Simbiosis entre aves y bacterias. Propiedades y modo de adquisición de la microbiota intestinal de críalos y urracas, y de la existente en la glándula uropigial de abubillas

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Simbiosis entre aves y bacterias. Propiedades y modo de adquisición de la microbiota intestinal de crías y urracas, y de la existente en la glándula uropigial de abubillas

Memoria presentada por Mª Magdalena Ruiz Rodríguez para optar al Grado de Doctora en Ciencias Biológicas por la Universidad de Granada.

Los directores

Juan J. Soler **Manuel Martín-Vivaldi**

La tutora La doctoranda

Carmen Zamora Muñoz **Mª Magdalena Ruiz Rodríguez**

Granada, 2007

"Una bacteria pesa $1x10^{-10}$ gramos. Una ballena azul pesa $1x10^{8}$ gramos. Pero una bacteria puede matar una ballena… tal es la adaptabilidad y versatilidad de los microorganismos comparados con los llamados "organismos superiores" que seguirán sin duda colonizando y alterando la superficie de la tierra mucho tiempo después de que el resto de habitantes hayan desaparecido para siempre". **Bernard Dixon**

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Acuarelas portada: **Sara C. Sánchez Martínez**

A mis padres y a Mª Jo

A Rafa

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CONTENIDOS

MANUSCRITOS INDIVIDUALES

RESUMEN

El objetivo principal de esta tesis es profundizar en el conocimiento de las interacciones entre aves y microorganismos en condiciones naturales desde una perspectiva ecológica y evolutiva. Para ello, hemos estudiado (i) relaciones en las que las bacterias puedan proporcionar un beneficio a su ave hospedadora, y (ii) la influencia del ambiente en la adquisición, por parte de aves salvajes, de bacterias simbiontes.

De acuerdo con la importancia de la microbiota intestinal en el desarrollo de los pollos en aves nidícolas, encontramos que la diversidad bacteriana de la cloaca en pollos de críalos (*Clamator glandarius*) y urracas (*Pica pica*) está relacionada con la condición física al abandonar el nido y con la respuesta inmune a un antígeno inocuo (PHA) respectivamente, en las que estudios previos demostraron relación con la probabilidad de supervivencia y reclutamiento.

En las abubillas (*Upupa epops*) hemos trabajado en la novedosa relación con enterococos que colonizan la glándula uropigial de hembras y de pollos durante su estancia en el nido. En esta época la secreción que estos producen, en comparación con la de machos, se vuelve oscura y con un fuerte mal olor. De esta secreción oscura, pero no de la blanca (de hembras no reproductoras y de machos), hemos aislado enterococos productores de bacteriocinas (péptidos antimicrobianos), y además hemos comprobado la actividad antimicrobiana directamente de la secreción. Al aislar las colonias y comprobar su actividad antimicrobiana por separado, detectamos una variación en dicha actividad ligada al perfil genético de la cepa.

Estudiamos además la posible influencia de las bacterias aisladas de la glándula sobre sus características y las de la secreción que produce, así como los posibles beneficios que dichas bacterias proporcionarían al hospedador a través de la producción de bacteriocinas inhibidoras del crecimiento de patógenos microbianos. Hemos detectado mediante microscopía a los enterococos en determinados compartimentos de la glándula uropigial, y además, los resultados de la inyección experimental de antibióticos para eliminar a los enterococos de la glándula sugieren que los cambios de tamaño que se producen en la glándula y en las

propiedades de su secreción, tanto de las hembras como de pollos, están mediados por la presencia de estas bacterias. Mediante tests *in vivo* e *in vitro*, hemos comprobado un beneficio directo de las bacteriocinas sobre el éxito de eclosión (directamente relacionado con el éxito reproductor) y sobre inhibición de la actividad queratinolítica en bacterias degradadoras de plumas. Todos estos resultados sugieren que las abubillas y los enteroccos que habitan en sus glándulas uropigiales mantienen una relación mutualista con beneficios para el ave en forma de defensas frente a patógenos.

En la adquisición y establecimiento de la microbiota digestiva influyen factores tanto ambientales como filogenéticos. En aves nidícolas, la adquisición de bacterias depende de los aportes alimenticios de los padres, por lo que analizando la microbiota intestinal de pollos de dos especies filogenéticamente muy alejadas pero criadas en el mismo ambiente (nido) se podría estudiar el componente filogenético de las comunidades bacterianas del digestivo. Este estudio lo realizamos en el sistema parasito de cria – hospedador formado por la urraca y el críalo. De acuerdo con la existencia de un componente filogenético, hemos encontrado que, aunque no existen taxones bacterianos específicos, la microbiota intestinal de los pollos de ambas especies difiere. Además, comprobamos diferencias interespecíficas en la anatomía intestinal que podría influir en la implantación de distintas bacterias. Debido a que la microbiota intestinal está directamente implicada en el aprovechamiento energético de la comida, las diferencias interespecíficas detectadas podrían tener consecuencias en el aprovechamiento energético del alimento por los pollos parásitos y, por tanto, en la cantidad necesaria para un desarrollo óptimo de los pollos parásitos.

Por otro lado, estudiamos también la importancia del ambiente en la adquisición de las bacterias de la glándula uropigial de la abubilla. En la mayoría de los individuos analizados, se encontró que tenían cepas similares, y además, pollos del mismo nido compartían la misma cepa o cepas muy similares de la bacteria simbionte. Mediante experimentos de intercambio de pollos de 2-3 días entre nidos, aun con la glándula sin desarrollar, pusimos de manifiesto la influencia del nido (tanto en el que habían nacido como en el que se criaron) en la adquisición de las cepas de bacterias simbiontes detectadas en sus secreciones uropigiales.

ABSTRACT

The goal of this PhD is to increase the knowledge of the interactions between birds and microorganisms under natural conditions from an ecological and evolutionary point of view. To do that, we studied (i) relationships in which bacteria could provide a benefit to their avian hosts, and (ii) the influence of the environment in the acquisition of symbiotic bacteria by wild birds.

Considering the importance of the gut microbiota in the nestling development, we found in great spotted cuckoos (*Clamator glandarius*) and in magpies (*Pica pica*) that the physical condition and cell-mediated immune response to an inocuous antigen (PHA) (two factors that previous studies found to be related to the survival and recruitment probability of nestlings at fledging) is related to their bacterial communities respectively.

In hoopoes (*Upupa epops*), we investigated a novel research idea about the mutualistic relationship between females and nestlings focusing on enterococci that colonize the uropigial gland during their nesting stage. In the breeding season, the uropygial secretion produced by nestlings and females become dark and stinky compared to that of the males. From this dark secretion (but not from the white one of males or of non-breeding females outside of the breeding season), we have isolated enterococci, which produce bacteriocins (antimicrobial peptides), and we also proved the antimicrobial activity directly from the secretion. We isolated and tested each colony activity, and detected a variation of the antimicrobial activity which was related to the strain genetic profile.

We studied the influence of those bacteria over the gland and its secretion characteristics, and the hypothetical benefits that those bacteria could provide to their hosts through the production of bacteriocins that could inhibit the growing of pathogen microorganisms. By using the microscope, we have detected the enteroocci in some sections of the gland. Moreover, after experimentally injecting antibiotics to eliminate the enterococci, our results suggest that bacteria are responsible for some of the changes of the gland and its

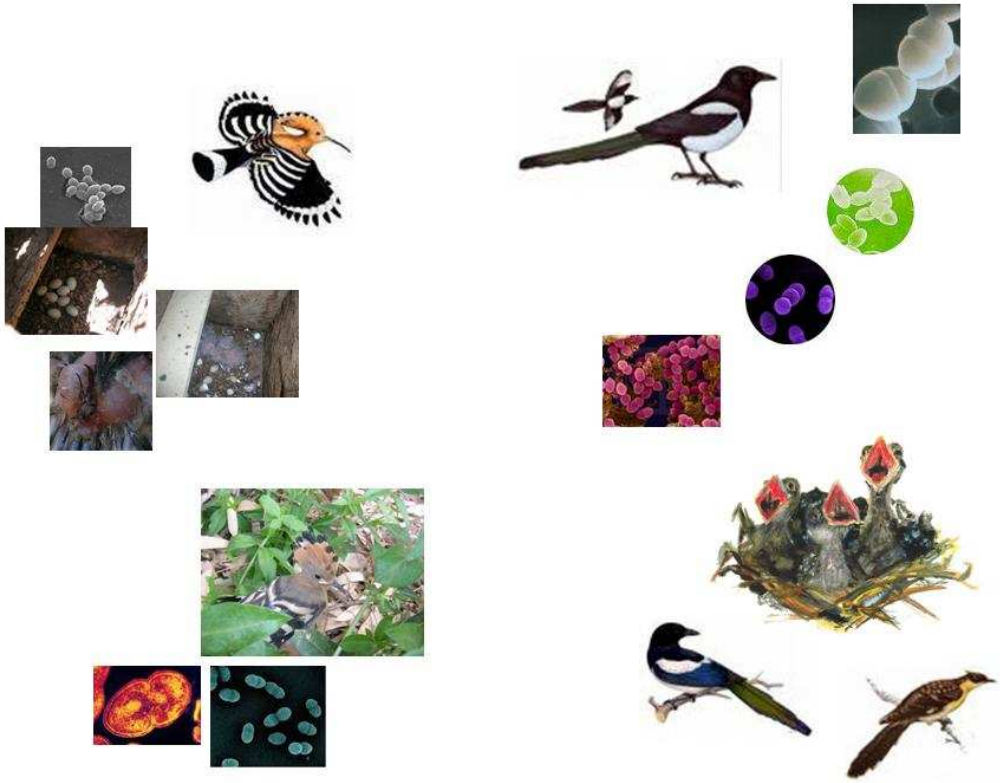
secretion characteristics in both nestlings and females. After performing tests *in vivo* and *in vitro*, we found a direct benefit of the bacteriocins for the hatching success (directly related to the reproductive success) and the inhibition of the keratinolytic activity of feather-degrading bacteria. Those results suggest that hoopoes and bacteria living in their uropygial glands maintain a mutualistic relationship which benefit to the birds by defending them from pathogens.

Environmental and phylogenetic factors are involved in the acquisition and establishment of the gut microbiota. In altricial birds, bacteria acquisition depends also in the food carried by their parents. Therefore, by analyzing the intestinal microbiota in two phylogenetically distant species, but reared in the same environment (i.e., the nest), the genetic component of the bacterial communities in the digestive tract could be studied. We performed this work in the brood parasite-host system which comprises of the magpie and the great spotted cuckoo. According to the existence of the genetic component, we found that, although there are no species-specific taxons, the gut microbiota is different in the two species. Moreover, we found interspecific differences in the digestive tract anatomy, which could influence the establishment of several bacteria.Due to that the intestinal microbiota is directly implicated in the energetic use from food, interspecific differences detected could have consequences in the energety obtained by the parasite nestlings and, therefore, in the amount of food necessary for an optimal development of parasites.

Finally, we studied the importance of the environment in the acquisition of bacteria from the uropygial gland in hoopoes. Most individuals have the same strains, while nestlings sharing the nest also share the same strains of symbiotic bacteria. By cross-fostering experiments with 2-3 days old nestlings, the urpygial gland of which is yet not developed, we found an influence of the original and rearing nests in the acquisition of their symbiotic bacterial strains living in their uropygium.

INTRODUCCIÓN

GENERAL



INTRODUCCIÓN

Las interacciones entre organismos y en particular los procesos coevolutivos han jugado un papel fundamental en la diversificación y organización de la vida, siendo el principal motor de la evolución y responsable de la biodiversidad existente en el planeta (Thompson 1999a). De hecho, las principales presiones selectivas a las que están sujetos los organismos vivos son diversos factores bióticos que incluyen parasitismo y depredación, pero también mutualismos en los que organismos simbiontes (es decir, que conviven estrechamente con el hospedador) favorecen de una u otra manera el desarrollo, supervivencia y reproducción de otros seres vivos. Estas relaciones entre organismos de la misma o distinta especie dan lugar a procesos coevolutivos, definidos como los cambios evolutivos recíprocos que ocurren entre especies o grupos de individuos como consecuencia de sus interacciones (Thompson 1986).

Los procesos coevolutivos no suelen ser estáticos. Por el contrario, las relaciones entre las partes se parecen a una "carrera de armamentos" en la que las modificaciones de uno inducen cambios en el otro. Cambios que a su vez provocan más cambios en el primero, y así se van moldeando alianzas o relaciones ecológicas entre especies que pueden ser filogenéticamente muy distantes (Thompson 1998). La idea de coevolución apareció reflejada por primera vez en el "Origen de las Especies" de Darwin (1859), quien al hablar de plantas e insectos polinizadores utilizó el termino "coadaptación". Fue a partir del trabajo de Flor (1942) sobre patógenos en plantas cuando comenzaron los estudios sobre los cambios evolutivos recíprocos y, en 1958, Mode desarrolló el primer modelo matemático utilizando el término "coevolución". Su uso aumentó a partir del trabajo de Ehrlich & Raven (1964) sobre plantas e insectos que se alimentan de ellas, pero fue Janzen (1980) quien proporcionó la primera definición restrictiva del proceso de coevolución. Según este autor, el proceso coevolutivo se define por las siguientes características: (1) *Especificidad*, la evolución del carácter se debe a presiones selectivas que impone el caracter de la otra especie, (2) *Reciprocidad*, ambos caracteres

deben evolucionar conjuntamente, y (3) *Simultaneidad*, ambos caracteres evolucionaron al mismo tiempo.

Los procesos de coevolución tienen lugar tanto en **relaciones antagonistas**, como ocurre por ejemplo entre depredadores y sus presas o entre parásitos y sus hospedadores, como en **relaciones mutualistas**, como la que tiene lugar entre las plantas con sus polinizadores. A su vez, los procesos coevolutivos pueden ser **difusos** si las interacciones se dan conjuntamente entre multitud de especies, o **restrictivos** si los procesos ocurren entre dos o pocas especies. Por otro lado, las relaciones coevolutivas suelen no ser simétricas en el sentido de dependencia de una especie de otra (Bascompte *et al* 2006), y un mismo organismo puede mantener relaciones coevolutivas estrechas con más de una especie, como es el caso de algunas plantas leguminosas que presentan estrechas y sofisticadas relaciones con rizobios bajo tierra y, al mismo tiempo, sus flores las presentan con insectos polinizadores (Thompson 1999a).

Debido al papel principal de las interacciones entre especies y los procesos coevolutivos en la evolución de la biodiversidad, la comprensión de su dinámica es de gran interés en biología evolutiva. Por otra parte, actualmente, la influencia del hombre sobre los ecosistemas terrestres es más que evidente y, en parte debido a sus efectos en las interacciones entre organismos, los efectos sobre la biodiversidad y las relaciones coevolutivas que se dan en muchos de los ecosistemas terrestres pueden ser devastadores. Además, el conocimiento de estos procesos en general y de aquellos que tienen lugar entre parásitos y hospedadores en particular, podrían ser aplicados en vigía de la conservación y en intentos de solucionar algunos problemas contemporáneos como la resistencia a antibióticos de algunas cepas bacterianas patógenas, o a la lucha biológica contra plagas y enfermedades (Thompson 1999b).

LOS MICROORGANISMOS Y SU IMPORTANCIA EN PROCESOS DE COEVOLUCIÓN

Debido a la ubicuidad y abundancia de los microorganismos, los organismos superiores están constantemente en contacto, y por tanto interactuando con ellos. Los microorganismos aparecieron hace más de 3.7 billones de años, y se definen como aquellos organismos que miden menos de 0.1 mm de diámetro (Jessup *et al*. 2004). Incluyen a procariotas y virus, pero la

mayoría pertenecen al reino Bacteria, considerado como una importante fuerza selectiva que ha actuado sobre la evolución de los organismos eucariotas (Xu & Gordon 2003). Es en el estudio de este grupo de procariotas y sus interacciones con aves donde se ha centrado el trabajo llevado a cabo en esta tesis.

Como consecuencia de las continuas interacciones eistentes entre microorganismos, éstos han desarrollado mecanismos para excluir a sus competidores consistentes en la segregación de compuestos antibióticos que impiden el crecimiento de cepas competidoras (Riley & Wertz 2002 a y b). Este mecanismo es aprovechado por algunos organismos eucariotas con los que mantienen relaciones ecológicas y/o coevolutivas los cuales, gracias a las sustancias antibióticas producidas por sus organismos simbiontes, disminuyen la colonización por microorganismos patógenos (por ejemplo en hormigas (Currie *et al*. 1999), o en calamares (Barbieri *et al*. 1997)). El estudio de las interacciones coevolutivas podría por tanto tener un enfoque aplicado, ya que los microorganismos son importantes candidatos para la "bioprospección", práctica que consiste en la búsqueda de nuevos compuestos biológicos que puedan usarse tanto en la medicina como en la industria (Horner-Devine *et al*. 2004). Según Keller & Zengler (2004), hay aún un reservorio enorme e inexplorado de compuestos naturales que podrían usarse como fuente para el desarrollo de nuevos medicamentos.

Las bacterias constituyen una importante herramienta de estudio de procesos evolutivos debido, entre otras razones, al corto tiempo de generación, a la gran diversidad existente y a la facilidad de manejo de los cultivos de miles o millones de individuos (Steinert *et al*. 2000), que permite apreciar cambios en las frecuencias génicas y adaptaciones a condiciones ambientales en un corto espacio de tiempo.

Además, son muchas las bacterias que viven en **simbiosis** con otros organismos hospedadores, dando lugar a procesos coevolutivos más o menos estrechos entre ellos. Estos organismos les proporcionan las condiciones óptimas de pH, temperatura y presión osmótica para su desarrollo (Madigan *et al*. 2003). Desde el punto de vista del hospedador, dependiendo de las ventajas o inconvenientes que le puede suponer la convivencia con el simbionte, existe una gradación en el tipo de relación (simbiosis) que mantiene con los microorganismos. Los

efectos que estas relaciones tienen en los hospedadores pueden oscilar desde un extremo en el que existe un alto beneficio mutuo (**mutualismo**), hasta el otro extremo con elevados efectos negativos para el hospedador (**parasitismo o antagonismo**) (Steinert *et al*. 2000). Existe un término intermedio (**comensalismo**) en el que se considera que los efectos de la convivencia son neutros o de poca relevancia para el hospedador. Sin embargo, los límites entre los tres tipos de simbiosis son bastante difusos y, además, variaciones en determinadas variables ambientales pueden hacer que el balance entre costos y beneficios de la relación oscile, pudiendo pasar por ejemplo de relaciones típicamente comensales a relaciones de parasitismo (Hoeksema & Bruna 2000; Gomulkiewicz *et al*. 2003) (ver Fig. 1). La relación antagonista-mutualista puede incluso variar entre los mismos taxones en distintas áreas geográficas o dependiendo de condicionantes ambientales (Thompson 1998).

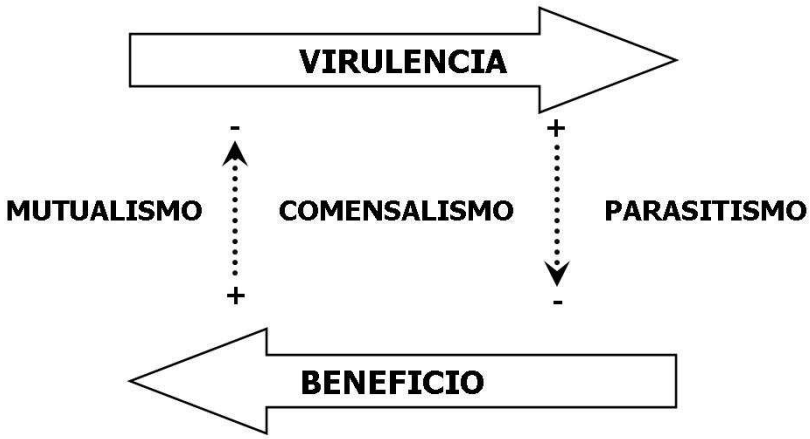


Fig. 1. *Esquema del tipo de relación simbiótica según los efectos que cause el microorganismo sobre el hospedador. Modificado de Steinert et al. (2000).*

Esta labilidad en el grado de la interacción es muy característica de algunas especies de bacterias intestinales, las cuales en condiciones normales forman parte de la microbiota intestinal típica del hospedador colaborando en la función digestiva, pero que ante determinados cambios ambientales pueden actuar como patógenas oportunistas (Fudge 2001). De cualquier forma, aunque los límites entre los distintos tipos de simbiosis no son claros, la

discriminación entre asociaciones mutualistas y antagonistas (parasíticas) tiene claras ventajas ya que permite la distinción entre procesos evolutivos que favorecen o previenen las asociaciones simbióticas.

