, P= 0.029; experimental nests mean (SD) =1.3 % (1.9 %); control nests mean (SD) = 5.8 % (7.4 %)).

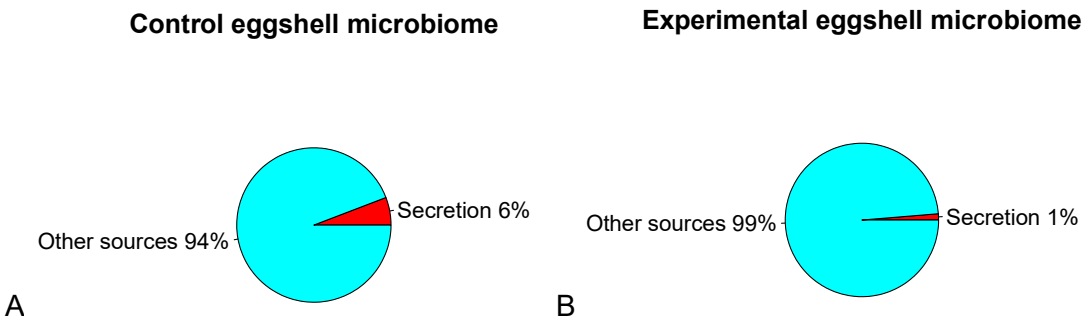

Figure 1. Fraction of all reads in eggshells belonging to OTUs shared with uropygial secretion (red) and other sources (light blue), in both control and experimental samples in normal nest environments.

The bacterial richness in the eggshells did not vary according to the treatment applied to the female (Mann-Whitney U-test; N = 27, U= 96, P = 0.604; experimental mean (SD) = 1124.6 (478.9), control mean (SD) = 1209.9 (573.5)). That was the case also for phylogenetic diversity (Mann-Whitney U-test; N = 27, U= 102, P = 0.414; experimental mean (SD) = 7.17 (4.70), control mean (SD) = 7.65 (3.86)) and composition of the community (Adonis, $F_{1,26}$= 1.394, P = 0.241).

The representation of the 100 most abundant OTUs in a heatmap using only one breeding attempt per female (Fig. 2) shows that certain OTUs tend to appear more in control than in experimental samples and *vice versa*. The frequencies of 10 OTUs were significantly different between treatments using a Mann-Whitney U test (8 were more frequent in control samples and 2 more frequent in experimental samples). Those OTUs were identified as *Psychrobacter* (class *Gammaproteobacteria*), three unknown genera of family *Clostridiaceae* and another one of family ML1228J-1 (class *Clostridia*), in addition to one unknown genus of family *Actinomycetaceae* (class *Actinobateria*) in the control samples and *Brevibacterium* (class *Actinobacteria*) and an unkown genus of family *Xanthomonadaceae* (class *Gammaproteobacteria*) in the experimental samples.

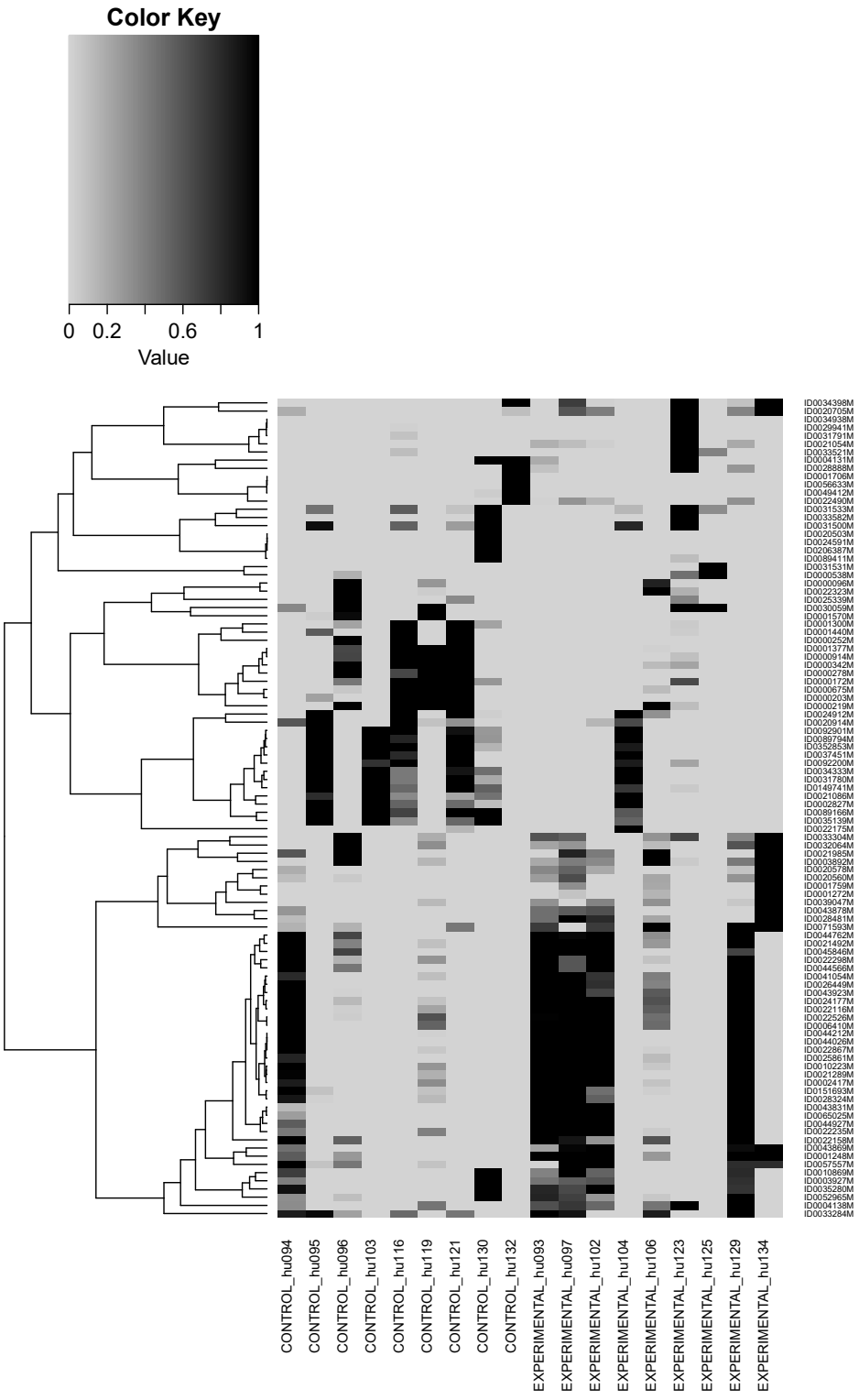

Figure 2. Heatmap showing the 100 most abundant OTUs in our samples after filtering only one representative per nest. Some OTUs appear to be more represented in control samples and others in experimental samples.

2. Evaluation of the effect of an increased risk of infection within the hole-nest

There were no significant differences between pairs of eggs from the same clutch stratified by treatment with hen rotten egg either in richness (Wilcoxon signed rank test; N = 58, W = 270, P = 0.265) or in phylogenetic diversity (Wilcoxon signed rank test; N = 58, W = 193, P = 0.609). The overall composition of the bacterial community did not vary between eggshell treatments either (Adonis, $F_{1,57}$= 0.275, P = 0.943), suggesting that our manipulation was effective in causing a contamination reaching all eggs in the clutch. Therefore, we used only the treated egg of each clutch for subsequent analyses.

The origin of the bacterial OTUs found in eggshells was evaluated, but in this case taking into account both the bacteria from the uropygial secretion and the hen rotten egg mixtures (Fig. 3). The experimental contamination was found in 96.6 % of the samples (N = 29), while the secretion influence was detected in 100 % of the control samples (N = 19) but only in 50 % of the experimental samples (N = 10). In both experimental and control clutches, the experimental contamination accounted for an equivalent proportion of the populations found in the eggshells (T-test; N = 29, T = -0.51, P = 0.616; experimental nests mean (SD) =14.2 % (17.5 %); control nests mean (SD) = 10.8 % (16.3 %)). Moreover, the female treatment had a significant effect in the proportion of bacteria in the eggshell coming from the secretion (T-test; N = 29, T = 2.71, P = 0.013; experimental nests mean (SD) =5.5 % (6.5 %); control nests mean (SD) = 20.7 % (22.6 %)).

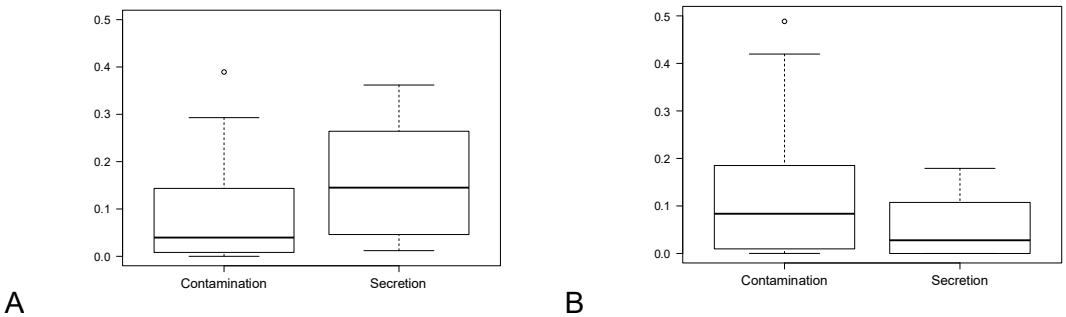

Figure 3. Boxplot showing the distribution of the proportion of the eggshell microbiome that comes from the experimental and from the uropygial secretion in both control (A) and experimental (B) nests

When the relationship between the proportion of the eggshell microbiome due to the uropygial secretion of the female and to the experimental contamination were compared (Fig. 4), a tendency to reduced percentages of experimental contamination in controls was found once the secretion influence reached certain threshold (around 2 %). That effect, however, did not reach statistical significance (ANOVA, $F_{1,27} = 2.52$, $P = 0.124$).