Relaciones parasíticas

En general, el parasitismo se considera una fuerza selectiva de primer orden que influye, por ejemplo, en la evolución de las estrategias vitales de las especies, en la elección de pareja y en otros procesos de selección sexual (Hamilton & Zuk 1982, Møller *et al*. 1999), así como en la selección de nutrientes (Price *et al*. 1986).

Son numerosos los ejemplos de bacterias patógenas en la naturaleza. Por ejemplo en aves, *Salmonella sp*. puede causar graves trastornos intestinales, mientras que *Mycobacterium avium* puede producir tuberculosis (Friend & Franson 1999). Concretamente en aves silvestres, tras examinar las causas de muerte en 116 fringílidos, alrededor del 90% resultaron deberse al contagio de *Salmonella typhimurium* y *Escherichia coli* (Pennycott *et al*. 1998; Pennycott *et al*. 2002). Además, Pinowski *et al*. (1994) pusieron de manifiesto que la causa de muerte de embriones en huevos de gorrión se debía en un 70% a infecciones causadas por microorganismos como *E. coli* o *Staphylococcus epidermitis.* Por otro lado, los efectos de algunas bacterias descomponedoras de queratinas afectan al desarrollo y características de las plumas, por lo que estas bacterias pueden tener mucha importancia en la evolución del grupo (Muza *et al*. 2000).

El principal mecanismo defensivo contra patógenos es el sistema inmunitario, de donde evolucionó como consecuencia directa de las presiones selectivas ejercidas por parásitos (Wakelin 1996). Es un sistema dinámico en el que la mayoría de las células que constituyen la respuesta inmunitaria pueden migrar a los tejidos donde se requiere su acción para aislar o eliminar a los agentes patógenos (Pruett 2003). Consta de un componente innato formado por células fagocitarias y algunos anticuerpos naturales (que constituyen un sistema de alerta ante cualquier tipo de infección), y un componente adquirido formado por linfocitos T (generalistas, que reaccionan ante la presencia de sustancias extrañas) y linfocitos B (específicos), que ajustan su respuesta al tipo de patógeno gracias a procesos de "memoria antigénica" (Roitt *et al*. 2001).

Aunque las principales defensas antibacterianas son las respuestas inmunitarias adquiridas, otras sustancias menos específicas que forman parte del sistema inmunitario innato constituyen la primera barrera frente al contagio bacteriano. Entre estas, se destacan las angiogeninas (Hooper *et al*. 2003), las β-defensinas (Sugiarto & Yu 2004) y las lisocimas, que también pueden ser transferidas a la descendencia, antes del desarrollo del sistema inmune del propio embrión, aumentando de esta forma las posibilidades de supervivencia durante el desarrollo embrionario (Saino *et al*. 2002). Además de estas sustancias, existen otras producidas por glándulas exocrinas (células epiteliales que liberan su secreción al exterior, nunca al torrente sanguíneo) con propiedades antibióticas frente a bacterias y hongos patógenos. Algunos ejemplos son el caso de las glándulas sebáceas en humanos (Marples 1969), las glándulas metapleurales de hormigas cortadoras de hojas (Bot *et al*. 2002), o la glándula uropigial de las aves (Shawkey *et al*. 2003).

También, algunos animales utilizan sustancias producidas por otros organismos en su defensa frente a patógenos. Esta práctica está ampliamente extendida en la naturaleza y, cuando se excluyen sustancias inorgánicas, se define como **automedicación** (Newton 1991; Clayton & Wolfe 1993; Lozano 1998). Esta medicación, o la adquisición de sustancias defensivas, puede ocurrir a través de la ingesta de alimentos, generalmente plantas, cuyos productos del metabolismo secundario tienen propiedades antibióticas (ver ejemplos en Lozano 1998). Estos productos exógenos también se pueden utilizar directamente en los nidos como hacen las hormigas de la madera (*Formica paralugubris*) (Christe *et al*. 2003) y los estorninos (*Sturnus vulgaris*) (Clark & Mason 1985; Clark 1990), o bien aplicándose la sustancia en el tegumento como los coatís, que se untan con resina de árboles (Gompper & Hoylman 1993), o algunas aves que lo hacen con hormigas (conocido como "anting", Ehrlich *et al*. 1986).

El uso de sustancias exógenas producidas por otros organismos se podría considerar incluido dentro de un conjunto de comportamientos de higiene que reducen la probabilidad de sufrir parasitismo en general o contagio bacteriano en particular. Estos comportamientos han sido revisados en varios artículos y monografías (Hart 1990; Hart 1992; Moore 2002), como por ejemplo los baños de sol o de arena (Clayton 1999), la evitación de vectores o de individuos contaminados, y la desparasitación o acicalamiento. El comportamiento de acicalamiento en

aves es muy importante ya que, por un lado implica la eliminación física de los parásitos de la piel y el plumaje (Clayton 1991) y, por otra, implica en muchos casos la utilización de secreciones de la glándula uropigial que, como hemos expuesto anteriormente, pueden poseer sustancias que inhiben el crecimiento bacteriano.

Relaciones mutualistas

Las relaciones mutualistas entre bacterias y animales están muy extendidas en la naturaleza, siendo las principales ventajas que las bacterias simbiontes proporcionan a sus hospedadores el suministro de nutrientes o de sustancias defensivas. Quizás la más común sea la existente entre las comunidades bacterianas intestinales y sus hospedadores. Estas bacterias proporcionan a los animales alimento directamente o a través de la digestión de macromoléculas no aprovechables por el hospedador. El otro tipo de mutualismo se refiere a las relaciones con bacterias que, mediante producción de sustancias químicas, proporcionan a sus hospedadores defensas frente a depredación o infección por organismos patógenos. A continuación se analizan con más detalle las implicaciones de cada uno de estos dos tipos de sistemas y de la dinámica de estos dos tipos de mutualismos con bacterias.

Bacterias intestinales y su importancia en procesos evolutivos

La función nutricional que muchas de las bacterias mutualistas desempeñan en sus hospedadores se basa en la transferencia de moléculas de bajo peso molecular, previamente digeridas por los microorganismos, y que son esenciales para el hospedador. De hecho, algunos de los nutrientes necesarios en animales no se obtienen directamente de la comida, han de ser procesados previamente por la microbiota intestinal. Por lo tanto, la efectividad de la comunidad bacteriana en la digestión y absorción de energía está directamente relacionada con la condición fenotípica del individuo (ver por ejemplo Glunder 2002; Moreno *et al*. 2003; Xu *et al*. 2003; Engberg *et al*. 2004). Otro papel importante que desempeñan las bacterias intestinales es el de protección frente a patógenos, ya que constituyen una barrera protectora a nivel intestinal

que impide la colonización por microorganismos patógenos invasores mediante mecanismos de exclusión bacteriana principalmente (Price *et al*. 1986).

Hasta el momento existen muy pocos estudios sobre la importancia de la microbiota intestinal en animales salvajes. En el caso de los vertebrados, es bien conocida la relación simbiótica que mantienen los mamíferos herbívoros con las bacterias del rumen (revisado en Kamra 2005). En aves silvestres, se han detectado experimentalmente efectos negativos (Potti *et al*. 2002) y positivos (Moreno *et al.* 2003) de algunas bacterias intestinales en el crecimiento de los pollos. También se han descrito relaciones entre la presencia de determinadas bacterias y la calidad fenotípica de los pollos antes de abandonar el nido (por ejemplo en Mills *et al*. 1999), pero no está claro hasta qué punto esas comunidades bacterianas son determinantes del éxito (probabilidad de reclutamiento) de los individuos que las albergan. Un aspecto que se sabe que afecta a la probabilidad de reclutamiento tanto de los adultos (Soler *et al*. 1999a) como de los volantones de aves salvajes (Cichon & Dubiec 2005; Moreno *et al*. 2005) es su capacidad de respuesta inmune medida como el nivel de respuesta de los linfocitos T. Por otro lado, varios estudios con otros modelos de animales predicen una relación entre características de la comunidad bacteriana intestinal y la capacidad de respuesta inmune (Cebra 1999, Umesaki y Setoyama 2000, Lanning *et al*. 2000, 2005, revisado en Lombardo 2007), por lo que la asociación entre microbiota bacteriana y eficacia biológica del hospedador quedaría establecida. Sin embargo esa hipótesis no se ha comprobado en aves salvajes.

Bacterias mutualistas como defensas frente a patógenos y depredadores

Algunas bacterias proporcionan a sus hospedadores protección frente a depredadores y parásitos. Algunos ejemplos de estas funciones los encontramos en el caso de bacterias asociadas al calamar *Loligo pealei*, que aportan protección a sus huevos frente a patógenos bacterianos (Barbieri *et al*. 1997); o las bacterias que proporcionan resistencia a parasitoides en áfidos (Wernegreen & Moran 2000). Quizás, el ejemplo más conocido de simbiosis de este tipo entre invertebrados y bacterias tiene lugar entre las hormigas cortadoras de hojas, que conviven con una bacteria simbiótica (género *Pseudonocardia*) que produce un antibiótico capaz de inhibir

el crecimiento de un hongo (género *Escovopsis*) que parasita su alimento y destruye por completo sus colonias (descrito en Currie *et al*. 1999). Otro ejemplo son bacterias en secreciones orales de escarabajos, que inhiben el crecimiento de hongos patógenos (Cardoza *et al*. 2006).

Aunque todos los ejemplos conocidos de este tipo de mutualismo corresponden a relaciones con animales invertebrados, los vertebrados también son buenos candidatos a desarrollar asociaciones con bacterias similares a las encontradas en hormigas, pulgones o escarabajos como las citadas arriba. Por ejemplo, las glándulas exocrinas involucradas en la producción de sustancias que se extienden sobre el cuerpo podrían ser lugares ideales para albergar bacterias que sinteticen sustancias beneficiosas en la lucha frente a patógenos, parásitos o depredadores. Las glándulas sebáceas de los mamíferos y la glándula uropigial de las aves producen sustancias que estos animales extienden por su superficie corporal para proteger el pelo y las plumas. También existen varias glándulas responsables de segregar las capas externas de los huevos en peces, anfibios, reptiles y aves, que podrían albergar bacterias beneficiosas. Sin embargo, en muy pocos casos se ha investigado si estas glándulas en general (McFall-Ngai 2002) y la glándula uropigial en particular albergan bacterias simbiontes que pudieran producir sustancias beneficiosas responsables, por ejemplo, de las propiedades antimicrobianas de las glándulas uropigiales (Jacob & Ziswiler 1982; Shawkey *et al*. 2003).

GLÁNDULA UROPIGIAL

La glándula uropygial (GU) es por tanto uno de los principales órganos presentes en las aves con posibilidades de constituir el escenario de una asociación mutualista con bacterias productoras de sustancias defensivas. Se trata de un complejo glandular holocrino (es decir, la célula se destruye para liberar su contenido) situada en el integumento de las aves. Constituye su única glándula cutánea, aparte de las glándulas sebáceas del oído externo y las glándulas anales (Jacob & Ziswiler 1982), y es exclusiva de este grupo. Se sitúa dorsalmente en la base de las plumas que constituyen la cola, en la región sinsacrocaudal, sobre las vértebras caudales libres, donde se aprecia externamente como una prominencia sobre la cola (ver Fig. 2l). La forma externa y el tamaño relativo de la GU varían dependiendo de la especie (Fig. 2a-2k). En

general, está formada por dos lóbulos separados por un tabique (Jacob & Ziswiler 1982, Fig. 2g). Contiene además una papila nítidamente separada de los lóbulos por un istmo (Fig. 2l, 2m). Los lóbulos contienen el tejido secretor activo, y la secreción sale al exterior a través de un sistema de conductos que se introduce en la papila y llega hasta su extremo, que se abre en la superficie corporal. Hay dos o más conductos que desembocan en la papila, excepto en abubillas, donde todo desemboca en un único conducto (Jacob & Ziswiler 1982, Fig. 2l, 2m).

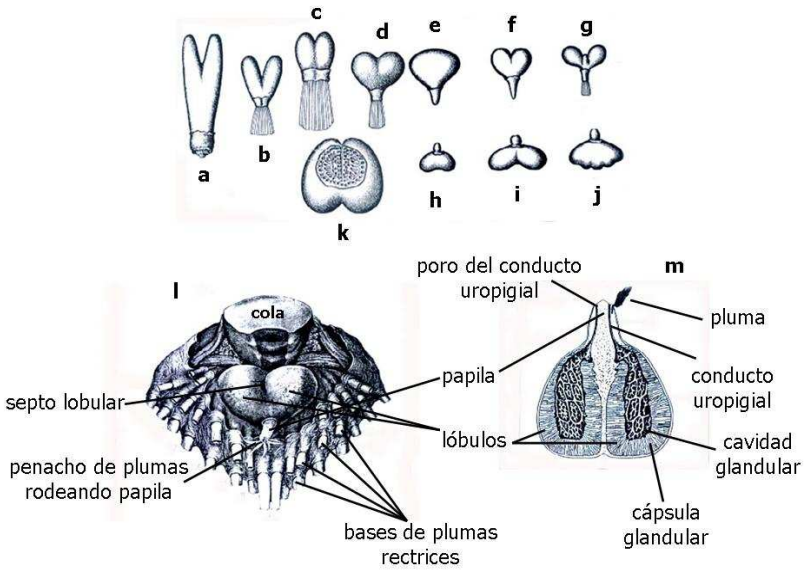


Fig. 2. ***Glándula uropigial***: ***3a-3k***: *variabilidad externa de la glándula (visión dorsal) en distintas especies (a, Spheniscus demersus, x 1,5; b, Gavia arctica, x 1; c, Ciconia ciconia, x 1,5; d, Aquila chrysaetos, x 1,5; e, Athene noctua, x 3; f, Steatornis caripensis, x 2; g, Upupa epops, x 4; h, Passer montanus, x 3; i, Cinclus aquaticus, x3; j, Turdus merula, x 3; k, Pelecanus onocrotalus, x 1,5).* ***3l****: Vista dorsal de una disección de la glándula de un pollo (Gallus gallus) de la raza Leghorn.* ***3m****: sección frontal de glándula.*

SECRECIÓN UROPIGIAL

La secreción uropigial (SU) está controlada por hormonas esteroides (Jacob *et al*. 1979). En general, es bastante variable en su composición química dependiendo de la especie de ave (Jacob & Ziswiler 1982; Burger *et al*. 2004; Gebauer *et al*. 2004; Montalti *et al*. 2005). Suele ser bastante espesa, de color blanquecino y naturaleza sebácea. Se compone principalmente de monoésteres de alcoholes alifáticos y ácidos grasos; también puede contener diferentes tipos de diésteres, triésteres, glicéridos y, en menor cantidad, esteroles (por ejemplo, colesterol), e incluso ciertos hidrocarburos (por ejemplo, escualeno) como ocurre en algunos Anseriformes (Jacob & Ziswiler 1982).

La secreción es esparcida por las aves en su plumaje periódicamente (lo que se conoce como "preening") y se le han atribuido diferentes funciones no excluyentes entre sí cuya importancia varía en distintos tipos de aves. Por ejemplo, las aves al acicalarse con la secreción pueden impermeabilizar y flexibilizar las plumas (Jacob & Ziswiler 1982), eludir depredadores a través del mal olor (Steyn 1999; du Plessis com. pers.), conseguir la vitamina D (Elder 1954), colorear o maquillar sus plumas (Piersma *et al*. 1999; Zampiga *et al*. 2004; Delhey *et al*. 2007; Amat com. pers.), intervenir en la producción de feromonas (Kolattukudy & Rogers 1987) o, debido a sus propiedades antimicrobianas (Shawkey *et al*. 2003), prevenir enfermedades y contagio de distintos agentes patógenos.

En algunos grupos de aves se han detectado cambios en la composición de la GU relacionados con la época reproductora. En el ánade real, los monoésteres que tienen durante todo el año son reemplazados por diésteres (de menor a mayor peso molecular) en hembras en esta época (Jacob *et al*. 1979; Kolattukudy *et al*. 1987), mientras que en varias especies de correlimos (Fam. Scolopacidae) ocurre igual en ambos sexos. La función propuesta para este cambio en la composición de la SU es la cripsis olfatoria durante la estancia en el nido ya que la menor volatilidad del diester dificultaría la detectabilidad por los depredadores (Reneerkens *et al*. 2002). Estos cambios estacionales parecen estar mediados por cambios hormonales (Sandilans *et al*. 2004a; Sandilans *et al*. 2004b).

Últimamente, se ha prestado especial atención al posible papel defensivo de las SU frente a patógenos, sobre todo de plumas. El principal componente de las plumas es la queratina, que es la proteína estructural que les confiere una alta estabilidad mecánica y resistencia a la degradación gracias a su compacta estructura mantenida por fuertes enlaces a través de puentes de cisteína (Parry & North 1998; Sangali & Brandelli 2000). Sin embargo existen numerosos organismos capaces de degradar la queratina y sobre los que las SU podrían actuar. Por ejemplo, se ha comprobado que las palomas (*Columba livia*) a las que se les extirpaba la GU se les degradaba el plumaje mucho mas rápido que a las que se les mantenía (Moyer *et al*. 2003), posiblemente debido a la acción de la secreción uropigial frente a malófagos (Moyer *et al*. 2003) o frente a agentes microbianos degradadores queratinolíticos (Muza *et al*. 2000; Pugh &

Evans 1970; Jacob *et al*. 1997; Bandyopadhyay & Bhattacharyya 1999; Shawkey *et al*. 2003). Sin embargo, hasta el momento ningún estudio ha planteado que las propiedades defensivas de la SU puedan ser debidas a la acción de bacterias simbiontes.

TIPOS DE SUSTANCIAS DEFENSIVAS PRODUCIDAS POR LOS SIMBIONTES. BACTERIOCINAS

Como consecuencia de la competencia por el hábitat y los recursos, los microbios han desarrollado a lo largo de la evolución un importante arsenal para luchar contra sus competidores (Riley & Wertz 2002a). En general, estos mecanismos consisten en la secreción de sustancias tóxicas, con una amplia variabilidad en origen, composición y espectros de acción. Entre ellas se encuentran las bacteriocinas, que son péptidos de pequeño tamaño, naturaleza anfílica/hidrófoba, cargados positivamente a pH fisiológico (Cleveland *et al*. 2001), de síntesis ribosómica y excretados fuera de la célula (Diep & Nes 2002). Las bacteriocinas fueron descubiertas por André Gratia en 1925, que las aisló de *E. coli*, viendo que en presencia de determinadas cepas el crecimiento de otras se inhibía. Posteriormente, se comprobó que la producción de bacteriocinas estaba ampliamente extendida entre las bacterias, tanto Gram negativas (Riley & Wertz 2002a) como Gram positivas (Jack *et al*. 1995).

Tradicionalmente se ha considerado que las bacteriocinas tienen un espectro de acción relativamente estrecho, siendo activas sólo frente a especies muy próximas filogenéticamente (Riley & Wertz 2002b). Sin embargo, en el único estudio realizado para comprobar el espectro de acción de las bacteriocinas de todas las cepas bacterianas de una determinada comunidad, se ha encontrado que aunque todas las bacteriocinas matan a las bacterias de la propia especie productora, en más de la mitad de los casos actúan también frente a otros taxones, y en algunos casos el espectro de acción es muy amplio (Riley *et al*. 2003). Siendo así, las bacteriocinas y sus cepas productoras pueden constituir una herramienta de lucha frente a especies de bacterias patógenas tanto para animales que establezcan simbiosis con ellas, como para los humanos en distintos tipos de aplicaciones prácticas. Así, por ejemplo, en aves de corral, concretamente en codornices, experimentos de laboratorio han demostrado que determinadas bacteriocinas

producidas por *E. faecium* pueden inhibir el crecimiento de otras bacterias potencialmente patógenas, como es el caso de *Salmonella spp.* (Laukova *et al*. 2003; Strompfova *et al*. 2003). Existe una bacteriocina (la nisina, producida por *Lactobacillus lactis lactis*) cuyo uso en alimentación fue aprobado por la Organización Mundial de la Salud en 1969, y se utiliza como conservante de alimentos, ya que impide su colonización por bacterias patógenas o descomponedoras. Pero existen más bacteriocinas, segregadas también por bacterias del ácido láctico (aquellas cuyo producto de la fermentación de los hidratos de carbono es el ácido láctico), con las mismas propiedades cuyo uso en la industria alimentaria se ha propuesto también (O'Sullivan *et al*. 2002; Topisirovic *et al*. 2006). Además, en los últimos años, se están considerando las bacteriocinas como posibles alternativas al uso de antibióticos en medicina y agricultura (revisado en Kirkup 2006), lo que podría solucionar el importante problema de la evolución de resistencias a antibióticos por bacterias patógenas (revisado recientemente en Wright 2007).