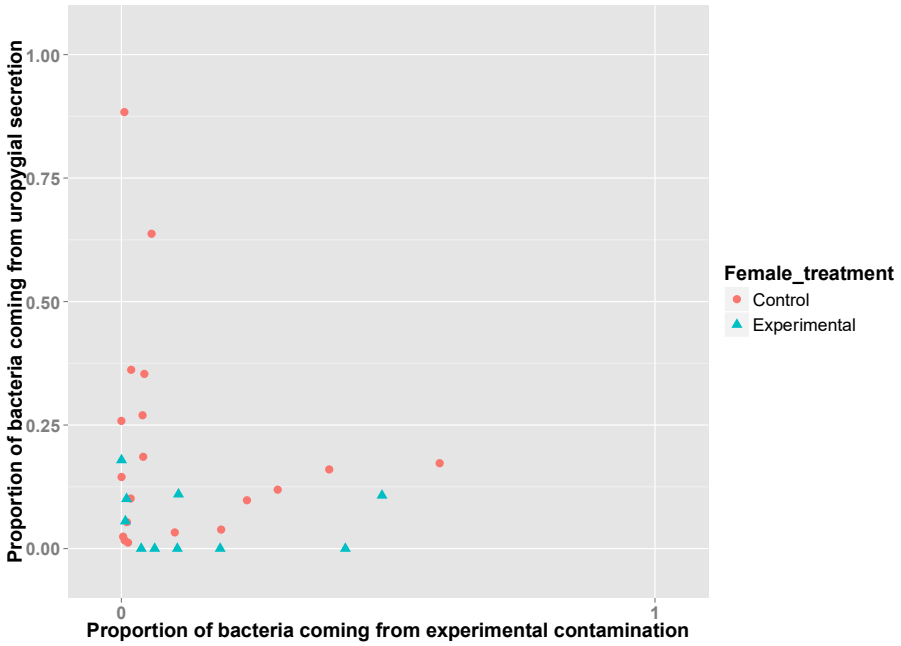

Figure 4. Correlation between proportion of bacteria coming from the experimental source of contamination and from the uropygial secretion in the eggshells. Experimental nests are represented in blue and controls in pink.

No effect of the female treatment was detected for richness (Mann-Whitney U-test; N = 29, U = 119.5, P = 0.261; experimental mean (SD) = 1685.4 (386.9), control mean (SD) = 1875.2 (424.9)), phylogenetic diversity (Mann-Whitney U-test; N = 29, U = 85, P = 0.668; experimental mean (SD) = 15.10 (4.68), control mean (SD) = 14.52 (4.96)) or community composition (Adonis, $F_{1,28}$= 1.364, P = 0.220).

3. Effect of eggshell bacteria on hatching success

The experiment reducing female access to glands did not cause a decrease in hatching success (One-way ANOVA; 2010: $F_{1, 24}$ = 0.139, p = 0.713; 2012: $F_{1, 27}$ = 0.032, P = 0.859). However, the analysis of the best combination of eggshell OTUs explaining hatching success selected two strains (33521M and 89411M) for

samples of 2010 (rho = 0.177, P < 0.05) and four strains (25339M, 1248M, 20503M and 43143M) for samples of 2012 (rho = 0.269, P <0.001). In both cases we performed a multiple regression with their abundances as predictors to determine their relative association with the hatching success. In 2010 these predictors did not explain a significant proportion of hatching success variance (Multiple Regression, R = 0.297, $F_{2, 27}$ = 1.11, P = 0.346) while in 2012 the combination of three of the four OTUs showed a significant effect, and two of them showed strong opposite associations with hatching success, Fig. 5). However, the abundance of these OTUs was not different between female treatments (One-way ANOVA, 1248M: $F_{1, 27}$ = 0.004, P = 0.954; 20503M: $F_{1, 27}$ = 0.082, P = 0.776).

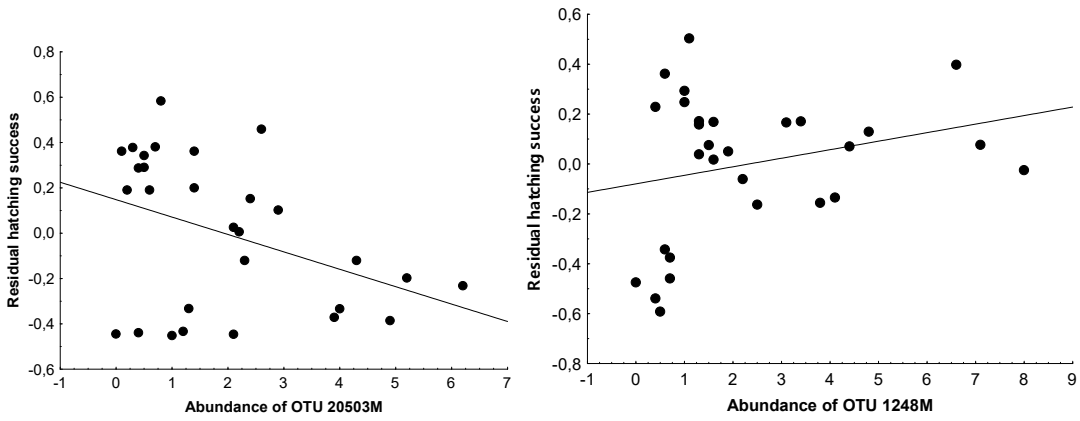

Figure 5. Relationships between the abundance of the two eggshell OTUs that best explain hatching success and the residual hatching success of clutches after correcting for the other predictors retained in a multiple regression model.

DISCUSSION

The present work aimed to determine the effect of the uropygial bacterial contribution to the eggshell microbiome, and the effect of the bacteria that reach the eggshell on hatching success. The experimental manipulation of the female access

to the gland reduced the influence of the uropygial bacteria on the composition of the eggshell microbiome. This is the first time that the whole contribution of the uropygial bacteria to the eggshell community has been experimentally demonstrated, and our results confirm the link between uropygial bacteria and bacteria found in the eggshells suggested in previous work based on culture-dependent techniques for a particular bacterial group (i.e. enterococci) (Martín-Vivaldi *et al.*, 2014). However, the portion of the eggshells' microbiome that was of uropygial origin was not high enough to produce a significant effect of the experiment either in the whole diversity or the structure of the community when considering all the OTUs detected together (10056 OTUs). Nevertheless, a group of 10 of the 100 most frequent OTUs had a presence on eggshells significantly affected by the manipulation of female access to its uropygial gland, suggesting that part of the dominant members of the eggshell microbiome depend on the use of female secretion to smear the eggs. There was, however, a large proportion of the eggshell community coming from other unknown sources. Previous studies with the pied flycatcher (*Ficedula hypoleuca*) have demonstrated that along incubation the eggshell bacterial loads of this species are correlated with the cloacal bacteria of the female (Ruiz-De-Castañeda *et al.*, 2011). In the hoopoe, the influence of the bacteria present in the nest material on the eggshell community has also been experimentally demonstrated (Martínez-García, 2015), and these could be potential origins of the rest of the microbiome found here. As inoculating only two eggs with an external source of bacteria caused all the eggs in the clutch to reach a similar contaminant influence, bacterial dispersal and transmission occurs within the nest environment. These results are agree with those seen by other authors in nest environments (Brandl *et al.*, 2014; Martínez-García, 2015). This process affects equally potential pathogens and beneficial bacteria from any nest source, and in

fact the difference between one and the other may just be a matter of context (Pérez-Brocal *et al.*, 2013). More research may be done in this sense, as knowing the origin of symbiotic and pathogenic bacteria may provide us not only with information about the bacteria acquisition, but also about the evolutionary processes that can lead to the assemblage of complex bacterial communities in hosts.

In a previous study with the same experimental approach used here to impede the female access to the uropygial secretion, a reduction of bacterial input from the uropygial secretion reduced hatching success (Martín-Vivaldi *et al.*, 2014). Individual fitness may be determined by trophic interactions (i.e. predation), climatic influences and food availability among other factors (Iles *et al.*, 2013; Martín-Vivaldi *et al.*, 1999). Moreover, the influence of uropygial bacteria in the eggshell may be masked by other factors directly determining the eggshell bacterial loads (Soler *et al.*, 2015). Here, by means of a contaminated hen egg mixture applied onto the eggs, we produced an increased risk of infection in the hole-nest environment to try to increase the effects of the experimental manipulation, thus highlighting the role of the uropygial secretion bacteria in the eggshell microbiome. In this scenario with increased risk of infection we found a greater influence of the uropygial secretion on the eggshell microbiome, a trend to strains of uropygial source and environmental contaminants to be inversely related, and an effect of particular strains on the hatching success of clutches, showing that the eggshell microbiome is an important component of hoopoes breeding success, and that the uropygial gland loaded with symbionts exert an influence on this community.

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DISCUSIÓN

En la naturaleza los microorganismos aportan numerosos beneficios a los organismos con los que conviven formando simbiosis (Moran, 2006). El papel que los simbiontes pueden tener en la fisiología, la ecología y la evolución de sus hospedadores resulta de vital importancia para mantener unas funciones y relaciones con el entorno adecuadas. En el caso de la abubilla, la presencia de bacterias en su glándula uropigial se ha asociado previamente con beneficios en la protección frente a patógenos (Ruiz-Rodríguez *et al.*, 2009, 2012), que en el momento de la reproducción se traduce en un aumento del éxito de eclosión de los huevos y por tanto en un aumento de su éxito reproductor (Martín-Vivaldi *et al.*, 2014; Soler *et al.*, 2008). Estas bacterias pueden resultar esenciales para entender la ecología y la evolución de la abubilla.

En esta tesis, mediante el uso de diferentes técnicas moleculares (que han demostrado ser una herramienta poderosa en el estudio de comunidades bacterianas complejas; ver sección de INTRODUCCIÓN) se ha descrito por primera vez la comunidad microbiana completa presente en la secreción uropigial de la abubilla. En ese sentido, un primer enfoque basado en librerías de los ADNs ribosomales y/o secuenciación directa de amplicones ha permitido determinar que en la secreción uropigial hay una compleja comunidad bacteriana formada principalmente por microorganismos anaerobios estrictos y facultativos (como cabría esperar de un hábitat cerrado como es la glándula uropigial) que por sus características culturales no habían sido detectadas por los tradicionales métodos de cultivo empleados con anterioridad (CAPÍTULO I). Paralelamente, el uso de técnicas de *fingerprinting* permitió determinar también que esta comunidad era bastante constante en todos los individuos analizados, aunque se encontraron diferencias entre las comunidades de las poblaciones de cría en cautividad y silvestres. Este hallazgo supone que las bacterias de la glándula uropigial deben estar influidas de alguna forma por el modo de vida del ave, y en concreto por factores ambientales como la alimentación o el lugar de invernada (CAPÍTULO I).

La comunidad bacteriana de la secreción uropigial de la abubilla es mucho más compleja de lo que inicialmente se había propuesto. Una segunda aproximación para el estudio de esta comunidad se ha realizado mediante la aplicación de técnicas de secuenciación masiva y utilizando dos tecnologías pirosecuenciación y secuenciación por síntesis y al mismo tiempo estudiar la dinámica poblacional durante el ciclo reproductor del animal, que ya se sabía que cambia en función del sexo y el estado reproductor (Martín-Vivaldi *et al.*, 2009).