Sin embargo, en los estudios realizados hasta el momento de simbiosis mutualistas de animales con bacterias en las que la ventaja para el hospedador es la obtención de sustancias activas frente a patógenos, los compuestos identificados como responsables del efecto antibiótico no han sido bacteriocinas sino de otro tipo (por ejemplo tyrosol (4-hydroxyphenethyl alcohol) o isatin (2-3-indolenedione) en los huevos de dos especies de crustáceos (Gil-Turnes *et al* 1989; Gil-Turnes & Fenical 1992; revisado en Piel 2004)), por lo que no se sabe hasta qué punto este tipo de sustacias producidas por las bacterias pueden ser la base de asociaciones mutualistas con animales.

Dinámica de los mutualismos con bacterias. Influencia del ambiente

Un aspecto importante del funcionamiento de las relaciones mutualistas entre animales y bacterias, con gran influencia sobre múltiples aspectos de la evolución de los organismos involucrados en ellas, es la forma en que se constituye y mantiene esa asociación, incluyendo la forma en que se accede a los simbiontes (por ejemplo Lombardo *et al*. 1999), la selección de los más adecuados (por ejemplo Visick *et al* 2000) o el control que se pueda ejercer sobre ellos (revisado en Frank 2003, Lombardo 2007). En el caso del sistema digestivo y su microbiota

asociada está claro que la comunidad de simbiontes es resultado de componentes tanto ambientales como genéticos (Stevens & Hume 1998). Las aves presentan adaptaciones digestivas a procesar su dieta típica (Duke *et al*. 1997; Klasing 1999) y, además, su microbiota dependerá de las características del sistema digestivo (anatomía, pH, etc., ver Stevens & Hume 1998) que, en muchos casos, son caracteres específicos. También, se sabe que existe convergencia adaptativa en la morfología del sistema digestivo de especies filogenéticamente distantes pero con dietas similares (King & McLelland 1984; Ziswiler 1985). Por tanto, la microbiota digestiva debe también de tener un componente filogenético asociado a la morfología del intestino de distintas especies que, a su vez, debe de influir en el aprovechamiento del alimento. Sin embargo, distintas características del sistema digestivo, incluyendo su microbiota, tienen un fuerte componente ambiental como demuestra la similaridad encontrada entre pollos de dos especies diferentes de páridos cuando son criados en el mismo ambiente (Lucas & Heeb 2005), siendo capaz de adaptarse a los cambios nutricionales a lo largo de su ciclo de vida (Diamond 1991). Existen algunas especies de aves en las que la estrategia reproductora implica cambios más o menos drásticos en la dieta de los individuos, como sucede en los parásitos de cría. En estas especies se deberían encontrar adaptaciones especiales para disponer de la microbiota adecuada en cada fase de su vida. Sin embargo, hasta el momento no existe ningún estudio de las peculiaridades de la composición de la microbiota intestinal y su modo de obtención en estas especies.

En muchos casos de mutualismos entre animales y bacterias, un largo proceso coevolutivo ha llevado a que los dos componentes de la simbiosis, en muchas ocasiones, no puedan "vivir" uno sin el otro (mutualismos obligados, Douglas 1994). Si las bacterias se han hecho dependientes de las sustancias que le provee el hospedador, o requieren los habitáculos que este construye para ellas, como es el caso de simbiosis intracelulares, es posible que sea ese el único lugar donde se encuentren. En estos casos la adquisición del simbionte por los individuos hospedadores dependerá estrictamente de una transmisión desde otros individuos portadores, ya sea por transmisión horizontal desde coespecíficos no parientes, o vertical de madres a hijos (Lombardo 2007). En el caso de la transmisión vertical existe un predominio del

componente genético en la determinación de las comunidades bacterianas presentes en los individuos de la especie hospedadora como es el caso simbiontes relacionados con la defensa frente a patógenos entre colonias de hormigas (Currie *et al* 1999), o de algunos simbiontes digestivos, por ejemplo en escarabajos (Fukatsu y Hosokawa 2002) y hormigas (Sauer *et al* 2000). La estrecha coevolución entre hospedador y simbionte en estos sistemas ha llevado en muchos casos a que los linajes de ambos hayan evolucionado en paralelo (Sauer *et al* 2000, Hosokawa *et al* 2006), si bien puede haber casos de coespeciación que no se deben a la transmisión vertical (revisado en Herre *et al* 1999). Aunque en principio dichas especializaciones extremas se ha asumido que estaban restringidas a endosimbiontes intracelulares, los estudios de Hosokawa y colaboradores con escarabajos (2006) y de Currie y colaboradores con hormigas cortadoras de hojas (Currie *et al* 2006, Poulsen *et al* 2005, aunque falta construir la filogenia de las bacterias) sugieren que se pueden dar también en simbiontes extracelulares.

Cuando existe la transmisión horizontal, sin embargo, la especialización suele ser menor, aunque dicho modo de transmisión puede tener implicaciones evolutivas sobre diversos aspectos de la vida de los hospedadores. Si la transmisión ha de producirse desde otros coespecíficos, y además son necesarias varias inoculaciones, la dependencia de los simbiontes puede condicionar la evolución de la vida en grupo y de comportamientos sociales complejos (Lombardo 2007). Por el contrario, si los simbiontes están ampliamente disponibles en el ambiente, y son reclutados de él por los individuos de la especie hospedadora en su proceso de maduración (como por ejemplo en calamares que usan bacterias lumínicas, Nyholm *et al* 2000), la vida en grupo no se ve favorecida (Lombardo 2007). En este caso, la influencia del ambiente en la determinación de las comunidades de simbiontes es esencial, a pesar de que los hospedadores puedan seleccionar qué cepas de las disponibles tienen éxito en la implantación (Visick *et al*. 2000).

Hasta el momento no se ha estudiado ningún sistema de mutualismos con simbiontes especializados en la defensa en vertebrados, y específicamente en aves, por lo que no hay antecedentes sobre la importancia relativa que podrían tener los tipos de transmisión de los simbiontes que pudieran albergar los órganos de estas especies dedicados al cultivo de bacterias

productoras de sustancias defensivas. Probablemente la importancia relativa de un componente genetico (comunidades heredadas de progenitores) o ambiental (comunidades adquiridas de otros individuos de la misma especie o del medio que los rodea) dependa, entre otras cosas, de que la asociación se mantenga estable de forma constante, o de que tenga lugar sólo en una parte del ciclo. Por ejemplo, en el calamar *Loligo palei* con bacterias que ayudan a proteger los huevos (Barbieri *et al* 1997) que no se transmiten verticalmente (Barbieri *et al* 2001), el hecho de que las ventajas del mutualismo sólo sean útiles para la reproducción, ha podido favorecer que la asociación sólo se produzca en la madurez sexual y, por tanto, que los simbiontes no se hayan podido especializar en permanecer siempre vinculados al hospedor. Por el contrario, en las hormigas cortadoras de hojas o los pulgones, la presencia del simbionte es ventajosa en todo el ciclo del animal, y de ahí una asociación permanente, especialización, y transmisión directa entre hospedadores obligada.

OBJETIVOS

En esta tesis nos planteamos varios objetivos encaminados a aumentar los conocimientos existentes sobre evolución de mutualismos entre aves y bacterias en los dos tipos principales de estas simbiosis que funcionan en animales: con bacterias digestivas y con bacterias defensivas, y desde los dos puntos de vista abordados en la introducción: (1) determinación de las ventajas de la asociación para las aves, y (2) dinámica de la relación en lo que se refiere al modo de adquisición de los simbiontes, particularmente de qué modo influyen el componente filogenético y el ambiental en la comunidad de simbiontes albergada. Para ello utilizamos dos sistemas de aves-bacterias cuyas peculiaridades la hacen ideales para este estudio: (a) el formado por un ave parásita de cría (el críalo, *Clamator glandarius*), su hospedador (la urraca, *Pica pica*) y las bacterias intestinales de ambos; y (b) el de la abubilla y las bacterias que habitan en su glándula uropigial.

I. EVIDENCIAS DE LA EXISTENCIA DE MUTUALISMOS. VENTAJAS OBTENIDAS POR LOS HOSPEDADORES.

Como se ha revisado en la introducción, las evidencias existentes sobre relaciones mutualistas entre aves y bacterias en las que se ponga claramente de manifiesto el efecto de la relación sobre el éxito del ave son escasas. En el caso de simbiosis en el digestivo, son muy pocos los estudios que han trabajado con aves silvestres, y en cuanto a las simbiosis defensivas, no se ha estudiado ninguna en vertebrados. El primer objetivo global de esta tesis consiste por tanto en obtener evidencias de que las relaciones de simbiosis estudiadas pueden ser consideradas mutualismos en los que los hospedadores obtienen ventajas de la asociación, y evaluar en qué medida esas ventajas son determinantes de su éxito reproductor o probabilidad de reclutamiento a la población reproductora.

(**a) Aparato digestivo de aves parásitas de cría y sus hospedadores.**

Una de las maneras de evaluar el beneficio a largo plazo que las aves obtienen de sus simbiontes es comprobar el efecto de la presencia de esos simbiontes sobre la capacidad de respuesta inmune de sus hospedadores, ya que se sabe que, al menos la respuesta mediada por linfocitos T demostrada en test in vivo es un buen predictor de la probabilidad de reclutamiento en la población reproductora (Cichon & Dubiec 2005; Moreno *et al* 2005; Hidalgo-García 2006). Sin embargo hasta el momento ningún estudio ha abordado con aves salvajes la comprobación de dicha hipotética relación. En el caso de aves parásitas de cría, investigar la influencia de la comunidad bacteriana intestinal en el éxito de los pollos es especialmente interesante, porque podría ser un aspecto de gran importancia en el éxito de la propia estrategia de parasitismo, o en la selección de especie hospedadora. En el **manuscrito I** se analiza la relación de la comunidad bacteriana albergada por pollos de críalo y urraca que se han criado en el mismo nido en el éxito de dichos pollos, incluyendo como una medida de la probabilidad de reclutamiento a la población reproductora, la respuesta inmune mediada por linfocitos T a un antígeno inocuo (PHA) junto a su condición física al abandonar el nido. Nuestra hipótesis de partida es que la comunidad de simbiontes establecida en el intestino de ambas especies debe ser un determinante de su éxito por lo que deberíamos encontrar una relación entre la comunidad bacteriana intestinal y las medidas de condición física y capacidad de respuesta inmune (**predicción 1**).

(b) Glándula uropigial de la abubilla

Uno de los pocos casos descritos de posibles relaciones mutualistas entre vertebrados y bacterias que podrían conferir protección frente a patógenos y/o depredadores es el sistema presente en la glándula uropigial de dos especies de aves coraciiformes. Recientemente se ha puesto de manifiesto la presencia de bacterias en la glándula uropigial de la abubilla arbórea (*Phoeniculus purpureus),* perteneciente a la familia *Phoeniculidae* en la que se ha aislado una especie nueva de bacteria denominada *Enterococcus phoeniculicola* (Law-Brown & Meyers 2003) y la abubilla Europea (*Upupa epops*) perteneciente a la familia *Upupidae,* en cuya glándula se han

aislado cepas de *Enterococcus faecalis* (Martín-Platero *et al*. 2006). La secreción de la abubilla arbórea se ha comprobado que inhibe el crecimiento de varias especies de bacterias patógenas, entre ellas y con una actividad especialmente marcada *Bacillus licheniformis* (du Plessis *et al*. datos no publicados, citado en Burger *et al* 2004). Por otro lado, en la abubilla europea, se ha aislado también una bacteria simbionte de su glándula uropigial (*E. faecalis*, cepa MRR-10.3, Martin-Platero *et al*. 2006) que se comprobó que producía al menos dos **bacteriocinas** con un amplio espectro de acción incluyendo la bacteria degradadora de plumas *Bacillus licheniformis* (Martin-Platero *et al*. 2006). Estos estudios sugieren que las bacterias simbiontes detectadas en la glándula uropigial de estas especies de aves podrían proporcionar protección a sus hospedadores frente a patógenos. Phoeniculidos y Upupidos tienen en común que sus secreciones son oscuras, y emiten un fuerte y repulsivo olor que tradicionalmente se ha interpretado como un repelente hacia posibles depredadores (Cramp 1985, Ligon en Del Hoyo 1997), ya que podrían estar advirtiendo que son aves con un sabor desagradable (Dumbacher & Pruett-Jones 1996). Sin embargo, los compuestos químicos existentes en esas secreciones especiales también pueden ser importantes en la lucha frente a patógenos. En el caso de la abubilla europea, esas propiedades físicas de la secreción se producen tan solo en hembras y en pollos durante su etapa de estancia en el agujero que les sirve de nido (Cramp 1985), mientras que en abubillas arbóreas la secreción oscura se mantiene todo el año. En ambos casos coincide que las propiedades especiales de la secreción se mantienen durante el periodo que las aves pasan buena parte del día dentro de agujeros, ya que las abubillas arbóreas usan esos agujeros como dormideros comunales.

El hecho de que en la abubilla europea la secreción especial sólo aparezca de forma estacional hace a esta especie el objeto de estudio más útil para investigar la posible influencia de las bacterias de la glándula en las propiedades de la secreción y entender sus funciones. En las fases del ciclo en las que las secreciones no son oscuras se esperaría que la función fuera diferente y que no estuviera presente la bacteria. El hecho de que las secreciones sean especiales durante la estancia en el nido podría ser debido a un mayor riesgo de contraer enfermedades en

ese lugar por la acumulación de restos de excrementos, pollos muertos y presas de puestas anteriores, ya que las abubillas reutilizan los nidos frecuentemente.

Para comprobar la hipótesis de que las secreciones oscuras son el resultado de un mutualismo entre el ave y bacterias simbiontes, cuya función es defender al ave de patógenos, es necesario testar varias predicciones:

En primer lugar se esperaría una asociación temporal entre la presencia de bacterias simbiontes en la glándula, la actividad antimicrobiana de la secreción y las características especiales de la secreción. Es decir, las secreciones blancas (de hembras no reproductoras o de machos) deberían demostrar una capacidad antibacteriana menor que las marrones, y sólo en estas últimas deberían estar presentes las bacterias simbiontes (**predicción 2**). Esta posible asociación entre las propiedades de la secreción y la presencia de la bacteria se estudia en el **manuscrito II.**

Sin embargo una correlación entre la presencia de las bacterias y las propiedades de la secreción no es suficiente para demostrar que esas propiedades son debidas a la bacteria. Para comprobarlo es necesario manipular la presencia de la bacteria experimentalmente y ver el efecto de ese tratamiento en las propiedades de la secreción. Si la capacidad antimicrobiana de la secreción y sus propiedades físicas (color y olor) son debidas al simbionte esperaríamos que al eliminarlo de las glándulas las secreciones de hembras y pollos perdieran poder bactericida y se volvieran blancas e inodoras (**predicción 3**). Para comprobar esa predicción se realizó un experimento en el que se eliminaron las bacterias de las glándulas inyectando antibióticos, experimento que se presenta en el **manuscrito III** junto a un estudio descriptivo de la dinámica estacional natural de las propiedades de la glándula y la secreción en machos, hembra y pollos. En este estudio, sin embargo, no es posible detectar el efecto de la ausencia de las bacterias en la actividad antimicrobiana de la secreción, debido a que tras el tratamiento las secreciones de los individuos experimentales mantienen residuos del antibiótico inyectado. Por ese motivo es necesario obtener por otras vías evidencias de que la actividad de las secreciones oscuras es debida a las bacterias presentes en ellas.

Otro aspecto importante sería averiguar si existe una variación en la actividad antimicrobiana de distintas cepas aisladas de abubillas. En caso de que dicha se produzca, podríamos esperar que dicha variación estuviera relacionada con el perfil genético de las bacterias (**predicción 4**). Esa posiblidad se evalúa en condiciones de laboratorio en el **manuscrito IV**.

Por último, una cuestión fundamental para demostrar la existencia de un mutualismo que se ha seleccionado por las ventajas para el hospedador, es comprobar la consecución de beneficios por su parte que tengan que ver con el tipo de mutualismo hipotetizado. Es decir, deberíamos comprobar que la presencia de la bacteria simbionte en la glándula de las abubillas se traduce en beneficios para ellas en su lucha frente a patógenos. Dado que, que por el mismo motivo expuesto anteriormente, la eliminación de los simbiontes mediante antibióticos no es una vía válida para comprobar los efectos de su presencia sobre medidas de éxito que pueden ser afectadas directamente por el antibiótico. Debido a que las abubillas esparcen la secreción sobre el plumaje y, aparentemente, también sobre los huevos (ver **manuscrito III**), es esperable que la acción bactericida de la secreción se cumpla en la superficie de plumas y huevos. De hecho, varias de las bacterias que se ha comprobado in vitro que son sensibles a la bacteriocina producida por el simbionte de las abubillas son patógenas de plumas (*Bacillus licheniformis*) y potenciales patógenos de huevos (e.g. *Stafilococcus*) (Martín-Platero *et al*. 2006). Por ello, una forma de evaluar los efectos beneficiosos de las bacterias simbiontes de la glándula sobre las abubillas es comprobar el efecto de su acción sobre la degradación de las plumas y el éxito de eclosión de los huevos. En el primer caso esperaríamos que la presencia de la bacteria simbionte inhibiera la acción descomponedora de plumas por *Bacillus licheniformis* (**predicción 5**). Esta posibilidad es explorada con experimentos *in vitro* en el **manuscrito V**. En el segundo caso, si asumimos que la actividad antimicrobiana proporcionada por los simbiontes cuando la secreción se esparce por la superficie de los huevos es debida a su producción de bacteriocinas, deberíamos encontrar que cuando se inutilizan esas bacteriocinas experimentalmente por medio de proteasas, el riesgo de fallos de eclosión a causa de bacterias patógenas debería aumentar

(**predicción 6**). Para comprobarlo realizamos un experimento *in vivo* en el campo que se presenta en el **manuscrito II**.

II. DINÁMICA DE LA RELACIÓN AVES-BACTERIAS: INFLUENCIA DEL AMBIENTE Y MODO DE ADQUISICIÓN DE LOS SIMBIONTES.

El modo en que los hospedadores adquieren sus simbiontes mutualistas está íntimamente relacionado con el grado de especialización alcanzado en la relación. Una gran especialización normalmente va asociada a la monoespecificidad del simbionte, lo que asegura una mayor coincidencia de sus intereses con los del hospedador, y suele implicar la necesidad de transmisión vertical (Herre *et al* 1999). En muchos sistemas sin embargo el modo de adquisición de los simbiontes es por transmisión horizontal, entre individuos no relacionados o a partir del ambiente. Esta podría ser la mejor vía de obtención de simbiontes si los individuos experimentan condiciones ambientales cambiantes a lo largo de su vida en las que los simbiontes ideales puedan ser diferentes. Puede ser también la mejor forma de convivir con una asociación que conlleve costes si los beneficios netos se obtienen sólo en determinadas fases del ciclo de vida. Por ejemplo, en el caso de las bacterias de las hormigas cortadoras de hojas de los géneros *Atta* y *Acromyrmex* (Formicidae: Attini), a pesar de que el modo de transmisión del simbionte entre unidades reproductoras (colonias) es vertical, entre individuos de la misma colonia sucede de forma horizontal (Poulsen *et al* 2003). El albergar los simbiontes implica costes energéticos y sólo algunas obreras, y sólo durante una fase de su vida, presentan cultivos abundantes de dichas bacterias en su cutícula (Poulsen *et al* 2003). Por tanto, los balances de costes y beneficios para el hospedador pueden variar en distintos tipos de sistemas mutualistas y entre distintos tipos de transmisión del simbionte. A su vez, estos balances pueden variar a lo largo de la vida del hospedador, lo que podría influenciar el establecimiento de relaciones especializadas. En los dos sistemas estudiados en esta tesis, los condicionantes impuestos por el ciclo de vida de los hospedadores tienen implicaciones diferentes para el tipo de transmisión y el nivel de especialización alcanzado en la relación.

(a) **Bacterias digestivas en un parásito de cría**

Los sistemas formados por parásitos de cría y sus hospedadores permiten el estudio de la importancia del componente filogenético y del ambiental en la microbiota digestiva de dos especies lejanas filogenéticamente pero que, durante una etapa de sus vidas, comparten exactamente el mismo ambiente y tienen la misma dieta. Desde el punto de vista del parásito, además, la fase de su vida que transcurre en el nido de su hospedador implica la necesidad de adaptar su microbiota a un ambiente diferente al que se enfrentará de adulto, pues la dieta aportada por sus padres adoptivos y la que consumirá de adulto difieren. Es predecible, por tanto, que en el caso del parásito de cría el factor ambiental sea de gran importancia en la conformación de su microbiota intestinal si debe obtener el máximo rendimiento a la dieta de su especie hospedadora.

Estudiamos estos aspectos en el sistema parásito de cría – hospedador formado por el **críalo** y la **urraca**, dos especies filogenéticamente muy alejadas que, durante su etapa de pollo, comparten el nido y son alimentados por los mismos padres y con el mismo tipo de alimento por el que compiten durante el crecimiento. El críalo es un ave parásita de cría obligada (esto es, su descendencia es criada completamente por el hospedador) y, en Europa, es la urraca la especie de hospedador que es más frecuentemente parasitada (Cramp 1985). La urraca es omnívora, mientras el críalo adulto se alimenta exclusivamente de insectos, siendo especialista en orugas venenosas evitadas por la mayoría de aves. Son especies de distintos ordenes de aves (Cuculiformes vs Passeriformes) y, por tanto, filogenéticamente bastante alejadas. La dieta de los adultos de estas especies es diferente, por lo que el sistema digestivo de ambas especies también debe ser distinto. Sin embargo, como ya se ha expuesto anteriormente, los pollos de ambas especies se crían en el mismo ambiente, son alimentados por los mismos padres (urracas), con la misma comida, y con transferencia de saliva (factor altamente influyente en la transmisión de microbiota, ver Kyle & Kyle 1993). Con estas premisas, podría ocurrir que (i) los críalos presenten adaptaciones en su sistema digestivo y microbiota intestinal similares a los de la urraca que les permita aprovechar al máximo la dieta recibida durante su estancia en el nido, en cuyo caso deberíamos encontrar comunidades bacterianas similares en ambas especies

(**predicción 7)**. Otra posibilidad es que, (ii) debido a constricciones filogenéticas, su sistema digestivo y la microbiota asociada difiera de la de su hospedador (**predicción 8**). Esta segunda posibilidad implicaría que el aprovechamiento de la comida por el parásito sería menor que el de sus hermanastros. Ya que la comunidad bacteriana de la urraca debe estar adaptada a optimizar el alimento recibido por sus padres (Robbins 1983), los pollos de críalo necesitarían un aporte de alimento mayor que sus hermanos para un normal desarrollo. En el **manuscrito VI** estudiamos en detalle y comparamos tanto la anatomía del sistema digestivo como las comunidades bacterianas de las dos especies y discutimos sus posibles implicaciones en la relación parásito de cría – hospedador que mantienen.

(b) **Bacterias defensivas en la glándula uropigial.**

El sistema formado por la glándula uropigial de la abubilla y sus bacterias simbiontes posee condicionantes que, al mismo tiempo, permiten hipotetizar sobre la existencia de una transmisión directamente de la madre, o bien una adquisición de las bacterias presentes en el nido. Por una parte, la secreción es utilizada por las hembras para recubrir los huevos, que además tienen una cáscara visiblemente porosa (observación personal), lo que podría servir para transmitir los simbiontes de madre a embriones durante su desarrollo dentro del huevo, o en el momento de la eclosión. Por otra parte la madre convive estrechamente con los pollos en sus primeros días de vida, ya que los empolla hasta que son capaces de termoregular, tiempo en el que la glándula uropigial comienza a ser visible y a producir secreción (Cramp 1985) y en la que los simbiontes pueden fácilmente pasar de madre a hijo. Todas estas evidencias podrían apuntar a una transmisión vertical de los simbiontes, lo que facilitaría el desarrollo de una coevolución estrecha entre ave y bacteria (Herre *et al* 1999). Por otra parte, el hecho de que la cepa de *Enterococcus* aislada de la glándula de esta especie produzca bacteriocinas con un espectro de acción bactericida grande incluyendo bacterias degradadoras de plumas (Martín-Platero *et al* 2006), sugiere que su actividad puede ser de interés para las aves a lo largo de todo su ciclo de vida. Sin embargo, una peculiaridad notable de este sistema es que los cambios estacionales en las características de la secreción de la abubilla (color y olor) sugieren también

una estacionalidad en la relación con las bacterias (ver **manuscrito III**). Si la relación con la bacteria en la glándula no se mantiene a lo largo de todo el año de forma continuada, entonces las abubillas hembras deben adquirirla cada temporada antes del inicio de la reproducción. Bajo ese escenario se podría predecir una transmisión horizontal del simbionte, que no podría producirse ni siquiera entre individuos no relacionados, ya que todas las abubillas las perderían de una temporada de cría a la siguiente, y dependería de la presencia de la bacteria de interés en el ambiente. No obstante, puesto que el ámbito de actuación de la secreción especial de esta especie parece quedar restringido al nido, y en el interior del nido se produce el uso de ésta para la impregnación de las plumas de hembra y pollos así como de los huevos, es probable que el fondo del nido sea un reservorio importante de las bacterias de las abubillas que han criado o crecido en él. En esta especie es relativamente frecuente la reutilización de nidos por parte de la misma o diferentes hembras en intentos reproductores sucesivos en la misma o diferente estación de cría. Por tanto, una manera en la que las abubillas podrían acceder a los simbiontes sería la reutilización del nido donde tengan evidencias de que han criado otros individuos. No obstante, si esa es la forma en que las abubillas obtienen los simbiontes adecuados, la capacidad para incorporar bacterias del ambiente por parte de las abubillas es necesaria. Una forma de comprobar si esa capacidad existe es manipular las cepas disponibles en el ambiente que rodea a las abubillas, lo que puede conseguirse mediante experimentos de intercambios de pollos entre nidos. Si los pollos tienen la capacidad de incorporar las bacterias del ambiente esperaríamos que los transportados a otros nidos compartieran las cepas bacterianas albergadas en su glándula con los hermanos adoptivos en lugar de con sus hermanos genéticos (**predicción 9**). Alternativamente, los simbiontes adquiridos por los pollos directamente de sus madres podrían permanecer de alguna manera asociados al individuo, consolidándose la transmisión vertical iniciada durante la incubación y el empolle. Alternativamente, las bacterias capaces de formar la simbiosis con la glándula podrían ser conseguidas de cualquier lugar, lo que dificultaría la especialización. En el **manuscrito VII** se estudia la importancia del ambiente en que se crían los pollos de abubilla (es decir, el nido) en la adquisición de las bacterias por parte

del hospedador, realizando experimentos de intercambio de pollos entre nidos y determinación de las cepas desarrolladas por los pollos mediante análisis de RAPD.

MATERIAL Y MÉTODOS

I. ÁREA DE ESTUDIO

El trabajo de campo se llevó a cabo en la Hoya de Guadix-Baza (37º18´N, 3º11´W con urracas y críalos durante el año 2003, y 37º18´N, 3º08´W con abubillas los años 2003-2006. La zona de estudio está situada a 1000 m aproximadamente sobre el nivel del mar en la provincia de Granada, al sureste de la Península Ibérica (ver Fig. 3). Es una altiplanicie semi-árida con clima meso-Mediterráneo, donde la vegetación natural de porte arbóreo no es muy abundante. La mayoría del terreno se dedica a la agricultura, aunque hay algunas zonas adehesadas de encinas (*Quercus ilex*).

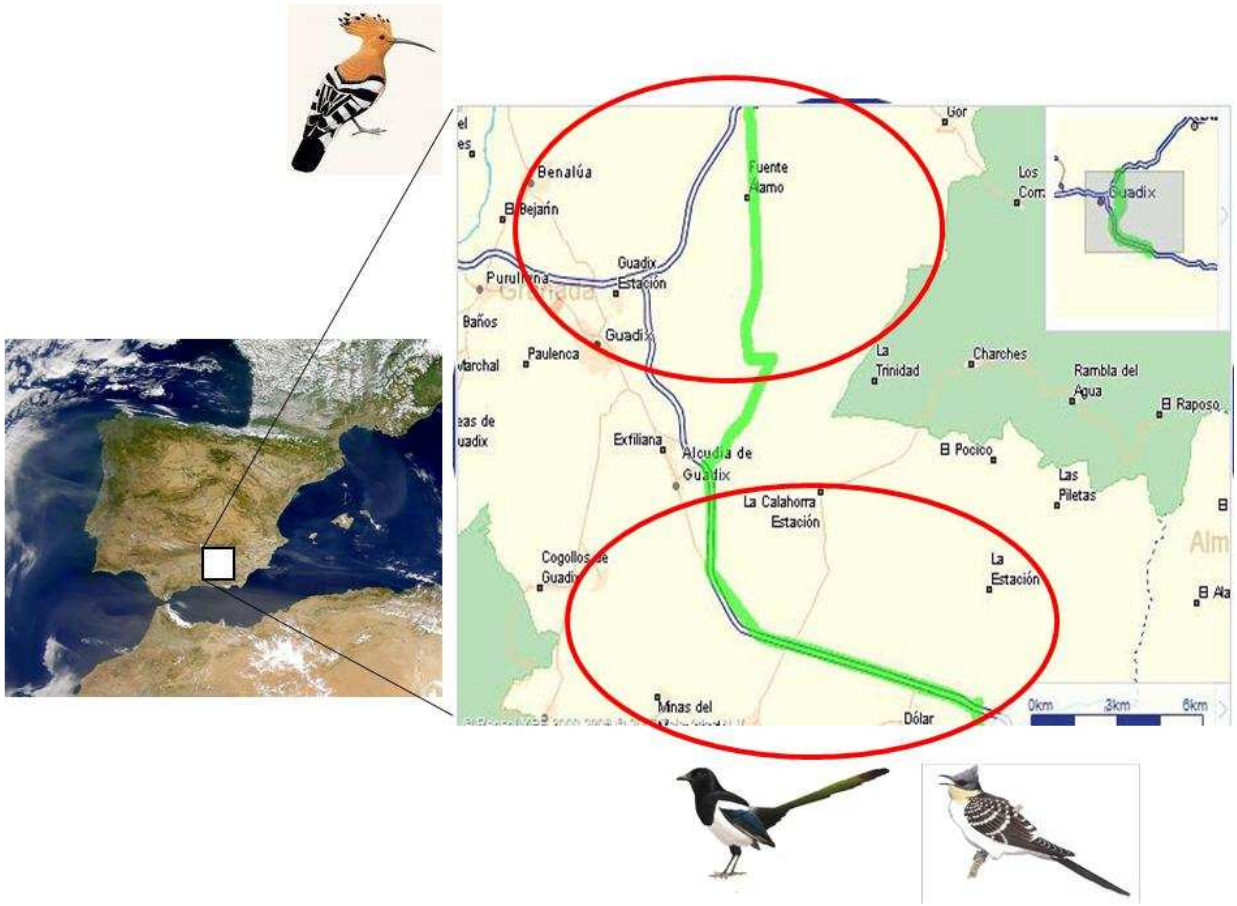


Fig. 3. *Área de estudio: arriba, zona de trabajo con abubillas; abajo, con urracas y críalos*

II. ESPECIES DE ESTUDIO

Esta tesis aborda el estudio de relaciones entre tres especies de aves y las comunidades de bacterias asociadas a su sistema digestivo y glándula uropigial. Las especies involucradas se presentan a continuación.

Urraca (O. Passeriformes, Fam. Corvidae, *Pica pica*)

Se encuentra por toda Europa y Norte de África, parte de Asia y de América. Es sedentaria, de alimentación omnívora oportunista. En nuestra área de estudio se distribuyen por una zona agrícola de secano donde los principales cultivos son almendros (*Prunus dulcis*, donde construyen la mayoría de sus nidos) y cebada (*Hordeum sativum)*.

Su época reproductora en la hoya de Guadix está comprendida entre finales de marzo y junio, y es frecuentemente parasitada por el críalo. Construye nidos voluminosos que indican calidad parental (Soler *et al* 1995a; De Neve *et al* 2004), y los críalos prefieren parasitar aquellos de mayor tamaño (Soler *et al* 1995a). Realiza puestas optimistas de unos siete huevos, aunque no todos ellos producen volantones, ocurriendo por tanto una reducción de pollada. La incubación suele comenzar con la puesta del cuarto huevo, dura unos 20 días y la lleva a cabo exclusivamente la hembra (Birkhead 1991). El periodo de estancia en el nido de los pollos es de unos 23 días, y son alimentados por machos y hembras con una dieta formada principalmente por insectos, aunque en la última etapa también incluyen granos verdes de cereal (Soler *et al*. 1995b).

Críalo (O. Cuculiformes, Fam. Cuculidae, *Clamator glandarius*)

Es un parásito obligado de cría, y su principal hospedador en Europa es la urraca. Es un ave migradora que cría en la Península Ibérica, Francia, Italia y Oriente Medio, en zonas semi-áridas. El resto del año vive en zonas tropicales y subtropicales del África subsahariana. Se alimenta de insectos, principalmente de orugas que suelen ser tóxicas para otras especies. En la temporada de cría, su alimento al sur de Europa se compone casi exclusivamente de procesionarias (*Thaumetopoea pityocampa*) (Cramp 1985).

La hembra, a veces con la ayuda del macho (Arias de Reyna 1998), realiza la puesta en nidos de urraca, muchas veces rompiendo algunos de los huevos de la urraca con lo que disminuye la competencia con pollos de hospedador (Soler *et al*. 1997). El periodo de incubación es de unos 16 días por lo que los pollos del parásito suelen eclosionar antes que los del hospedador. Esta pronta eclosión de los pollos de críalo les confiere una ventaja en tamaño respecto a los de urraca (Soler & Soler 1991) que, junto con la mayor agresividad del críalo en su comportamiento petitorio (Redondo & Zuñiga 2002), hace que sea preferentemente alimentado por los padres adoptivos (Soler *et al*. 1995b) lo que en muchos casos produce la muerte por inanición de los pollos de urraca. La dieta de los pollos de críalo en nidos de urraca no difiere de la de pollos de su hospedador (Soler *et al* 1995b) por lo que difiere bastante de la dieta de los críalos adultos.

Abubilla (O. Coraciiformes, Fam. Upupidae, *Upupa epops*)

Se distribuye por Europa, Asia y África. Busca el alimento en el suelo (Cramp 1985) y es insectívora, aunque en época de reproducción puede llevar al nido algunos reptiles de pequeño tamaño. En nuestro área de estudio nidifica principalmente en olivares (*Olea europaea*) de regadío y en dehesas de encinas (*Quercus ilex*). Cría en agujeros naturales, aunque también acepta cajas artificiales para su nidificación. Muchos de los resultados presentados en esta tesis provienen de eventos reproductores en cajas nido de corcho, colocadas en nuestra zona de estudio desde hace cinco años.

Su época de reproducción es bastante amplia, pudiendo oscilar según las condiciones ambientales de cada año desde final de febrero hasta julio (Martín-Vivaldi *et al*. 1999). Ponen una media de 7 huevos que empiezan a incubar desde el primer día, provocando una asincronía en la eclosión que, genera una marcada jerarquía de tamaño en la nidada lo que habitualmente provoca la muerte de los pollos más pequeños (40 % de los pollos nacidos, Martín-Vivaldi *et al*. 1999). Aunque la reducción de nidada es el principal determinante del número de pollos que vuelan en los nidos exitosos, con relativa frecuencia (36.4 % de nidos) hay fallos en la eclosión (Martín-Vivaldi *et al*. 1999). Durante la incubación, que dura una media de 17 días, la hembra

apenas abandona el nido y es el macho quien la alimenta. A los 7-8 días de la eclosión del primer huevo la hembra ya comienza a salir del nido y a buscar comida para, conjuntamente con el macho, alimentar a la descendencia. Los pollos se quedan en el nido hasta los 24-30 días de edad (Martín-Vivaldi *et al*. 1999).

Las abubillas no construyen nidos. Por el contrario, utilizan agujeros naturales en los troncos, a veces excavados por otras especies (e.g. pito real (*Picus viridis*) en nuestra zona de estudio). También utilizan huecos en casas abandonadas o entre montones de piedras. No meten ningún tipo de material en el nido y, al contrario que muchas especies de aves, cuando están criando a los pollos no retiran de forma exhaustiva los restos de comida, los huevos no eclosionados ni los pollos muertos de sus nidos en cavidades naturales (observación personal). No obstante, muchos de estos restos sí son sacados del nido con cierta frecuencia en las cajas-nido artificiales, quizás por la mayor facilidad de acceso a la entrada desde el habitáculo donde se encuentra el nido, que en nidos naturales. Por ejemplo, en olivos, el habitáculo de nidificación suele estar separado de la entrada por un largo corredor (observación personal). Incluso en las cajas nido, los excrementos se acumulan en un rincón del nido, y sólo son extraídos por la hembra después de su deposición y acumulación en él por los pollos. Esta falta de higiene podría suponer un foco de infección para la pollada.

Los nidos de abubilla tienen un olor característico, bastante fuerte y desagradable. Este olor es producido por la secreción de la GU, que tanto la hembra como los pollos producen durante su estancia en el nido, y que ha sido tradicionalmente interpretado como un repelente frente a depredadores (Cramp 1985, Kristin en del Hoyo 1997). Como está expuesto en la introducción, precisamente en las características de dicha secreción y su posible función se basan algunos de los objetivos de esta tesis.

Enterococos *(O. Lactobacillales, Fam. Enterococcaceae, Gen*. Enterococcus*).*

El género *Enterococcus* está formado por cocos Gram positivos que se alinean en cadenas cortas o en parejas. Son catalasa negativos (Devriese *et al*. 1992) y presentan un bajo contenido en

Guanina y Citosina (usualmente por debajo del 50%, Schleifer & Kilpperbalz 1984; Madigan *et al*. 2003). Son anaerobios facultativos, con una temperatura óptima de crecimiento de 35ºC, y un rango de 10 a 45ºC. Como metabolito final de la digestión de azúcares producen ácido láctico principalmente.

El género *Enterococcus* fue propuesto por primera vez por Schleifer y Kilpper-Bälz en 1984 para designar a las especies fecales del grupo D de Lancefield antes incluidas en el género *Streptococcus*: *S. faecalis* y *S. faecium*, ya que sus estudios genéticos demostraron que estaban bastante alejadas del resto de *Streptococcus*. Estudios quimiotaxonómicos y genéticos posteriores propusieron la escisión del género *Enterococcus* en nuevas especies como es el caso de *E. avium*, *E. casseliflavus*, *E. durans*, y *E. gallinarum* (Collins *et al*. 1984). Más recientemente se ha descrito la bacteria *E. phoeniculicola,* aislada en la abubilla arbórea verde (Law-Brown & Meyers 2003). Actualmente, el número de especies aceptadas dentro del género está por encima de 20.

Es un género de bacterias muy ubicuas que se localizan habitualmente en el intestino de animales, en plantas, suelo, agua y alimentos (revisado en Ballesteros 2004). En climas moderados, los enterococos desaparecen de las plantas durante el invierno y, probablemente debido al los insectos (Martin & Mundt 1972), reaparecen en la primavera, aumentando su frecuencia a medida que las plantas se desarrollan. Ni el suelo ni el agua parecen ser el hábitat natural de los enterococos, por lo que su aislamiento en este tipo de muestras, aunque es habitual, indicaría una contaminación a partir de animales y plantas (Devriese *et al.* 1992; Godfree *et al*. 1997). Por ello, la presencia de enterococos en las aguas es utilizada como indicador de contaminación fecal (Kuhn *et al*. 2003).

El grado de especificidad de hospedador para este grupo de bacterias es aparentemente muy variable ya que, por ejemplo, *E. phoeniculicola* sólo se ha encontrado en una especie de hospedador (abubilla arbórea verde), mientras que otras especies pueden encontrarse tanto en taxones animales como vegetales. Con diferencia, las especies más abundantes en cualquier ambiente son *E. faecium*, *E. faecalis* y *E. hirae* (revisado en Ballesteros 2004).

III. EXPERIMENTOS Y TOMA DE DATOS EN EL CAMPO Y EN EL LABORATORIO

Tanto para el estudio del sistema críalo-urraca como para el estudio de las abubillas se hizo un seguimiento exhaustivo de la reproducción, desde la búsqueda de nidos y la detección exacta del principio de la puesta y primera eclosión, hasta que voló el último pollo. Las urracas, en nuestra zona de estudio construyen sus nidos principalmente en almendros (*Prunus dulcis*) y encinas (*Quercus ilex*). Los nidos son muy conspicuos y fácilmente detectables antes del inicio de la puesta. Tanto los nidos de urraca como las cajas nido donde criaban las abubillas se revisaban cada semana. En ambas especies esta tasa de visitas permite conocer el día del comienzo de la puesta. Las visitas a los nidos, sin embargo, se hicieron mas frecuentes cuando los experimentos o las tareas relacionadas con los distitos objetivos lo requerían.

Evidencias de la existencia de mutualismos. Ventajas obtenidas por los hospedadores.