Mediante el uso de herramientas como la PCR cuantitativa y FISH hemos determinado la carga microbiana de las secreciones de las hembras y los pollos durante su estancia en el nido tienen, las cuales muestran un concentración mucho mas elevada que las secreciones de las hembras en invierno y fuera del periodo reproductor. En estos últimos casos es similar a la de los los machos (CAPÍTULO II). Se trata de la primera vez que se han descrito poblaciones microbianas en secreciones claras propias de los individuos que no pasan largos periodos en el interior del nido. A pesar de estas diferencias cuantitativas, la secuenciación masiva de amplicones del ADNr 16S ha revelado una composición del microbioma uropigial bastante invariable entre individuos de distintos sexos y condiciones reproductoras durante la estación de cría. El microbioma uropigial, está claramente representado por microorganismos anaerobios estrictos e incluidos en el grupo de los clostridios (filo *Firmicutes*, clase *Clostridia*), aunque también aparecen representantes de otros filos como los de *Proteobacteria*, *Bacteroidetes* y *Actinobacteria* (CAPÍTULO II). Los clostridios forman un grupo microbiano muy heterogéneo de microorganismos, anaerobios estrictos y con gran importancia en el mantenimiento de la homeostasis en sistemas simbióticos (Lopetuso *et al.*, 2013). Son capaces de producir ciertas sustancias con carácter antimicrobiano como ácidos orgánicos de cadena corta y compuestos volátiles (por ejemplo el ácido butanoico) que se había encontrado previamente en altas concentraciones en la la secreción uropigial (Martín-Vivaldi *et al.*, 2010). Estos compuestos, podrían tener también una relación directa en el incremento de tamaño de la glándula uropigial detectado en hembras reproductoras (Lopetuso *et al.*, 2013; Martín-Vivaldi *et al.*, 2009). El hecho de que la composición predominante de las comunidades uropigiales incluya bacterias que también se

encuentran con frecuencia en el hábitat del tracto gastrointestinal (tGI), junto con el hecho de que los microbiomas uropigiales difieran entre poblaciones de abubillas en cautividad y silvestres (que se diferencian principalmente en la alimentación), sugiere una posible colonización de la glándula a partir del tGI del animal, l de al menos parte de la comunidad bacteriana de la secreción (CAPÍTULO II). No obstante, la presencia de bacterias en las secreciones claras sugiere la existencia de un reservorio de microorganismos dentro de la propia glándula uropigial durante todo el año. Las hembras adultas podrían controlar de algún modo (presumiblemente mediante señales hormonales) el crecimiento de estas comunidades de bacterias de forma que las capacidades antimicrobianas de las cepas más beneficiosas sean aprovechadas durante la reproducción. Esta hipótesis estaría apoyada por el hecho de que durante la estación de cría las secreciones de hembras que no están reproduciéndose (pero que aún así han debido sufrir ciertos cambios hormonales propios de la estación) tienen una concentración de bacterias más parecida a la de las hembras que están incubando que a las hembras fuera de la época de cría (CAPÍTULO II). Además de las bacterias predominantes, otras poblaciones minoritarias como los géneros *Vagococcus* y *Parascardovia* podrían desempeñar un papel esencial en la función defensiva de estos simbiontes. Sin embargo esas especies “raras” en la comunidad suelen ser difíciles de detectar por métodos como la pirosecuenciación, como demuestra el hecho de que los enterococos (ampliamente estudiados por su aporte a las propiedades antimicrobianas de la secreción uropigial (Martín-Platero *et al.*, 2006; Ruiz-Rodríguez *et al.*, 2012) no se hayan detectado mediante esta metodología, aunque sí ha sido posible su detección como un grupo poco frecuente mediante FISH a partir de secreciones marrones de hembras y pollos. Por tanto, la secuenciación masiva aún puede requerir de técnicas adicionales para complementar estudios en este sentido (CAPÍTULO II).

La composición del microbioma de la glándula podría verse afectada por factores ambientales causando una estructura cambiante. La posibilidad de efectos de factores tanto espaciotemporales como climatológicos se ha examinado en el CAPÍTULO III. El resultado es una ausencia de influencia de los factores ambientales y geográficos (distancia entre nidos, población,

hábitat, avance de la estación, temperatura, humedad y precipitación) sobre la comunidad bacteriana de la secreción de la glándula uropigial, lo que indica que dicha comunidad es altamente invariable y estable frente a diversos factores ambientales (CAPÍTULO III). Esto implica que la comunidad bacteriana de la glándula uropigial ha debido coevolucionar con su hospedador hasta que ambos se han adaptado íntimamente. De hecho, parece que las hembras reproductoras presentan todas un núcleo de OTUs (*Operational Taxonomic Unit*, unidad taxonómica operacional) que podríamos denominar características de la glándula uropigial, y que son no sólo altamente prevalentes en nuestras poblaciones silvestres, sino que parecen ser bastante abundantes en todas las muestras estudiadas en general. Estos resultados también han sido demostrados por Martínez-García mediante ARISA (Martínez-García *et al.*, 2015). En concreto, 9 de estos OTUs constituyen cerca del 45 % de las secuencias de las muestras de secreción en promedio. Dichas OTUs, una vez más, se han identificado principalmente como bacterias anaerobias, e incluyen a los géneros *Coprococcus*, *Peptoniphilus*, *Parabacteroides* y algunos otros géneros no identificados de las familias *Clostridiaceae*, *Coriobacteriaceae* y *Actinomycetaceae* (CAPÍTULO III). A pesar de que muchas de ellas son anaerobias estrictas (*Coriobacteriaceae*, *Coprococcus*, *Peptoniphilus* y otros representantes de la clase *Clostridia*, así como *Parabacteroides*), aún podrían desempeñar una función en este sistema y en otras localizaciones corporales de la abubilla a las que serían transportadas mediante la secreción junto con sus productos metabólicos (Martínez-García *et al.*, 2015). Tanto los clostridios como *Parabacteroides* son microorganismos capaces de producir sustancias antimicrobianas, ya sean del tipo compuestos volátiles o de naturaleza específica como por ejemplo las bacteriocinas (Arellano *et al.*, 2000; Nakano *et al.*, 2006). Sin embargo el efecto de estas cepas no debe limitarse al aporte de sustancias antagonistas a la secreción uropigial. En concreto en los huevos, la presencia de unas criptas que se llenan rápidamente de secreción tras la puesta implicaría que estas bacterias podrían establecerse en presencia de un ambiente anaerobio donde crecer y ejercer su posible efecto beneficioso en cuanto al éxito de eclosión *in situ* (Martín-Vivaldi *et al.*, 2014; Soler *et al.*, 2008). No obstante, en la secreción uropygial también se han descrito bacterias anaerobias facultativas o incluso aerobias, como *Streptomyces* y algunos

representantes de la clase *Actinomycetaceae*, que podrían sobrevivir en estado latente (por ejemplo formando exosporas) dentro de la glándula, y posteriormente ejercer su función en otros hábitats corporales del ave como las plumas. Curiosamente, las actinobacterias a las que pertenecen estas bacterias son también conocidos productores de sustancias antagonistas (en este caso del tipo antibióticos) y su presencia en simbiosis defensivas ha sido descrita previamente (Visser *et al.*, 2011).

A pesar de que los factores ambientales incluidos en nuestros modelos estadísticos para la variación climatológica y geográfica de la comunidad bacteriana uropigial no dieron resultados significativos, el año si mostró un efecto consistente en la variación natural de dicha comunidad (CAPÍTULO III). En concreto, de los tres años muestreados (2010, 2011 y 2012), las diferencias aparecieron sólo en 2012. Dado que algunas de las variables climáticas de ese año se tuvieron en cuenta en nuestro estudio, las diferencias podrían ser debidas bien a otros factores meteorológicos no tenidos en cuenta en nuestros modelos (radiación solar, viento…) o bien a que dichas variables actúen una escala diferente a la medida en nuestro caso. Otra opción que no se puede descartar como posible fuente de variación en 2012 sería la influencia de los hábitos migratorios de nuestra población silvestre de abubillas (Reichlin *et al.*, 2012). Como ya se propuso en el CAPÍTULO I, la migración puede afectar al microbioma de las aves (Bisson *et al.*, 2009), y por tanto podría determinar cambios anuales en la composición de su comunidad bacteriana.

Aunque se ha descartado una fuerte influencia ambiental con respecto a los factores climáticos estacionales estudiados en el CAPÍTULO III, ya se sabe que la glándula uropigial y su secreción sufre cambios importantes cíclicos en las hembras durante el periodo reproductor, y dichos cambios parecen estar relacionados con la presencia de bacterias (Martín-Vivaldi *et al.*, 2009). Estos cambios no se refieren sólo a la época de cría en si, sino que tienen lugar desde la fase anterior al periodo reproductor hasta una vez completado el intento reproductor. Por tanto para estudiar el efecto de dichos cambios fue necesario estudiar, además de las secreciones oscuras de hembras reproductoras, secreciones blancas tomadas en los días previos y posteriores a la reproducción, en las que ya se había demostrado que la abundancia de

bacterias es al menos diez veces menor a la de las secreciones oscuras propias del periodo de estancia en el nido (CAPÍTULO II). Hemos comprobado que suceden profundos cambios de la comunidad microbiana entre las diferentes fases del ciclo anual incluso dentro de las hembras, llegando a sustituirse casi por completo una comunidad presente fuera del periodo reproductor por otra desarrollada durante la época de cría (CAPITULO IV). Los cambios entre fases son graduales, de forma que dentro del periodo de cría las fases más externas (puesta al principio y fase de volantones al final) también mostraron diferencias con el resto de fases de anidamiento (incubación, eclosión y fase de pollos) (CAPÍTULO IV). Estos cambios se corresponden bastante con los cambios anatómicos detectados en las hembras en las distintas fases, así como la variación en las propiedades de la secreción, por lo que en un futuro sería interesante estudiar los factores fisiológicos que pueden servir para impulsar los cambios microbiológicos por parte del ave. Este enfoque permitiría entender mejor la aparición de estos cambios y la función ecológica de los microbiomas asociados a distintos periodos, y ayudaría a distinguir entre la posibilidad de que los cambios de comunidades bacterianas impliquen existencia de diferentes presiones selectivas entre periodos o simplemente una ausencia de presión selectiva fuera de la época de anidamiento que favorecería el establecimiento de una comunidad menos regulada por el hospedador, como podría sugerir el aumento de diversidad encontrado en las muestras fuera del periodo reproductor.