(a) *Relaciones entre la comunidad bacteriana y la capacidad de respuesta inmune mediada por linfocitos T y la condición física del hospedador*

Las muestras bacterianas cloacales de urracas y críalos se tomaron unos días antes de que los pollos abandonaran el nido (con 16 días aproximadamente) asegurando, de esta forma, que estuviera completamente desarrollada. El muestreo de la comunidad bacteriana se realizó mediante lavados de la cloaca con 500 µl de tampón fosfato que, con ayuda de una pipeta automática, se introducía y extraía de nuevo varias veces en la cavidad cloacal. La muestra obtenida se depositaba en un tubo Eppendorf de 1.5 ml con 500 µl de tampón lítico para impedir el crecimiento bacteriano. A continuación se guardaban en frío (4-8 ^{o}C) hasta llegar al laboratorio donde se congelaban (-20^{o}C) hasta su posterior análisis molecular (ver mas adelante). De esta forma se inhibía inmediatamente el crecimiento bacteriano, impidiendo fenómenos de competencia y exclusión, y se aseguraba que al procesar las muestras la información obtenida se correspondiera con la comunidad bacteriana en el momento de la extracción (**manuscritos I** y **VI**).

Una vez en el laboratorio, el procesamiento de las muestras cloacales de críalos y de urracas consistieron en la extracción del ADN directamente de las muestras mediante el método

de Orsini (Orsini & Romano-Spica 2001) y su posterior amplificación usando la técnica RISA (Ribosomal Intergenic Spacer Analisis, ver García-Martínez *et al*. 1999 por ejemplo). Esta técnica amplifica la región intergénica del genoma comprendida entre los genes 16S y 23S del ADN ribosómico. Debido a que esta región del ADN bacteriano es bastante variable entre especies distantes, y conservativo en especies relacionadas, su análisis permite estimas de diversidad de comunidad bacteriana comparables entre muestreos de distintos individuos.

Tradicionalmente, las bacterias han sido clasificadas en base a criterios nutricionales (Keller & Zengler 2004), y la cuantificación de la diversidad microbiana se ha basado en observaciones microscópicas combinadas con las medidas de uso del sustrato (Ovreas 2000). Sin embargo, se estima que el 99% de los microorganismos no son cultivables (Amann *et al*. 1995) por lo que estas metodologías tradicionales parecen poco realistas. Gracias al reciente aumento en el uso de técnicas moleculares, la diversidad bacteriana se está estudiando directamente en las muestras sin la necesidad de realizar cultivos. Secuenciando los genes del ARN ribosómico obtenido de la extracción directa de ADN del medio a estudiar se pueden detectar grupos filogenéticos, los conocidos como "filotipos", pudiendo conocer la distribución y frecuencia de cada uno de ellos en comunidades naturales (Pace 1997). La metodología RISA, aunque no identifica especies, sí que permite diferenciar entre aislados que se agrupan en dichos filotipos o unidades operativas taxonómicas (OTUs, Operacional Taxonomic Unit, ver Fig. 4). Estos OTUs se consideran equivalentes a la categoría de "especie" a la hora de describir y comparar poblaciones y comunidades de bacterias (Ovreas 2000) (**manuscritos I y VI**).

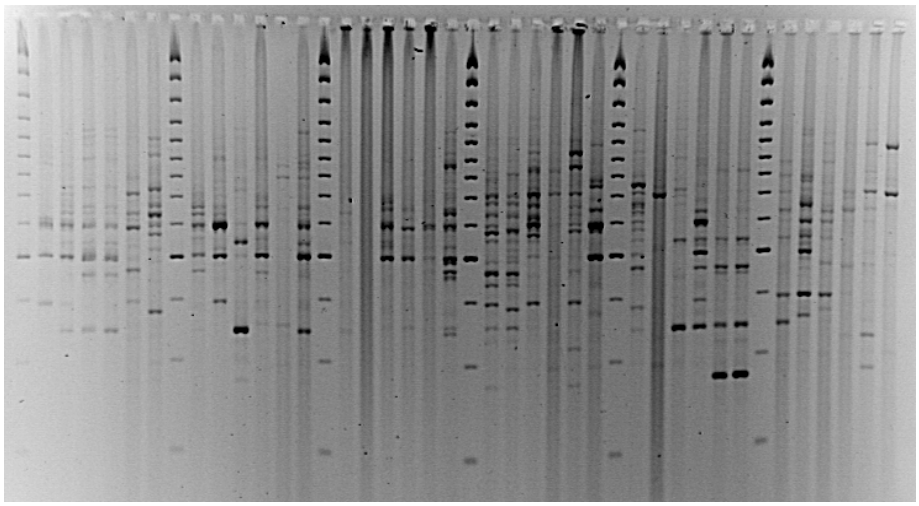


Fig. 4. *Resultados del RISA. En cada columna hay una muestra perteneciente a un pollo, y cada banda representa un OTU.*

Como variables indicadoras de la calidad fenotípica de los pollos de urraca y críalo, a la misma edad de la toma de muestras bacterianas (ver apartado anterior), por un lado, se les midió la respuesta inmune a un antígeno no patogénico (fitohemoaglutinina) que desencadena una respuesta inmunitaria mediada por linfocitos T, a la que también se suman macrófagos y algunas moléculas del HMC (e.g. Goto *et al*. 1978; Martin *et al*. 2006). Esta medida es muy utilizada en ecología evolutiva (Kennedy & Nager 2006) ya que es un buen indicador de la probabilidad de supervivencia de adultos y de pollos tras abandonar el nido (Soler *et al*. 1999b; Møller & Saino 2004; Moreno *et al*. 2005; Cichon & Dubiec 2005). Por otro lado, a todos los pollos se les tomaron medidas biométricas (peso y tarso) para calcular su condición física, variables que tradicionalmente se han considerado indicadoras de la probabilidad de reclutamiento de juveniles a la población reproductora (e.g. Perrins 1965) (ver **manuscrito I**).

(b) *Glándula uropigial de la abubilla.*

(b.1) *Estudio de la actividad antimicrobiana de la secreción*

(b.1.1.) Experimentos *in situ*: diferencias en la actividad antagonista entre secreciones blancas y oscuras.

De todos los individuos capturados, con la ayuda de una pipeta automática y puntas estériles que se introducían en la ampolla de la glándula, se extrajeron muestras de sus secreciones. Una

parte de la secreción (5µl.) se sembró *in situ* en placas de BHI en las que previamente se había inoculado la bacteria *Bacillus licheniformis* que, posteriormente en el laboratorio, se incubaba a 37ºC durante 24 h para comprobar si la SU inhibía crecimiento de los bacilos patógenos (Figura 4, **manuscrito II**). El resto de la secreción se introducía en tubos estériles y se guardaban en frío hasta su posterior análisis y procesamiento en el laboratorio (ver más adelante).

(b.1.2.) Experimentos *in vitro*: estudio de la variación entre cepas.

Con las secreciones recogidas en el campo, se realizaban siembras (5µl.) en placas de Petri con distintos medios de cultivo hasta que el conteo en placa de colonias aisladas fuera posible. Como medios de cultivo se utilizaban uno genérico, TSA, y uno específico de enterococos, KF. Todas las placas se incubaban a 37º C durante 24 h en el caso de TSA, y 62 h las de KF. De entre todas las colonias crecidas y aisladas en el medio general, se seleccionaban 5 al azar, que se volvían a sembrar en medio general líquido, el cual, después de un periodo de incubación de 24 h, se centrifugaba y se lavaba con agua destilada dos veces, de forma que conseguíamos aislar un volumen considerable de células con las que realizábamos los análisis genéticos. La extracción de ADN se hacía con ayuda del kit "Aquapure Genomic DNA" (BIO-RAD, España), y posteriormente se amplificaban mediante la técnica de RAPD (Fig. 5), con el "primer" M13. Esta técnica permite identificar perfiles genéticos de distintas colonias, y agruparlos por similaridad del patrón de bandas (ver **manuscritos IV** y **VII**). El método identifica variabilidad intraespecífica, por lo que las distintas cepas (perfiles de bandas que se agrupan juntos) pueden pertenecer a la misma especie bacteriana. Esto permite distinguir el origen de las cepas bacterianas presentes en los pollos del experimento incluso si los de distintos nidos son de la misma especie.

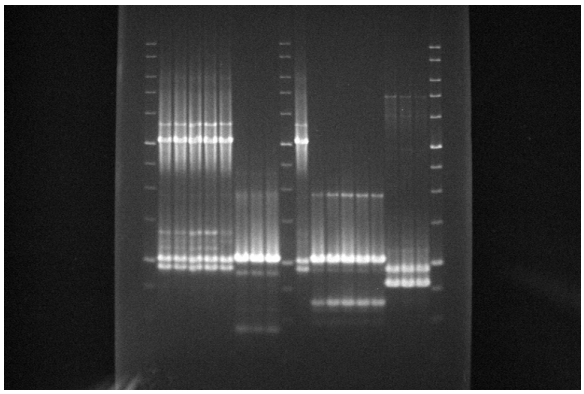


Fig. 5. *Resultado del RAPD. Cada columna corresponde a una colonia con un perfil genético de bandas.*

De las colonias aisladas de las muestras de secreción de los años 2003 y 2004 se estimó su actividad antimicrobiana frente a dos bacterias, *Enterococcus faecalis* y *Listeria inocua* (**manuscrito IV**). Estas pruebas de antagonismo se realizaron mediante la técnica de la doble capa (Gratia & Frederiq 1946), que consiste en depositar una capa de medio de cultivo previamente inoculado con la bacteria control (*E. faecalis* o *L. inocua*) sobre un cultivo en el que se encuentran ya desarrolladas las colonias de la bacteria de la que queremos conocer su actividad (ver figura 6).

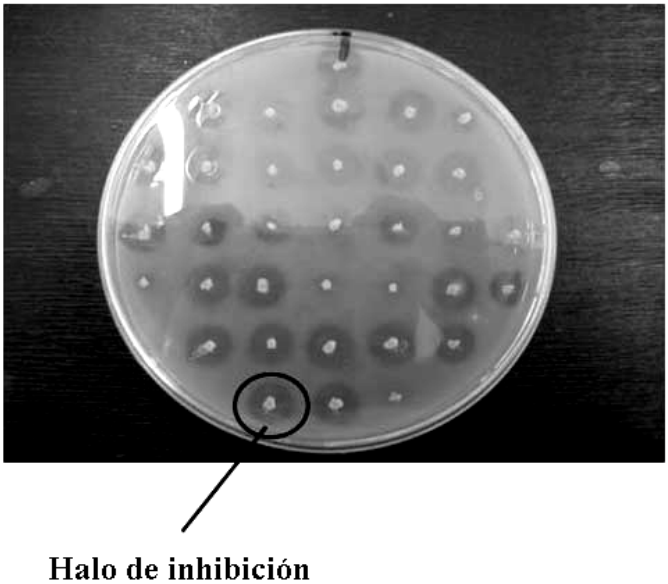


Fig. 6. *Placa de Petri en la que se han sembrado colonias aisladas de la SU, y posteriormente se cubrió con una sobrecapa con la bacteria indicadora.*

(b.2) *Influencia de las bacterias en el tamaño de la glándula uropigial de abubillas así como en el color y cantidad de la secreción que produce.*

En el campo se manipuló la comunidad bacteriana de la GU mediante la inyección de un antibiótico de amplio espectro (amoxicilina, Clamoxyl GlaxoSmithKline S.A.) para explorar sus efectos en características morfológicas de la glándula y de su secreción. En 2004 se manipularon hembras durante su periodo de estancia en el nido, mientras que los años 2005 y 2006 se manipularon los pollos. El antibiótico (0.04 ml) se inyectaba directamente en el tejido glandular de cada uno de los dos lóbulos principales (0.02 ml en cada lóbulo) y, desde ahí, fluía hasta la papila, lugar donde se encuentra almacenada la secreción junto a las bacterias simbiontes. Como control, a algunas hembras seleccionadas de forma aleatoria se les inyectaba solución salina. La selección de pollos controles y experimentales, sin embargo, se realizaba entre pollos hermanos que compartían el nido y teniendo en cuenta su peso corporal. Debido a que la jerarquía de tamaños de pollos que se establece en esta especie es muy acusada, los pollos se ordenaban según su tamaño, el tratamiento del pollo más pesado se decidía de forma aleatoria y el de los siguientes en la jerarquía se asignaba de forma alternativa al decidido para el primero (para mas información ver **manuscrito III**).

A todos los individuos adultos capturados (reproductores y no reproductores), así como a los pollos, se les tomaron medidas de la GU (detallado en el **manuscrito III**). Además, durante el experimento de inoculación de antibiótico a la GU, parte de la secreción se recogía directamente de la glándula con un capilar para, con la ayuda del Atlas de color de Küpper, valorar de forma objetiva su color (**manuscrito III**).

(c) *Beneficios directos de las bacterias simbiontes de la glándula uropigial sobre la abubilla hospedadora*

(c.1) *Influencia sobre el éxito de eclosión*

En el año 2005 tratamos de eliminar experimentalmente los hipotéticos efectos positivos de las bacterias simbiontes en la prevención de infecciones de embriones por agentes patógenos. Debido a que estos efectos positivos estarían mediados por las bacteriocinas producidas por los simbiontes, el experimento consistió en difundir proteasa (sustancia inhibidora de la

bacteriocina presente en la secreción) diluida en agua destilada en los nidos experimentales y sólo agua destilada en los nidos controles permitiendo un contacto prolongado de los huevos con estas secuencias acuosas. Para ello, el fondo de las cajas nido utilizadas por las abubillas se cubrió con una moqueta permeable por la parte superiori e impermeable por la parte inferior y de un color marrón oscuro que, posteriormente, se cubría parcialmente con el material disponible en el fondo de las cajas (cortezas de pino trituradas introducidas por nosotros al instalar las cajas o restos de nidos de otros años), y se perforaba con una aguja conectada a un catéter de unos 4 mm de grosor. El otro extremo de la sonda se sacaba al exterior y permitía la perfusión de proteasa o de agua en la moqueta que quedaba en contacto directo con los huevos de abubilla sin molestar a la hembra que, en la mayoría de los casos, permanecía incubando. Estas perfusiones se realizaban cada dos días, lo que aseguraba el continuo contacto de los huevos de esta especie con soluciones de proteasa, o directamente con agua, durante toda la incubación. También cada dos días eran visitados los nidos pertenecientes a un tercer grupo control de manipulación, al que no se le administraba ninguna sustancia, tan solo se les visitaba.

El mismo experimento se realizó en nidos de estornino (*Sturnus unicolor*) que nos sirvió como control del efecto de la proteasa directamente sobre los huevos, ya que en la GU del estornino no se han descrito bacteriocinas. Sin embargo, esta especie utiliza gran cantidad de material vegetal en sus nidos por lo que el uso de la moqueta no era efectivo. Por ello, cada dos días visitábamos los nidos y rociábamos los huevos con agua destilada o con solución de proteasa. El efecto de este experimento se buscó en los fallos de eclosión y, también, en la densidad de bacterias que se detectaban en las cáscaras de sus huevos antes de la manipulación y poco antes de la eclosión. Para ello, los huevos de ambas especies se limpiaron con la ayuda de hisopos mojados en agua destilada estéril y, posteriormente, los algodones con los restos de haber limpiado los huevos se guardaron en tubos eppendorfs que ya contenían 1 ml. de agua destilada estéril hasta su posterior siembra en el laboratorio, que solía ser en el mismo día. Tras mezclar bien la muestra del lavado de huevos, 5µl se sembraban en una placa de Petri con un medio de cultivo para enterobacterias (Hektoen). Estas siembras y el posterior cuenteo permite

una estima de la densidad de bacterias presentes en la cáscara de huevos de abubillas y de estorninos sometidos a distintos tratamientos (para mas información ver **manuscrito II**).

(c.2) *Influencia sobre la degradación de plumas*

Para determinar el efecto que las bacterias mutualistas de la abubilla podrían tener sobre organismos degradadores de las plumas, se hicieron experimentos *in vitro* en los que se estimó la degradación de plumas y de queratina sintética (principal componente de las plumas) sometidas a distintos tratamientos. Por un lado, los tratamientos experimentales consistieron en la siembra sobre plumas y queratina de una bacteria queratinolítica (*Bacillus licheniformis*), evaluándose la degradación que la bacteria producía en presencia y en ausencia de (1) una cepa de bacteria simbionte aislada de un pollo de abubilla (*Enterococcus faecalis* MRR-10.3) y de (2) la bacteriocina producida por ella. Estas muestras se cultivaron a 37º C durante 7 días en el caso de las plumas, y 16 en el caso de la queratina. Después de estos periodos, se obtuvieron fotografías de las plumas con un microscopio electrónico de barrido (Gemini 1530, Leo, Overkogen, Alemania, ver Figura 7). El nivel de degradación se estimó en una escala de 1 a 5 por investigadores que no conocían el tratamiento al que habían estado sometidas las plumas.

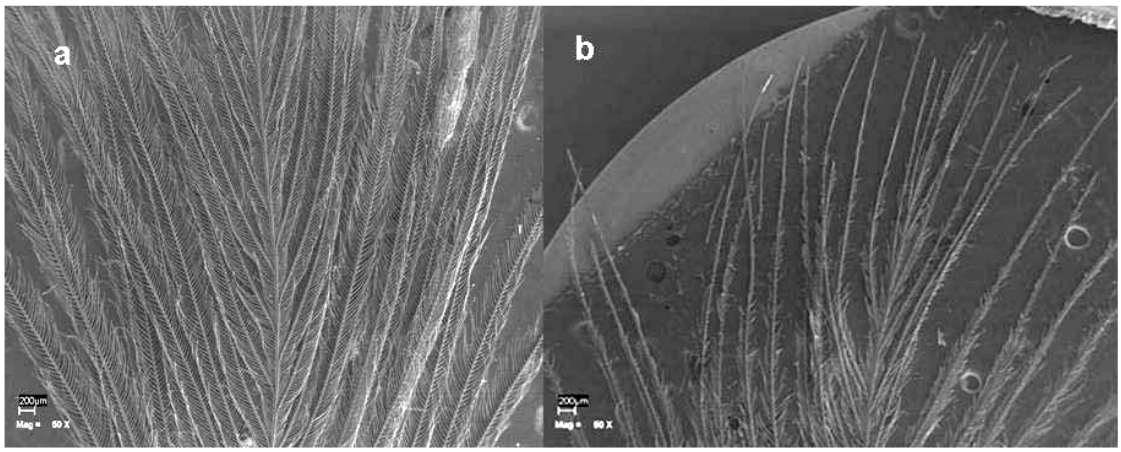


Fig. 7. *Ejemplo de (a) una pluma poco degradada y (b) una pluma muy degradada, fotografiadas a 60 aumentos.*

La estima de la degradación de muestras de queratina sintética sometidas a distintos tratamientos se calculó con la ayuda de un espectrofotómetro, estimando la absorbancia a una longitud de onda de 595 nm, donde se encuentra el pico de absorvancia para la queratina azul

sintética (Santos *et al*. 1996). Para más información sobre el diseño experimental y las estimas de degradación, ver el **manuscrito V**.

Dinámica de las relaciones entre aves y bacterias: Influencia del ambiente y modo de adquisición de los simbiontes por el hospedador.

(a) *Bacterias digestivas en un parásito de cría.*

Los experimentos llevados a cabo en el campo consistieron en hacer cambios de pollos de críalo y de urraca de 2 - 3 días de edad entre nidos de urracas parasitados y no parasitados. El objetivo de estos cambios era conseguir nidos en los que dos pollos de urraca y dos de críalo fueran criados por los mismos padres. Los pollos experimentales de ambas especies eran de la misma edad para evitar en gran medida la ventaja competitiva de los pollos parásitos y la muerte de los más pequeños. De este modo, podíamos estudiar la comunidad bacteriana en dos especies filogenéticamente distantes que han compartido el mismo ambiente desde que nacieron hasta que abandonan el nido (**manuscrito VI**). Los análisis de laboratorio de la comunidad bacteriana están descritos en el apartado I. a.

(b) *Adquisición de bacterias de la glándula uropigial de abubillas.*

En el año 2006 se llevaron a cabo experimentos de cross-fostering con pollos de abubillas. Estos cambios de pollos entre nidos se realizaron a edades tempranas (2-3 días de edad), antes de que ocurriera el exagerado desarrollo de sus GU. La caracterización y la identificación de las bacterias que se encontraban en sus glándulas poco antes de abandonar el nido, y la comparación con las que se encontraban en sus hermanastros (criados en el mismo pero nacidos en distinto nido) o hermanos (nacidos en el mismo pero criados en distinto nido), permite identificar la influencia del nido (es decir, nido de origen y/o nido destino). Por ejemplo, si las bacterias son más similares entre hermanos que entre hermanastros, nos indicaría que la adquisición del simbionte tiene lugar antes del experimento (en la formación de huevo o a través de su cáscara) (para mas información ver **manuscrito VII**). El procesamiento de las muestras en el laboratorio está descrito en el apartado b.1.2.

IV. TRATAMIENTO ESTADÍSTICO DE LOS DATOS

Para comparar la composición de las comunidades bacterianas digestivas de los pollos de urraca y de críalo se calcularon matrices de similaridad entre las muestras mediante el análisis informático de los geles obtenidos del método de RISA (manuscritos **I** y **VI**, Fig. 4). Para ello se utilizó el programa Gel Compare Software (Applied Maths, Kortrijk, Belgium), que asigna a cada banda (que a su vez se corresponde con un OTU) un tamaño determinado medido en pares de bases, considerando aquellas con un tamaño diferente como dos OTUs distintos. Las matrices de similaridad se construyeron mediante el coeficiente binario de Dice, que calcula las diferencias entre dos muestras (cada una correspondía a un pollo, ver Fig. 4) siguiendo la fórmula 2a/(2a+b+c), en la que "a" es el número de OTUs que ambas muestras comparten, "b" el número de OTUs presente sólo en la primera muestra, y "c" el número de OTUs presentes sólo en la segunda muestra. De este modo, el resultado de dicho coeficiente da lugar a las matrices de similaridad con valores oscilan entre 0 (si dos muestras no comparten ningún OTU) y 1 (si tienen exactamente la misma comunidad).

Con el fin de establecer relaciones entre las comunidades bacterianas con las variables especie del pollo y nido, construimos otras matrices binarias, con valores 0 y 1, en las que 0 implicaría ser de distinta especie o bien pertenecer a distinto nido, mientras que el 1 significaría lo contrario. Una correlación significativa positiva nos estaría indicando que para muestras de la misma especie o del mismo nido, las comunidades bacterianas son más similares entre ellas que con el resto de muestras (**manuscrito VI**). En cambio, las matrices de similaridad construidas para las variables indicativas de calidad (esto es, los índices de respuesta inmunitaria y de condición física) se calcularon dividiendo el valor más pequeño por el más alto, de forma que los valores de la matriz obtenida oscilan entre el 0 (para valores muy diferentes) y el 1 (para valores idénticos).

Por otro lado, los geles resultantes del análisis de RAPD (**manuscritos IV** y **VII**, Fig. 5) se analizaron con el programa Fingerprinting II Informatrix (Bio Rad, Hercules, CA). En estos geles, cada perfil de bandas corresponde a un clon bacteriano multiplicado a partir de una única colonia aislada de las secreciones. Cada banda representa un fragmento del genoma de ese clon

amplificado, por lo que el perfil completo representa una huella dactilar del genoma de cada clon que puede compararse con los de otras colonias aisladas para comprobar similitud y agruparlos en cepas.

El índice usado en este análisis fue el de correlación del producto - momento de Pearson, ampliamente usado en el análisis de similaridad entre dos muestras sometidas a RAPD (ver por ejemplo Rossetti & Giraffa 2005; Psoni *et al*. 2006; Morandi *et al*. 2006). En este caso, no sólo se consideran la presencia o ausencia de las bandas, sino que además se tiene en cuenta su intensidad. Esta intensidad está directamente relacionada con la cantidad de ADN amplificado, y por lo tanto, con la cantidad de ADN de la que se parte, lo que se tiene en cuenta para diferenciar las distintas cepas.

Las matrices se analizaron mediante el Test de Mantel, en el programa F-stat (Goudet 1995). El test de Mantel estima el grado de correlación entre dos matrices *X* e *Y*. La hipótesis nula de esta técnica (H_o) postula que las distancias/similitudes entre las variables de la matriz respuesta *Y* no están linealmente correlacionados con las correspondientes distancias/similitudes en la matriz modelo *X*. Se trata, por tanto, de evaluar si la asociación (positiva o negativa) es más robusta de lo que cabría esperar por puro azar. El estadístico del test de Mantel (Z_M) se calcula mediante la suma de los productos cruzados de los valores de las dos matrices de similitud/distancia, excluyendo la diagonal principal que sólo contiene valores triviales (en el caso de todas las matrices de similitudes calculadas en esta tesis, la diagonal sería siempre 1).

En los **manuscritos II, III**, y **V** los análisis se hicieron mediante modelos generales lineales (GLM, General Linear Model). El programa utilizado fue Statistica 6.0 (Statsoft 2001) Software.

RESULTADOS Y DISCUSIÓN

I. EVIDENCIAS DE LA EXISTENCIA DE MUTUALISMOS. VENTAJAS OBTENIDAS POR LOS HOSPEDADORES.

(a) *Relaciones entre la comunidad bacteriana y la capacidad de respuesta inmune mediada por linfocitos T y la condición física del hospedador* (**Manuscrito I**).

Encontramos que la comunidad bacteriana en urracas se relaciona significativamente con su respuesta inmune a la PHA, mientras que en los críalos se relaciona con su condición física. En estos últimos encontramos además una relación significativa entre la condición y su respuesta inmune que trabajos previos con mayores tamaños de muestra también la encontraron para urracas (Soler *et al*. 1999a). La comunidad bacteriana tiene un importante papel como procesadora del alimento, por lo que está directamente relacionada con el estado nutricional del individuo (Glunder 2002; Xu *et al*. 2003; Engberg *et al*. 2004) y con variables relacionadas con su calidad fenotípica (Moreno *et al*. 2003). Las variables indicativas de calidad individual pueden ser determinantes de la futura supervivencia y éxito reproductor en pollos (por ejemplo Alatalo *et al*. 1990 y Linden *et al*. 1992 sobre condición física; Christe *et al*. 2001 y Møller & Saino 2004 sobre respuesta inmune). En este trabajo, encontramos una relación significativa entre la respuesta inmune y condición física con la estructura de la comunidad bacteriana de los pollos que están apunto de abandonar el nido; por lo tanto, podríamos considerar a la comunidad bacteriana de un individuo (que a la vez, al igual que la condición fenotípica, estará relacionada con los nutrientes aportados por los padres) como predictora de su probabilidad de supervivencia futura.

El hecho de que características de la microbiota de críalos y de urracas se relacionen respectivamente con la condición física y con el grado de respuesta inmune puede ser debido a que ambas especies están sometidas a distintas presiones selectivas, y a que la energía y los nutrientes de los que dispone un pollo durante su crecimiento son limitantes existiendo un compromiso entre la inversión en desarrollo del sistema inmune y en crecimiento (Soler *et al*.

2003). Por ejemplo, los pollos de urraca se enfrentan a los parásitos generalistas y específicos, mientras que los críalos sólo se enfrentarían a los parásitos generales (Soler *et al*. 1999a). En este sentido, a las urracas les interesaría más invertir la energía obtenida en el sistema inmune para protegerse de una mayor tasa de parasitismo. En cambio, el críalo al poco tiempo de abandonar el nido ha de migrar miles de kilómetros, por lo que una priorización de inversión de recursos en condición física que le permita realizar este largo viaje podría tener sus ventajas.

(b) *Glándula uropigial de la abubilla*

(b.1) *Estudio de la actividad antimicrobiana de la secreción*

(b.1.1.) Experimentos *in situ*: diferencias en la actividad antagonista entre secreciones blancas y oscuras.

Al sembrar las secreciones oscuras en medios sólidos se obtuvo un elevado número de enterococos; en cambio, no creció ni una sola colonia de enterococos en las siembras de secreciones blancas. Además, encontramos que al inocular directamente la secreción oscura de hembras y de pollos sobre una placa sembrada conla bacteria *Bacillus licheniformis*, la SU inhibió el crecimiento de la bacteria. Las secreciones blancas por el contrario no inhibieron el crecimiento de *B. licheniformis* (ver figura 4 y **manuscrito II** para todos los detalles). Por lo tanto, se confirma que el cambio en el tipo de secreción en época reproductora supone un cambio en la capacidad de inhibición de los microorganismos potencialmente patógenos. Además, también comprobamos que las colonias de enterococos aisladas de las SU oscuras produjeron halos de inhibición (ver Fig. 8).

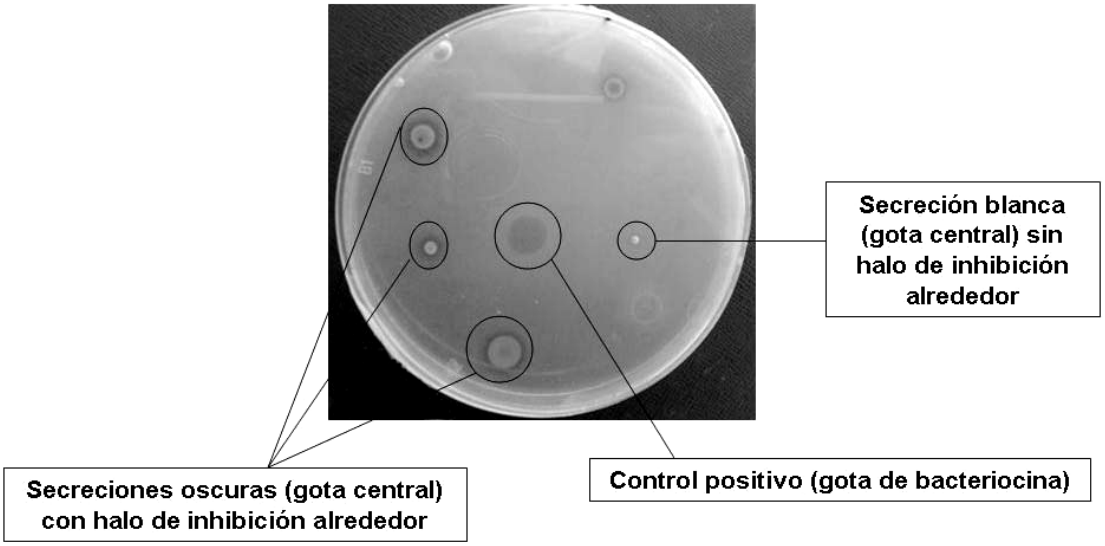


Fig. 8. *Placa de Petri en la que se ha realizado una prueba de antagonismo. Se aprecian halos de inhibición producidos por SU oscuras (rodeadas por un halo transparente), que es el lugar donde la secreción ha inhibido el crecimiento de B. licheniformis, y una muestra de SU blanca que no ha producido inhibición del bacilo.*

(b.1.2.) Experimentos *in vitro*: estudio de la variación entre cepas (**Manuscrito IV**).

Tras analizar los perfiles genéticos de las distintas cepas aisladas de la secreción uropygial de abubillas, y comprobar su capacidad de inhibición sobre dos cepas bacterianas (*E. faecalis* y *Listeria inocua*), hemos detectado variabilidad en su capacidad antibacteriana. Colonias agrupadas en la misma cepa (pertenecientes al mismo grupo genético deducido mediante el análisis RAPD, ver material y métodos y **manuscritos IV** y **VII**) tienen la misma actividad antagonista ante otras bacterias, mientras que hay diferencias en la actividad entre cepas pertenecientes a distintos grupos RAPD. Esto implica variabilidad en el potencial inhibidor de las cepas y, por tanto, en el valor de la bacteria para la abubilla hospedadora (Douglas 1998). Por ello, el carácter de capacidad antibacteriana de las cepas podría estar sometido a procesos de seleccionpor parte de las abubillas ya que en aquellos nidos en que vivan cepas con un mayor potencial inhibidor frente a patógenos, el éxito reproductor de las abubillas sería mayor (i.e. menos patógenos que comprometan el desarrollo de los pollos).

(b.2) *Influencia de las bacterias en el tamaño de la glándula uropigial de abubillas así como en el color y cantidad de la secreción que produce.*

Encontramos diferencias sexuales significativas en el volumen de la GU de individuos que aun no habían comenzado la reproducción (i.e. machos y hembras con SU blanca) (Fig. 9). Sin embargo, estas diferencias en tamaño no se tradujeron en diferencias en volumen de secreción almacenada en la glándula.

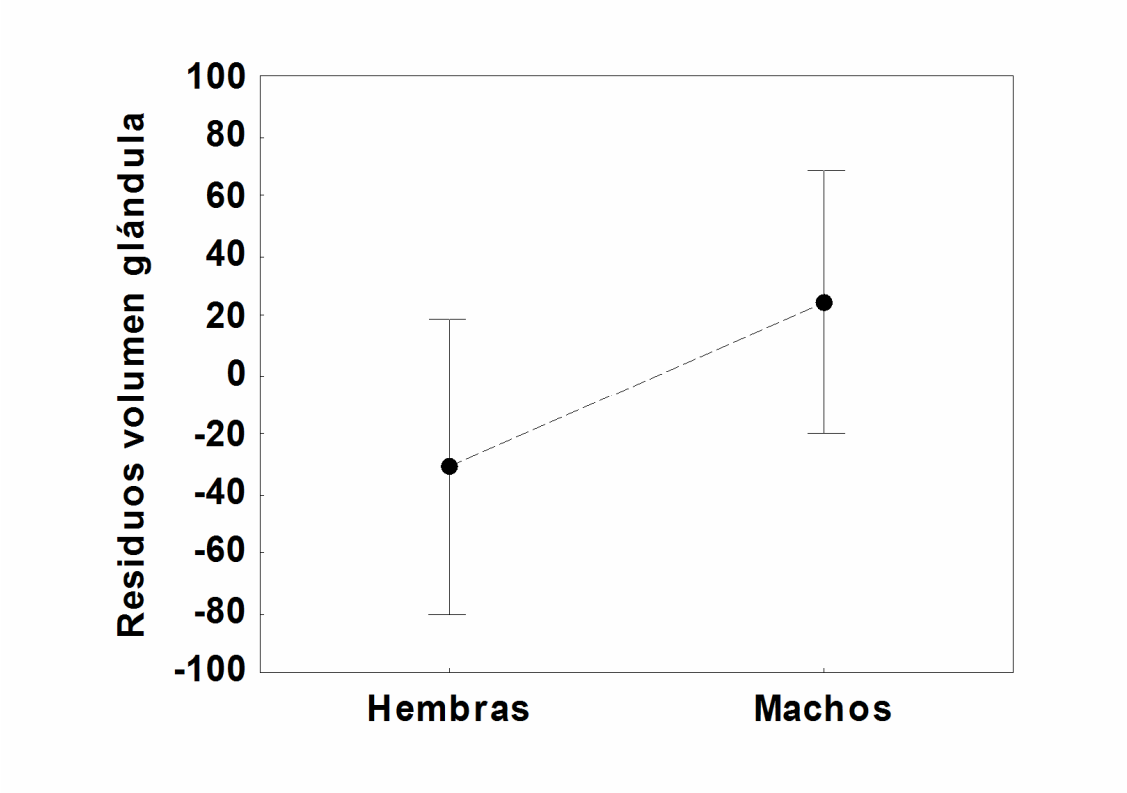


Fig. 9. *Resultado del GLM en el que se analizó el volumen de la glándula uropigial en ambos sexos en el período en que esta producía secreción blanca, corregido por el año y la longitud del tarso del individuo. Los puntos representan la media, y las barras el intervalo de confianza del 95%.*

En cambio, durante la reproducción, cuando la secreción de las hembras, pero no la de machos, es oscura y olorosa, las diferencias sexuales en el volumen de la GU eran significativas pero en sentido contrario (Fig. 10a). Además, el volumen de secreción producida por las hembras también es significativamente mayor que en los machos (Fig. 10b).

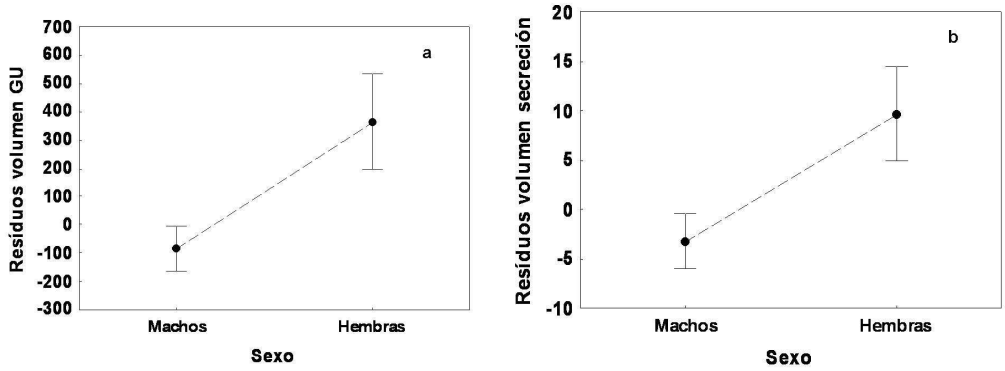


Fig. 10. *Resultado del GLM en el que se analizaron las diferencias en época reproductora entre (a) el volumen de la GU de hembras y machos, y (b) entre el volumen de la secreción producida por cada sexo. En ambos casos los análisis están corregidos por el año y la longitud del tarso del individuo. Los puntos representan la media, y las barras el intervalo de confianza del 95%.*

Por lo tanto, el cambio en las propiedades de la SU en hembras en la época reproductora viene acompañado por cambios morfológicos de la GU que, a su vez, se asocian con un mayor volumen de secreción. Estos cambios podrían desempeñar un importante papel en hembras durante su estancia en el nido.

El resultado de la eliminación experimental de las bacterias de la glándula, produjo los siguientes resultados:

Pollos

El tamaño de la GU de los pollos no se vio afectado por la eliminación experimental de los enterococos con el tratamiento con antibiótico; en cambio, sí se observó una diferencia significativa en el color de la secreción (en el nivel de magenta y de negro) entre los pollos experimentales y controles, siendo en estos últimos más oscura y menos rojiza (Fig. 11). También difirió significativamente el volumen de secreción producida por ambos grupos (Fig. 12).

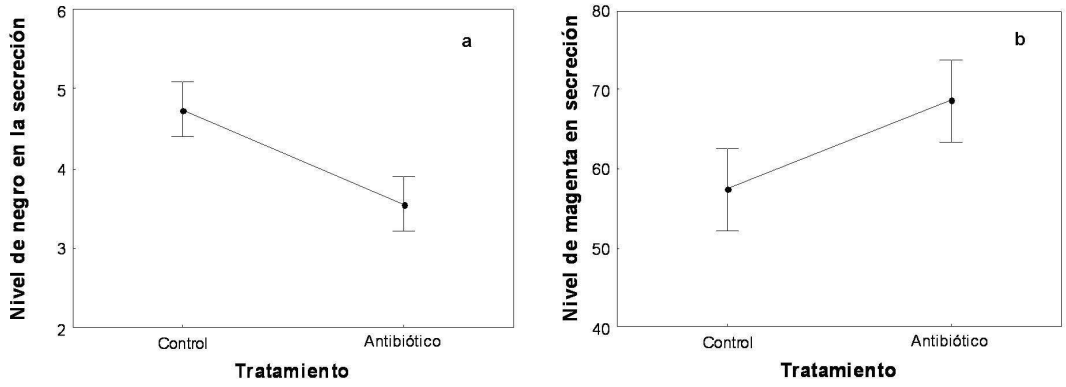


Fig. 11. *Representación de la media (puntos) con un intervalo de confianza del 95% (barras) del valor de nivel de (a) negro y (b) magenta según la escala del Atlas de color de Küppler, en pollos experimentales (inyectados con antibiótico) y controles (inyectados con solución salina).*

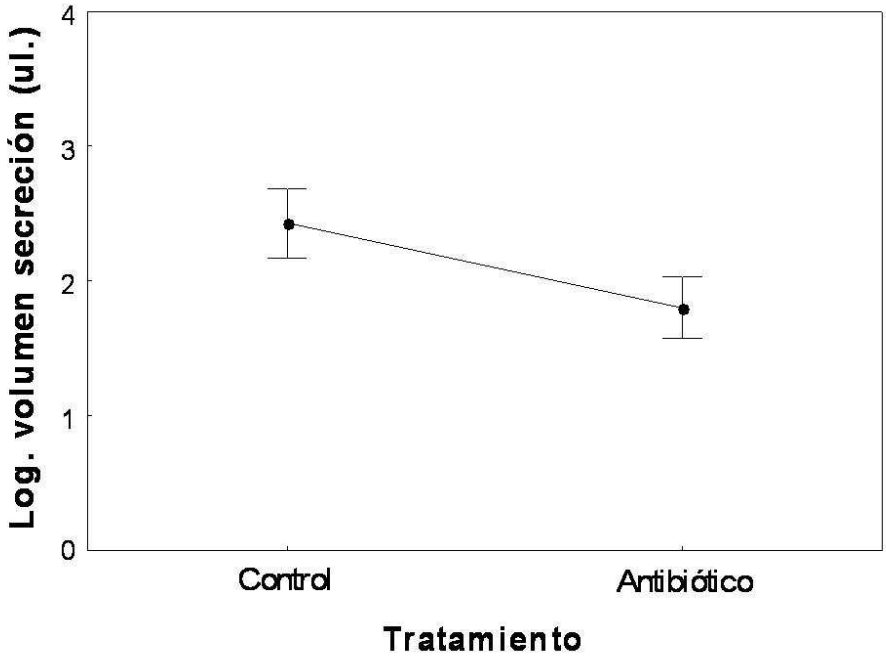


Fig. 12. *Representación de la media (puntos) con un intervalo de confianza del 95% (barras) del volumen de secreción producida por los pollos transformado logaritmicamente.*

Hembras

Se observó un efecto del tratamiento con antibiótico consistente en una disminución significativamente mayor de la altura de la GU entre medidas sucesivas en las mismas hembras (Fig. 13a), y también en el color de la SU, con un aumento que resultó significativo en el nivel de magenta (Fig. 13b).