Los cambios mas importantes que ocurren en el microbioma uropigial de las hembras durante la reproducción implican pasar de una secreción blanquecina en la que predominan las clases Gamma y *Betaproteobacteria* (filo *Proteobacteria*), y se encuentran además *Flavobacteria* (filo *Bacteroidetes*), *Bacilli* y *Clostridia* (filo *Firmicutes*) en menor proporción, a una comunidad claramente dominada por la clase *Clostridia* (filo *Firmicutes*) como las descritas en los capítulos anteriores (CAPÍTULO IV). Además de estos cambios a gran escala, como es lógico ocurre una sucesión de OTUs que aumentan (o disminuyen) relativamente su abundancia en la secreción uropigial a lo largo de los periodos muestreados. Dichas OTUs no son siempre las mismas, y esto hace pensar que podría haber cierta influencia ambiental en la composición de la secreción uropigial, de acuerdo con trabajos previos (Martínez-García, 2015;

Ruiz-Rodríguez *et al.*, 2014), aunque tampoco se descarta la influencia de la microbiota gastrointestinal (CAPÍTULO I) o la presencia de un reservorio de bacterias dentro de la propia glándula (CAPÍTULO II). Posiblemente una conjunción de todos estos factores sea la determinante de la composición final del microbioma uropigial, y conocer el origen de las bacterias que lo componen aportaría una información muy valiosa al estudio de la evolución de la simbiosis entre la abubilla y las bacterias uropigiales.

El principal contexto en el que parece tener importancia la secreción uropigial especial de las hembras de abubilla durante el periodo reproductor se centra en los huevos, sobre los que las hembras depositan activamente la secreción (Martín-Vivaldi *et al.*, 2014; Soler *et al.*, 2015) y en los que han evolucionado desarrollando estructuras y propiedades especiales para retenerla (Martín-Vivaldi *et al.*, 2014). Por ello una cuestión fundamental sobre la diversidad del microbioma de la secreción es conocer su influencia sobre la dinámica bacteriana en la superficie de los huevos. Nuestro experimento impidiendo el acceso de las hembras a su glándula uropigial demostró una reducción en las bacterias que provienen de la secreción uropigial sobre la cáscara de los huevos en los nidos experimentales con respecto a los controles y un efecto claro en un grupo de 10 OTUs, algunos de los cuales fueron más frecuentes en el grupo control, . lo que sugiere cierto efecto antagónico entre las bacterias de procedencia uropigial y otras bacterias que podrían colonizar el huevo (CAPÍTULO V). Esta hipótesis fue comprobada en una estación reproductora posterior, en la que el mismo experimento se aplicó a nidos en los que además se había incrementado el riesgo de infección por patógenos impregnando algunos huevos de cada puesta con una mezcla de huevo de gallina contaminado por exposición a ambientes patogénicos en el campo. Ambos tratamientos tuvieron efecto en las comunidades de la cáscara de los huevos, ya que en todos los nidos se encontró cierta influencia de bacterias provenientes del contaminante y prácticamente sólo en los controles apareció cierta influencia de bacterias uropigiales. En el primer caso, indirectamente hemos demostrado cierta transmisión de bacterias en el entorno del nido, de acuerdo con trabajos previos (Brandl *et al.*, 2014; Martínez-García, 2015), ya que tanto los huevos tratados como los no tratados presentaron niveles de

diversidad y composiciones de la comunidad bacteriana bastante similares (CAPÍTULO V). De nuevo se detectó una tendencia de efectos antagónicos de las bacterias de la secreción hacia las cepas provenientes del contaminante en la cáscara de los huevos cuando la influencia de la secreción aumentaba sobre cierto umbral (CAPÍTULO V). También se ha detectado el efecto directo de algunas cepas sobre el éxito de eclosión y por tanto el éxito reproductor de la abubilla, incluyendo tanto efectos positivos como negativos. Estos resultados muestran que las bacterias de la secreción uropigial en su conjunto pueden ser transmitidas y establecerse sobre la superficie de los huevos, de forman que podrían desplazar a otras bacterias potencialmente patógenas. Por otra parte el microbioma establecido en la cáscara de los huevos, independientemente de la procedencia de las cepas que lo conforman es un componente importante del éxito reproductor de la abubilla. Futuras investigaciones en este sentido serán de vital importancia para determinar no sólo qué bacterias específicas colonizan la cáscara de los huevos y su procedencia (algunas aproximaciones recogidas en Martínez-García, 2015), sino también las funciones concretas que dichas bacterias pueden llevar a cabo en dicho ambiente, y la confirmación de su relación con el éxito reproductor del ave (sugeridas para la porción cultivable de las bacterias uropigiales en Martín-Vivaldi *et al.*, 2014; Soler *et al.*, 2008), encontrada también aquí para otras no cultivables, que consolidaría nuestro conocimiento acerca de este sistema simbiótico.

El trabajo recogido en esta tesis ha permitido avanzar de manera notable en el conocimiento disponible sobre la composición taxonómica y la dinámica de la comunidad bacteriana asociada a la glándula uropigial de la abubilla (CAPÍTULOS I a IV), y ha puesto de manifiesto la interacción entre dos microbiomas de gran importancia para el ave (los de glándula y huevos) indicando que esta interacción es probablemente el contexto principal en el que se ha desarrollado y en el que se mantienen las presiones selectivas que explican este sistema ave-bacterias (CAPÍTULO V). Los resultados también abren nuevas posibles líneas de investigación para avanzar en el conocimiento de esta interacción, sobre todo al identificar los taxones candidatos a jugar un papel importante en esta simbiosis, y muestran que los diferentes métodos de estudio de las comunidades microbiológicas pueden aportar información

complementaria de gran importancia, especialmente cuando algunas especies clave pueden no ser las más abundantes y así pasar desapercibidas a aproximaciones globales como la secuenciación masiva.