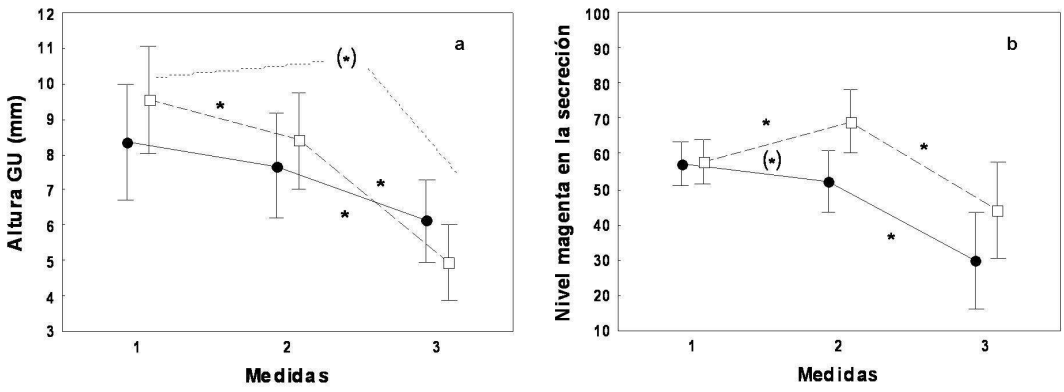


Fig. 13. *Representación de las medias (círculos negros, hembras experimentales inyectadas con antibiótico; cuadrados blancos, hembras controles inyectadas con solución salina) y los intervalos de confianza del 95% (barras) con respecto a (a) la altura de la GU y (b) el nivel de color magenta de la secreción (según la escala del Atlas de color de Küpler). Las medidas corresponden a (1) antes del tratamiento, (2) después de 2 inyecciones, y (3) 7 días tras la tercera inyección. * indica que existe una diferencia significativa entre medidas consecutivas del mismo tratamiento, mientras que (*) indica interacciones significativas entre el tratamiento con antibiótico y las medidas consecutivas.*

Además, en preparaciones microscópicas de cortes de GU de individuos encontrados recién muertos por depredación, como en secreciones extraídas en condiciones asépticas de individuos vivos y fijadas en glutaraldehido inmediatamente tras su obtención, encontramos que las bacterias se hayan físicamente en la secreción, y en el interior de la ampolla donde ésta se acumula. Tanto estas imágenes como los efectos del tratamiento con antibiótico son pruebas irrefutables de que existe una comunidad bacteriana albergada de forma natural en la GU y que además es responsable de algunas de las propiedades de la secreción (ver Fig. 14, y manuscrito II para más detalles).

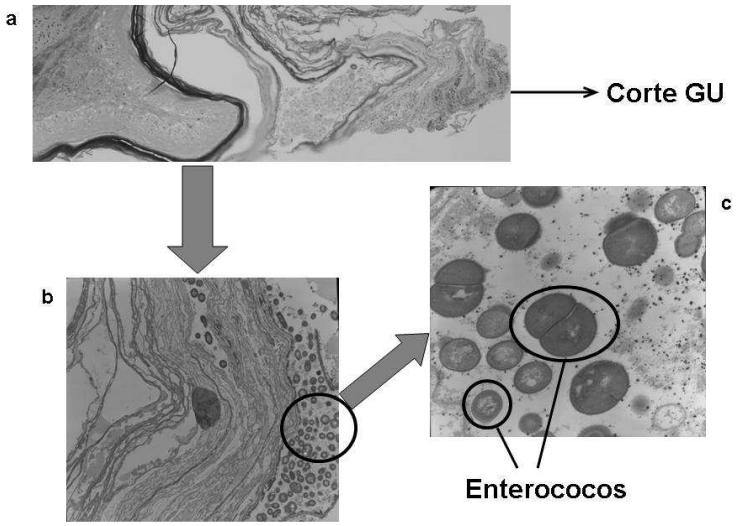


Fig. 14. *Fotos de microscopio electrónico de la glándula uropigial tomadas a (a) 40 aumentos, (b) a 5000 aumentos, dentro del círculo se pueden apreciar las bacterias, y (c) a 25000 aumentos, donde se pueden observar los enterococos.*

Considerando todos estos resultados, podemos concluir que las bacterias que viven en la GU de hembras y pollos de abubilla son las responsables, al menos en parte, de determinadas características tanto de la morfología de la GU como de la de su secreción. Este resultado coincide con el encontrado por Law-Brown & Meyers (2003) en la abubilla arbórea, donde también se comprobó que al eliminar experimentalmente las bacterias, las propiedades de la SU variaban, tanto en olor y color como en viscosidad.

(c) *Beneficios directos de las bacterias simbiontes de la glándula uropigial sobre la abubilla hospedadora*

(c.1) *Influencia sobre el éxito de eclosión (**Manuscrito II**).*

En las puestas en las que se inhibió experimentalmente la acción de la bacteriocina, encontramos que había un mayor número de bacterias en la superficie de los huevos (Fig. 15a), a la vez que una menor tasa de eclosión (Fig. 15b).

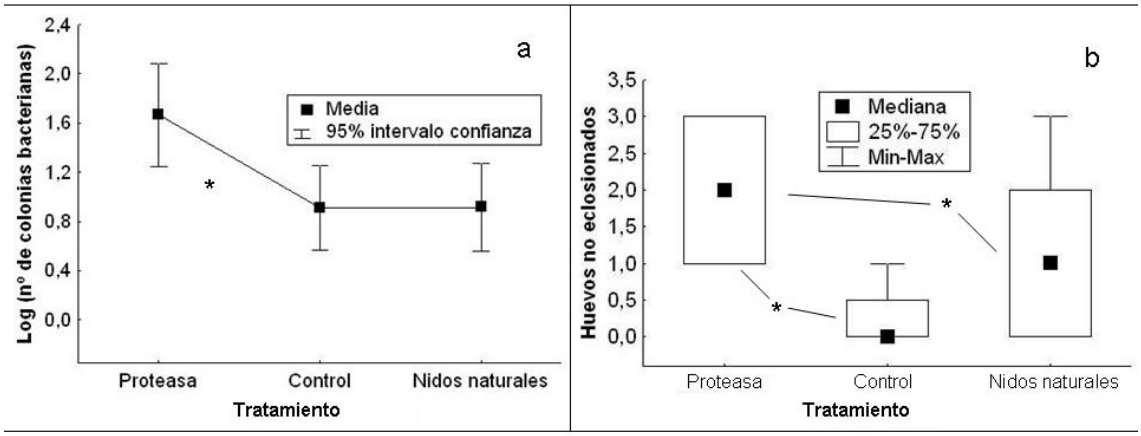


Fig. 15. *Diferencias entre puestas de abubilla controles (rociadas con agua), experimentales (rociadas con proteasa) y naturales (visitadas pero no rociadas) con respecto al número de colonias en la superficie de los huevos (a) y al fracaso de eclosión (b). * representa las diferencias que son estadísticamente significativas ($p<0.05$).*

En cambio, cuando analizamos los resultados del uso de la proteasa en el éxito de eclosión de los estorninos (especie control que consideramos para descartar un posible efecto indirecto de la proteasa en nuestro experimento), encontramos que no hubo ninguna diferencia ni en el número de bacterias ni en el éxito de eclosión entre las puestas experimentales y controles (ver detalles en **manuscrito II**).

Estos resultados sugieren que la bacteriocina producida por los enterococos que habitan en la GU de las hembras de abubilla, o de cualquier otra sustancia defensiva de naturaleza protéica que se segregue en la secrecion uropygial de la abubilla, tiene una influencia directa en el crecimiento de bacterias sobre la superficie de los huevos de esta especie, lo cual repercute en el éxito de eclosión de la puesta. Aunque no podemos descartar que otras sustancias de naturaleza proteica distinta a las bacteriocinas sean las responsables de nuestros resultados, éste junto con resultados del poder antimicrobiano de la secreción y de sus bacterias asociadas, sugiere que la asociación entre enterococos y abubillas podría ser el primer caso descrito hasta ahora de simbiosis mutualistas entre vertebrados y bacterias productoras de sustancias defensivas que habitan en un órgano corporal externo al digestivo.

(c.2) *Influencia sobre la degradación de plumas*

Tras analizar las estimas de degradación ocurridas en las plumas y en la queratina pura, encontramos que tanto la bacteria (*Enterococcus faecalis*) aislada de la SU de abubilla, como su bacteriocina inhiben el efecto queratinolítico de la bacteria *Bacillus licheniformis* s (Fig. 16).

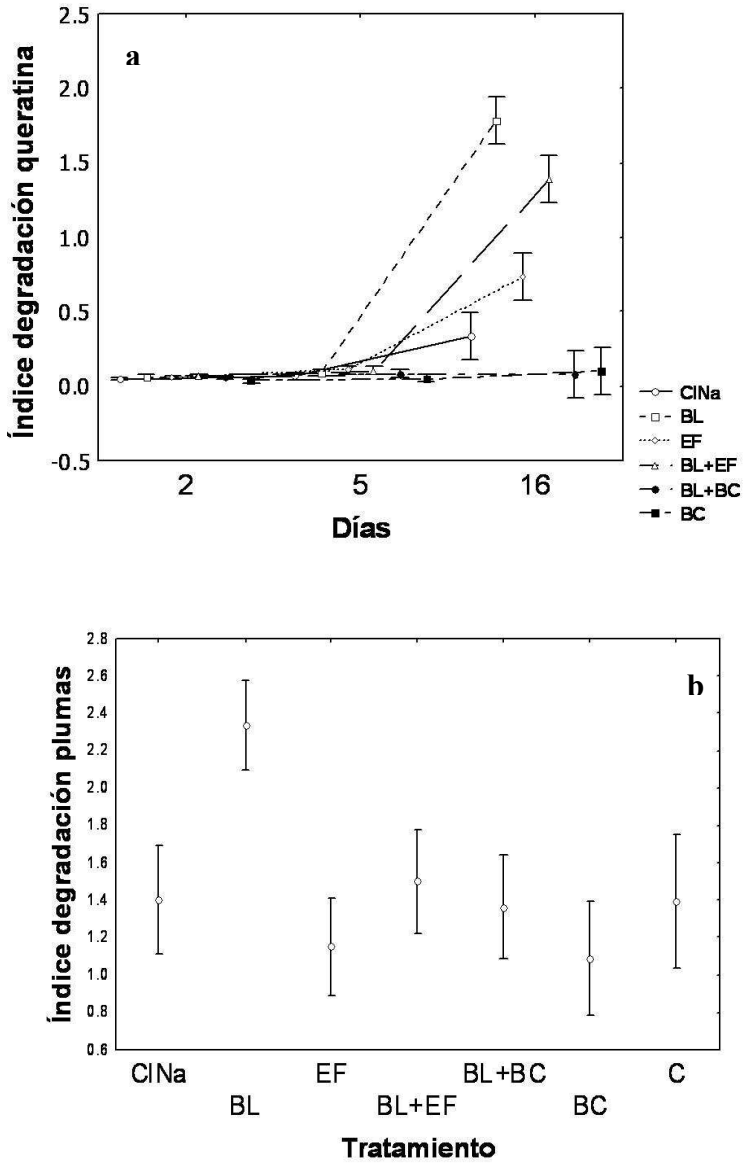


Fig. 16. *Medidas de degradación de (a) queratina (absorbancia, medida a una longitud de inda de 595 nanómetros, valores altos de absorbancia corresponden a niveles elevados de los productos de degradación de la queratina) en relación a los días de incubación y de (b) plumas (valores estimados entre 0-5, en una escala creciente del nivel de degradación); BL: B. licheniformis; EF: E. faecalis; BC: Bacteriocina; ClNa: solución salina; C: pluma control no incubada.*

El correcto desarrollo del plumaje de los pollos es de vital importancia para la capacidad de vuelo (Sttenheim 1976) y cualquier daño que pueda producirse en las plumas implicaría un empeoramiento de esta capacidad (Barbosa *et al*. 2002). Los resultados expuestos en este apartado indican que los pollos de abubilla podrían contar con un arma de lucha frente a potenciales patógenos de sus plumas, como es el caso de *B. licheniformis*. Además, hemos confirmado mediante grabaciones dentro del nido que los pollos realmente se untan la SU por el plumaje (Martín-Vivaldi *et al*. datos no publicados). De este modo, estarían "medicándose" contra parásitos de las plumas, lo que les ayudaría a completar el desarrollo de éstas durante su estancia en el nido de un modo óptimo.

II. Dinámica de las relaciones entre aves y bacterias: Influencia del ambiente y modo de adquisición de los simbiontes por el hospedador.

(a) *Bacterias digestivas en un parásito de cría.*

Como era de esperar de dos aves alejadas filogenéticamente y con dietas distintas, encontramos diferencias anatomícas en su sistema digestivo (Fig. 17). Mientras que el de urracas es más largo, y presenta un segmento intestinal separando los dos lóbulos del hígado, el de los críalos presenta un ciego muy patente que no aparece en urracas, y sus dos lóbulos hepáticos permanecen unidos.

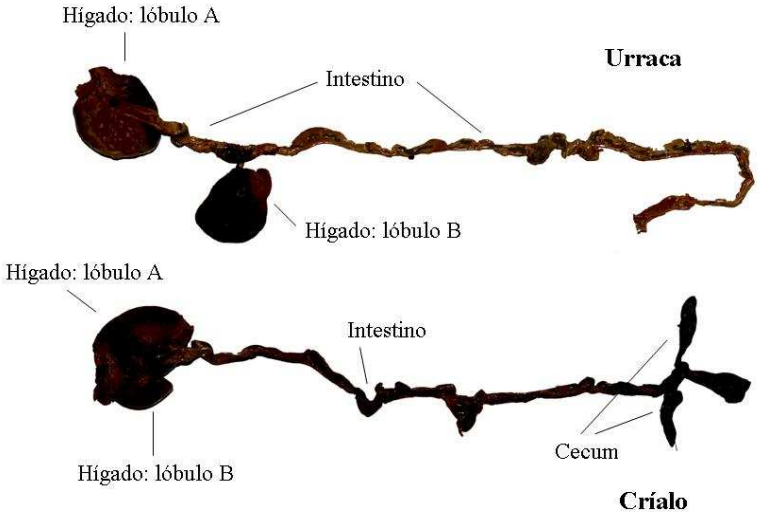


Fig. 17. *Arriba, intestino de pollo de urraca; abajo, intestino de pollo de críalo*

En congruencia con estas diferencias morfológicas, encontramos diferencias significativas entre la microbiota intestinal de ambas especies que eran independientes del nido en que se crían o del que nacieron los pollos. Aunque casi todos los OTUs aislados se encontraron en ambas especies, la frecuencia de cada OTU fue muy variable entre ellas (Fig. 18) dando como resultado comunidades bacterianas diferentes.

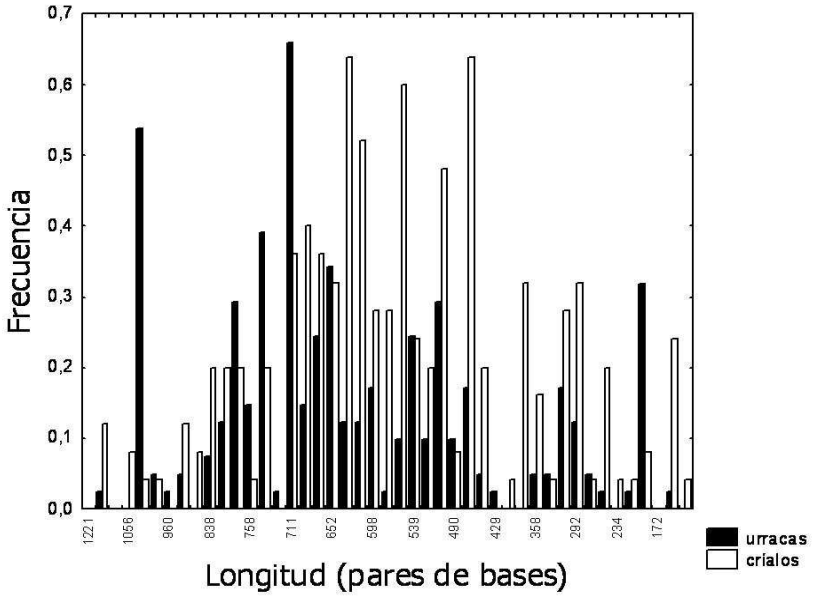


Fig. 18. *Representación de la frecuencia de cada OTU en ambas especies.*

De acuerdo con la importancia de un componente ambiental explicando la flora bacteriana de aves silvestres sugerido en trabajos anteriores (por ejemplo en Lucas & Heeb 2005), no encontramos especies bacterianas exclusivas de críalos o de urraca. Sin embargo, sí que encontramos diferencias en las comunidades bacterianas de las dos aves lo que se podría explicar por constricciones filogenéticas que incluyen la morfología del sistema digestivo (Stevens & Hume 1998).

La comunidad bacteriana de una especie estará optimizada para maximizar la energía obtenida a partir de los nutrientes en que se basa su dieta. Además, los padres al alimentar a los pollos les transfieren componentes importantes de la microbiota con la saliva (Kyle & Kyle 1993) que pueden no tener el mismo efecto en pollos parásitos de cría. Estas diferencias interespecíficas implicarían un distinto aprovechamiento de la comida aportada por los padres de urraca entre sus pollos y los parásitos y que, por tanto, un pollo de críalo necesitaría más comida para obtener una misma cantidad de energía que los pollos de urraca. Sin embargo, las diferencias entre crialos y urracas se debieron a una mayor riqueza de OTUs (diversidad bacteriana) (Fig. 19) en los pollos de críalo, lo cual podría ser una adaptación a la vida parásita ya que una mayor diversidad bacteriana podría permitir una degradación de un abanico más amplio de nutrientes (Loreau 2001). De este modo, alternativamente a las dificultades que le supondría al críalo no compartir las comunidades bacterianas con su hospedador, el albergar una mayor riqueza bacteriana en su intestino le podría asegurar el aprovechamiento de un amplio rango de nutrientes de distintos hospedadores potenciales.

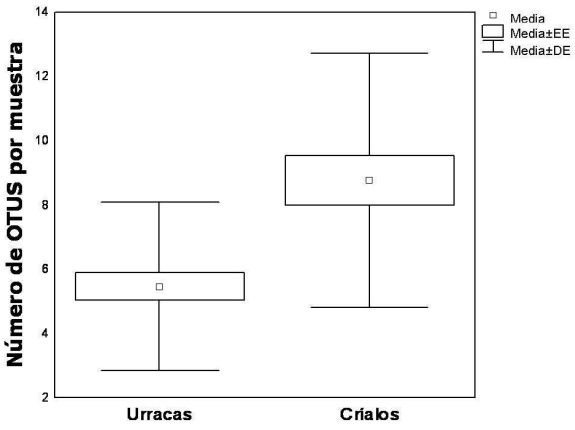


Fig. 19. *Comparación de la riqueza de OTUs en cada especie de ave. (EE: Error Estándar); DE: Desviación Estándar).*

(b) *Adquisición de bacterias de la glándula uropigial de abubillas*. (**Manuscrito VII**).

En primer lugar, nuestros resultados indican que las colonias de enterococos aisladas de un mismo individuo corresponden a perfiles genéticos similares, lo que ocurre también a nivel de nido. El hecho de que los simbiontes de un mismo individuo estén altamente emparentados entre sí se suele considerar como una garantía de cooperación entre ellos, ya que disminuye la competencia, lo cual revierte en un mayor beneficio hacia el hospedador (Frank 1997; Currie *et al*. 2006).