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CONCLUSIONES GENERALES

1) El uso de dos técnicas de *fingerprinting*: TTGE y ARISA, ha permitido establecer la existencia de un perfil genético característico en la comunidad bacteriana de la secreción uropigial de la abubilla con muy poca variación entre los individuos que están en fase reproductora.

2) Mediante el uso de herramientas como la PCR cuantitativa y el FISH hemos determinado que las secreciones de las hembras y los pollos durante su estancia en el nido tienen una carga microbiana más elevada que las secreciones de las hembras en invierno y de los machos durante todo el año.

3) La caracterización completa del microbioma de la secreción de la glándula uropigial de abubillas en la época reproductora usando técnicas de secuenciación masiva (pirosecuenciación y secuenciación por síntesis) ha revelado una elevada diversidad bacteriana. Este complejo microbioma está mayoritariamente representado por microorganismos anaerobios estrictos y pertenecientes al grupo de los clostridios (filo *Firmicutes*, clase *Clostridia*). Además de este grupo mayoritario, se han encontrado poblaciones de los filos *Proteobacteria*, *Bacteroidetes* y *Actinobacteria,* aunque en proporciones inferiores.

4) La importancia relativa de los distintos filos encontrados varía a lo largo del ciclo reproductivo. Así, en el periodo previo a la reproducción, los

microorganismos más abundantes en la glándula uropigial de las hembras pertenecen a los filos *Proteobacteria*, *Bacteroidetes* y *Firmicutes* (clases *Bacilli* y *Clostridia*). Conforme avanza el intento reproductor, las poblaciones de los dos primeros grupos van disminuyendo progresivamente frente al de *Clostridia*, volviendo de nuevo a las proporciones del estadío pre-reproductor una vez que los pollos abandonan el nido. Esta dinámica cíclica del microbioma de la glándula sugiere la existencia de presiones selectivas que favorecen un cambio de funcionalidad del sistema durante la estancia de la hembra en el nido.

5) Aunque el perfil genético del microbioma de la secreción de las hembras durante esa fase es bastante uniforme, se han detectado variaciones en su composición relacionadas con la cría en cautividad y el año de muestreo. Sin embargo no se ha encontrado ningún efecto de factores ambientales geográficos, ni de variables meteorológicas.

6) Se ha demostrado experimentalmente que la microbiota de la secreción uropigial de la abubilla contribuye a la composición de la comunidad bacteriana establecida en la superficie de la cáscara de los huevos. A partir de cierto umbral, esta contribución puede resultar beneficiosa en cuanto que se relaciona con una disminución en el nivel de colonización del huevo por bacterias potencialmente patógenas provenientes de una fuente experimental de contaminación.

7) La abundancia de algunas de las cepas bacterianas presentes en la cáscara de los huevos está asociada al éxito de eclosión de la puesta, incluyendo efectos positivos y negativos. La identificación de estas cepas y muchas otras presentes en esta comunidad que pertenecen a grupos habitualmente involucrados en simbiosis mutualistas entre animales y bacterias, va a permitir abordar el estudio de sus dinámicas particulares y su funcionalidad, para así avanzar en la comprensión del funcionamiento y la evolución de este sistema.

CONCLUSIONS

1) The usage of two fingerprinting techniques: TTGE and ARISA, allowed to establish the existence of a genetic profile that characterizes the bacterial communities of the uropygial secretion of the hoopoe, which show very little variation among reproductive individuals.

2) The use of techniques such as qPCR and FISH yielded the conclusion that female and chick secretions while nesting have higher microbial loads than female secretions in winter and male secretion all over the year.

3) The whole characterization of the microbiome of the uropygial gland of hoopoes during breeding by means of high-throughput techniques (pyrosequencing and sequencing by synthesis) has shown a high bacterial diversity. This complex microbiome is mainly represented by strict anaerobic microorganisms within the clostridia group (phylum *Firmicutes*, class, *Clostridia*). In addition to this main group, populations of the phyla *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* have been found, but in less proportion.

4) The relative importance of the different phyla found varies along the reproductive cycle. This way, during the pre-breeding phase the more abundant microorganisms in the uropygial gland of females belong to the phyla *Proteobacteria*, *Bacteroidetes* and *Firmicutes* (classes *Bacilli* and *Clostridia*). As the breeding attempt begins, the populations of the first two

groups start a progressive decrease against *Clostridia*, going back to the proportions of the pre-breeding phase once the nestlings leave the nest. This cyclic dynamics of the gland microbiome suggests the existence of selective pressures that favor a functional change of the system during the stay of the female within the nest.

5) Even though the genetic profile of the female secretion microbiome during this phase is quite uniform, variations in its composition related with captive breeding and year of sampling have been detected. However, an effect of geographical environmental factors or meteorological variables has not been found.

6) It has been experimentally demonstrated that the microbiota of the hoopoe uropygial secretion contributes to the composition of the bacterial community established on the eggshell surface. Starting at a certain threshold, this contribution may be beneficial as it is related to a reduction on the level of egg colonization by potential pathogenic bacteria from an experimental source of contamination.

7) The abundance of certain bacterial strains in the eggshells is related to hatching success of the clutch, including both positive and negative effects. The identification of these strains and much others within this community (pertaining to groups commonly involved in mutualistic symbioses between animals and bacteria) is going to let the study of their particular dynamics

and their functionality, thus allowing an advance in the understanding and evolution of this system.