El experimento de cambio de pollos llevado a cabo en 2006 reveló que las colonias aisladas de individuos trasladados de nido presentan un perfil genético similar al de las colonias aisladas de sus hermanos biológicos, criados en distinto nido, y hermanastros, pero diferente al de las de individuos de otros nidos con los que no han estado en contacto. Estos resultados parecen indicar que el nido en que crecen los pollos juega un importante papel en la adquisición de bacterias simbiontes de la glándula uropigial, y que las glándulas se han podido infectar tanto en el nido en el que los pollos han nacido como en el que crecen. Además sugieren que los pollos de abubilla adquieren las cepas antes de tener la GU completamente desarrollada, aunque puede adquirir otras cepas simbiontes mas tarde durante el desarrollo.

CONCLUSIONES

1) La comunidad bacteriana intestinal está significativamente relacionada con determinadas variables fenotípicas del pollo hospedador que, a su vez, son indicativas de la probabilidad de supervivencia. Estos resultados sugieren que la microbiota asociada al sistema digestivo de críalos y de urracas es un factor que afecta a su calidad fenotípica.

2) La secreción uropigial oscura de abubillas tiene actividad antimicrobiana en hembras y pollos durante su estancia en el nido, cuando se han detectado las bacterias. En cambio, en las secreciones blancas de machos y hembras fuera de la estación reproductora no se han detectado ni bacterias ni actividad antimicrobiana.

3) El potencial inhibidor de bacterias patógenas por parte de los enterococos aislados de abubillas varía entre cepas. Esto supone una variabilidad en los beneficios que los enterococos simbiontes pueden aportar a su hospedador por lo que podrían existir mecanismos de selección de las cepas más eficaces por parte de las abubillas hospedadoras.

4) La eliminación experimental de los enterococos simbiontes reveló que estos son los responsables de la morfología de la glándula uropigial en abubillas, así como de la cantidad y color de la secreción que esta produce. Además, comprobamos que dichas bacterias viven dentro de los tejidos glandulares.

5) Los enterococos que viven en la glándula uropigial, a través de la secreción de bacteriocinas, podrían tener beneficios directos para las abubillas. La disminución del éxito de eclosión de los huevos cuando se inutilizan las bacteriocinas mediante proteasas sugiere que esos compuestos están impidiendo el crecimiento de bacterias patógenas en la

cáscara y la infección del embrión. También hemos comprobado que las bacteriocinas producidas por las bacterias simbiontes inhiben el crecimiento de especies con actividad queratinolítica e impiden en gran medida la degradación del plumaje.

6) Los pollos de críalo y urraca criados en el mismo nido difieren tanto en la anatomía del tubo digestivo como en las comunidades bacterianas que este alberga. Los críalos presentan un mayor número de taxones bacterianos que su hospedador. Constricciones filogenéticas podrían explicar las diferencias detectadas en la estructura de la microbiota intestinal de ambas especies.

7) En la población de abubillas estudiada, la técnica de anásis de RAPD nos permitió distinguir varias cepas de enterococos aislados de sus secreciones uropigiales. Para un mismo individuo, y para individuos de un mismo nido, las colonias aisladas suelen pertenecer al mismo grupo RAPD. Además, resultados experimentales sugieren que la adquisición de la bacteria es anterior al desarrollo de la glándula, y que durante el crecimiento otras cepas de la bacteria simbionte pueden también colonizar la glándula.

CONCLUSIONS

1) Gastrointestinal bacterial communities are significantly related to some phenotypic variables which, at the same time, are good predictors of the probability of survival. Therefore, according to previous results, the microbiota associated to the digestive tract in both magpies and great spotted cuckoos is an indicator of their phenotypic quality.

2) The dark uropygial secretion of hoopoes showed antimicrobial activity in females and nestlings during their nesting stage, when bacteria were detected. However, in the white secretions of males and non-breeding females no bacteria or antimimcrobial activity was detected.

3) The potencial inhibition capacity of pathogenic bacteria in enterococci isolated from hoopoes is a variable character which is associated to the strain genetic profile. This fact implies variability in the benefits that symbiont enterococci could provide to their hosts. Thus, it could have mechanisms of selection for those more efficient strains by host hoopoes.

4) By experimental ellimination of the symbiotic Enterococci, we found that they are responsible of the morphology of the uropygial gland, as well as of the quantity and colour of its secretion. Moreover, we revealed the existence of those bacteria inside the gland tissues.

5) Enterococci living in the hoopoes uropygial gland, through the secretion of bacteriocins, have direct benefits for hoopoes. The decrease of eggs hatching success when the bacteriocins are inhibited with proteases suggests that this component is avoiding the growing of pathogen bacteria in the shell and embryo infection. We also proved that bacteriocins produced by symbiotic bacteria inhibit the growing of keratinolytic bacteria, preventing from feather degradation.

6) Great spotted cuckoos and magpie nestlings reared in the same nest have different digestive tract anatomy and bacterial communities. Great spotted cuckoos have a greater number of bacterial taxons than their host. Phylogenetic constrains could explain those differences detected in the gut microbiota between the two species.

7) In the hoopoes population studied, the RAPD analysis allowed us to distinguish some enterococci strains isolated from their uropygial secretions. In the same individual, or individuals from the same nest, the strains detected used to be in the same RAPD group. Moreover, cross-fostering experiments revealed that the acquisition of the bacteria is previous to the gland development, and that during the growing other symbiotic bacterial strains could also colonize the gland.

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I

STRUCTURE OF THE CLOACAL BACTERIAL COMMUNITY PREDICTS IMMUNOCOMPETENCE AND PHYSICAL CONDITION OF MAGPIES AND GREAT SPOTTED CUCKOO NESTLINGS

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ABSTRACT

Gut bacterial community is of prime importance for optimal food digestion and, therefore, for nutritional condition of avian nestlings, which predicts recruitment. Consequently, the bacterial community may be considered as a predictor of the future survival and recruitment of young birds. To explore this hypothesis, we studied the structure of the cloacal microbiota, by using RISA procedure, in two avian species sharing environmental conditions during growth, the avian brood parasite great spotted cuckoo (*Clamator glandarius*), and their main host in Europe, the magpie (*Pica pica*), and compared the two. We also explore variables related to immunocompetence (i.e. levels of immune response to an innocuous antigen, phytohemagglutinine) and physical condition (i.e. residuals of body mass versus tarsus length) of nestlings, which have been shown to predict survival and recruitment in birds. According to the hypothesis, we found significant positive relationships between microbiota structure and nestling phenotypic traits. Both magpie and cuckoo nestlings sharing their microbiota were those with similar immune response and physical condition index. We discuss a possible association between bacterial communities and variables related to the probability of post-fledging survival and recruitment of birds, as well as possible reasons explaining magpie-cuckoo differences in the nutritionally conditioned variables better associated with the bacterial community.

INTRODUCTION

Early development is a crucial determinant of fitness in many animals and, therefore, factors that affect not only offspring growth but also variation in adult phenotypic quality due to environmental experience during growth are of prime importance in evolutionary ecology (Lindstrom 1999). In birds, it is known that body-mass at fledging (e.g. Perrins 1980; Alatalo *et al*. 1990; Linden *et al*. 1992), as well as body condition and immunocompetence (e.g. Horak *et al*. 1999; Christe *et al*. 2001; Møller & Saino 2004), are good predictors of individual survival. In particular, local immune response to the non-pathogenic antigen phytohemagglutinine (hereafter PHA response) of adults (Hidalgo-García 2006) and fledglings (Cichon & Dubiec 2005; Moreno *et al*. 2005) predicts their survival to the next breeding season better than any other phenotypic trait (i.e. body mass and condition, laying date, etc.). The PHA response is usually positively related to body mass and nutritional condition of nestlings (e.g. Soler *et al*. 1999; Tella *et al*. 2000; Christe *et al*. 2001; Moreno *et al*. 2005), variables that have traditionally been considered to be reliable indexes of survival probability of avian nestlings about to fledge (e.g. Tinbergen & Boerlijst 1990). However, this association with survival could be influenced by the relationship between immunity and body mass (Cichon & Dubiec 2005; Moreno *et al*. 2005). The PHA response is a nutritionally conditioned trait (Alonso-Alvarez & Tella 2001), not only because the development of the immune system is costly in terms of energy and nutrients that otherwise could be used for further development of other phenotypic traits (e.g. Lochmiller & Deerenberg 2000; Norris & Evans 2000; Bonneaud *et al*. 2003), but also because it depends on the availability of essential micronutrients, such as sulphur amino acids and vitamins (see e.g. Grimble & Grimble 1998; Klasing 1998; Redmond *et al*. 1998; Siegel *et al*. 2000; Kidd 2004), which are directly acquired from the diet, or after the digestion process.

Most nutrients that nestlings need for development and growth are, however, not directly provided by their parents, but indirectly acquired after digestion of food by the gastrointestinal microbiota. It is known that bacterial community structure in the intestinal tract has a strong environmental component (Lucas & Heeb 2005) and, therefore, varies between individuals of the same species. In addition, intestinal microbiota is related to individual

nutritional condition (see for example Glunder 2002; Xu *et al*. 2003; Engberg *et al.* 2004) and phenotypic quality (Moreno *et al.* 2003). Moreover, basic research has shown a link between the gastrointestinal tract, immunocompetence, and nutritional status (see Hammarqvist 2004) and, therefore, gastrointestinal microbiota may be the direct link explaining the relationship between nutritional condition and PHA response (i.e. T-cell-mediated immunity) detected in wild birds. If so, a relationship between intestinal microbiota and nutritional conditioned traits, such as PHA response and body condition, should be detected. Data supporting this prediction could indicate an important role of gastrointestinal microbiota in nestling's probability of recruitment.

While pathogenic bacteria may compromise the investment in growth (Potti *et al*. 2002), reduce probability of survival (Mills *et al*. 1999), or even kill their hosts (Nutall 1997), non-pathogenic bacteria in the digestive tract may have important benefits for hosts. These benefits include those directly related to their important function in the digestive process, including the acquisition and storage of nutrients optimising their use from food intake (Xu & Gordon 2003; Bäckhed *et al.* 2004) and the synthesis as well as absorption of essential nutrients (e.g. Barnes 1972; Stevens & Hume 1998). Moreover, non-pathogenic bacteria in the gut may competitively exclude potential pathogens (Hooper *et al*. 1998), influence local immune response (Cebra 1999; Umesaki & Setoyama 2000), and alter the diversification of the antibody repertoire (Lanning *et al.* 2000, 2005). Therefore, a good background exists for predicting a relationship between bacterial community structure in the intestinal tract and body condition or immunocompetence of nestlings. While there is some evidence of a positive relationship between the gut microbiota and nestling growth and development in wild birds (Lombardo *et al.* 1996; Moreno *et al.* 2003), little is known about the predicted relationship between microbiota and immunocompetence of nestlings.

Here, by using Ribosomal Spacer Analysis (RISA) (García-Martínez *et al*. 1999), we estimated the bacterial community structure of nestlings of the great spotted cuckoo (*Clamator glandarius*) and that of its main host in Europe, the magpie (*Pica pica*) a few days before leaving the nests. These two species, despite exploiting the same resources (growing in the same environment, thus sharing parents and food), are phylogenetically distant, and fledgings of

each species differ in using resources, given that, after leaving the nest, cuckoos migrate a long distance, while magpies stay in their territories.

Bacterial community structure was used to explore its possible relationship with variables related to nestling phenotypic quality, including (i) physical condition (residuals of body mass after correcting for the effect of tarsus length; see Schulte-Hostedde *et al*. 2005), and (ii) level of immune response to an innocuous antigen (phytohemagglutinin-P). To break the possible association between the environment during development and the characteristics of gastrointestinal bacterial community that at the same time may have a phylogenetic component (Ruiz-Rodríguez *et al.* submitted), we experimentally prepared most nests with two magpies and two great spotted cuckoos of the same age and similar weight when nestlings were 2-3 days old. In addition, this experimental approach allowed us to control partially for possible intra- and inter-specific differences in diet and food intake due to nestling hierarchy and early hatching of parasitic nestlings (Soler & Soler 1991; Soler *et al*. 1995).

MATERIAL AND METHODS

The study area was the Hoya de Guadix (37°18'N, 3°11'W), southern Spain, at approximately 1000 m a.s.l. The vegetation is sparse, with some holm oaks (*Quercus rotundifolia*) and many orchards of almond trees (*Prunus dulcis*) in which magpies nest (see Soler 1990 for a better description of the study area). Parasitism of magpies by the great spotted cuckoos is quite common in the area (see Soler & Soler 2000).

Field work and experimental procedure

At the beginning of the breeding season of 2003, we identified magpie nests and visited them twice a week to detect laying date, the start of incubation, and parasitism by the great spotted cuckoo. The incubation period of great spotted cuckoo eggs averages 4 days shorter than that for magpie eggs and, thus, in most parasitized nests the great spotted cuckoos hatched earlier than magpie nestlings (Soler 1990). Moreover, magpies preferentially feed the larger nestlings in the nest and, therefore, most magpie nestlings die from starvation in parasitized nests (Soler &

Soler 1991; Soler *et al*. 1995). To avoid such age differences, we manipulated the nests by exchanging the cuckoo, but also magpie nestlings and leaving two cuckoos and two magpie nestlings of the same age per experimental magpie nest. Since, on average, more than 2 magpie nestlings hatch in non-parasitized nests, the remaining nestlings were joined in other magpie nests (maximum 7 nestlings per nest) which were not used for the experiment. All nestling exchanges were performed soon after hatching, when nestlings were 1-3 days old.

After loss due to predation and nestling starvation, or samples that failed in subsequent laboratory work, we analysed 41 magpies and 26 great spotted cuckoos from 27 nests, from which 19 nests were experimental (i. e. artificial mixing of cuckoos and magpies), and 8 were natural (i. e. non-parasitized magpie nests in which nestlings were not exchanged). From the 19 nests, we collected data for the two species in 11 nests (16 magpies and 17 cuckoos), while samples from a single species were collected in the other 8 nests (9 cuckoos and 2 magpies).

Bacterial sampling, Body Condition and T-cell Mediated Immune Response

About four days before fledging, when nestlings were 16-17 days old, we ringed, measured and weighed them. The right and left tarsus lengths were measured and mean values were used in the analyses. We took bacterial samples from the cloaca of all the nestlings raised in magpie nests, by injecting and repipetting two or three times 500 µl of sterile phosphate buffer (Na_2HPO_4 0.1 M and NaH_2PO_4 0.1 M, pH 7.4) in the cloaca, using sterile tips and automatic pipettes. Afterwards, we immediately lysed the bacterial cells in the field by adding 500 µl of lysis buffer (Tris HCl 50mM, 1% SDS, EDTA 2mM, NaCl 100mM). Samples were kept cool (i.e. 1-3 ^{o}C) for a few hours and later stored in the lab at -20 ^{o}C until molecular analyses.

Finally, as a variable related to immunocompetence of nestlings, skin swelling elicited by injection of the mitogen phytohemagglutinin (PHA-P, Sigma Chemical Co.) was measured. This is commonly used in evolutionary ecology to estimate T-cell-mediated immunity (Kennedy & Nager 2006), although it also reflects other components of the immune system such as MHC molecules (e.g. Goto *et al*. 1978; Martin *et al*. 2006). Briefly, following a well-established protocol, we injected fledglings subcutaneously in the right wing web with 0.5 mg of PHA dissolved in

0.1 ml of physiological saline solution (Bausch & Lomb Co.). The left wing web was injected with 0.1 ml of saline solution. We measured the thickness of each wing web at the injection site with a digital pressure-sensitive micrometer (Mitutoyo, model ID-CI012 BS; to the nearest 0.01 mm) before injection and 24 h afterwards. The T-cell-mediated immune response or wing-web index was then estimated as the change in thickness of the right wing web (PHA injection) minus the change in thickness of the left wing web (Lochmiller *et al*. 1993). Measurements of each wing web on each occasion were repeated three times, and the mean value was used in subsequent analyses.

Laboratory analysis

To analyse the bacterial community in each nestling, we extracted DNA from 200 µl of each cloacal sample. First, samples were thermally shocked to further lyse the cells, and then DNA was extracted following the protocol of Orsini & Romano-Spica (2001), slightly modified. Shortly, after the addition of 400 µl of a buffer prewarmed at 65ºC (Tris HCl 10 mM, EDTA 1 mM, sodium acetate 0.3 mM and 1.2% polyvinylpyrrolidone), the DNA was purified by the phenol-chloroform procedure. Finally the DNA was precipitated with isopropanol overnight at -20 ºC. After washing 3 times with 80% ethanol, the DNA was re-suspended in TE buffer pH 8 (Tris HCl 10mM and EDTA 1mM). Subsequently, we used the ribosomal intergenic spacer analysis (here after, RISA) method to amplify the spacer region between the 16S and 23S rRNA genes in the ribosomal operon. This fragment is extremely variable in both sequence and length for the different prokaryotic species, due to the presence of several functional units within them such as tRNA genes (García-Martínez *et al*. 1999). The primers used were S-D-Bact-1522-b-S-20 and L-D-Bact-132-1-A-18 (Ranjard *et al*. 2000). The polymerase chain reaction was performed in 50 µl, with 100 ng of DNA, 1 x PCR buffer (QIAGEN), 2 mM $MgCl_2$, 0.1 mg/ml BSA, 0.5 µM of each primer, 150 µM of each dNTP and 1 U Taq polymerase (QIAGEN). The amplification reaction was performed using an initial denaturating at 94ºC for 3 min, followed by 25 cycles at 94ºC for 1 min, 55ºC for 30 sec, 72ºC for 1 min, and a final extension at 72ºC for 5 min. (Ranjard *et al*. 2000). PCR products were subsequently quantified with a fluorimeter DynaQuant

(Hoefler) after staining with Hoechst Dye diluted to 1/10000. To separate the PCR products (200 ng), we used a 2% Metaphor agarose (FMC Bioproducts) gel electrophoresis for 4 h at 150 V. Each band in the gel corresponds to one Operative Taxonomic Unit (OTU), also called phylotype, which is assumed to be a taxonomic unit (i.e. species; Atlas & Bartha 1997). OTU richness of each sample was estimated by the total number of bands present in one individual.

Statistical analyses

The resulting gels were analysed with GEL COMPARE II (Applied Maths, Kortrijk, Belgium). A similarity matrix was built using pairwise comparisons among RISA profiles (i.e. individuals). The similarities were calculated using the Dice's binary coefficient: $2a/(2a+b+c)$, where a is the number of OTUs in common for the two samples, b is the number of OTUs present only in the first sample, and c is the number of OTUs present only in the second sample. The two species were analysed separately because of the marked differences in the bacterial community structure among them (Ruiz-Rodríguez *et al*. submitted).

We also prepared similarity matrices for each variable hypothetically related to the bacterial community (physical condition and immune response), but also for variables that could mask the predicted relationships (nest of rearing, parasitized, or non-parasitized nest, see below). For continuous variables (i. e., immune response and physical condition), similarity matrices were performed, between two nestlings, dividing the lowest value by the highest one, thus the more similar immunocompetence or condition in two nestlings, the closer to 1 is this value in the matrix. For discrete variables, binary matrices were built by using 1 when the two nestlings shared identity (e.g. same nest), and 0 otherwise.

Physical condition was independently estimated for great spotted cuckoo and magpies nestlings as residuals of body mass after correcting for tarsus length (Magpies: $R = 0.76$, $F_{1,39} = 54.21$, $p < 0.0001$; Great spotted cuckoos: $R = 0.53$, $F_{1,23} = 9.23$, $p = 0.0058$). These residuals were not significantly correlated with wing length (Magpies: $R = 0.29$, $F_{1, 39} = 3.72$, $p = 0.06$; Great spotted cuckoos: $R = 0.29$, $F_{1, 23} = 2.2$, $p = 0.15$) and, thus, we used them as an index of body condition.

The relationships between matrices were studied using Mantel's test as implemented in FSTAT (Goudet 1995), which estimates partial correlation coefficients as well. Statistical significances were estimated by Monte Carlo procedure after 10000 permutations, which provide associated p-values to the correlation coefficients.

Because nestlings within the same nest share the same rearing environment and, thus, the use of nestlings as independent data points may imply a pseudo-replication problem, when significant correlations arose, analyses were run again. However, in these models we included identity matrices of nest of rearing and nest of origin (i.e. pairs of nestlings being of the same or different nests). Matrices of differences in the type of nests from which the nestlings were reared (i.e. experimental or natural nests) were also included in the model.

Given that bacterial richness (i.e. number of OTUs) may influence estimations of similarity coefficients (see above), we also estimated matrices of absolute differences in the number of OTUs detected at the level of nestlings as well as nests, and included this information as additional independent variables. However, results did not qualitatively differ from those where this information was not included (results not shown). Thus, here we present results from models that did not include differences in number of OTUs as an additional independent variable.

RESULTS

We detected a total of 45 different OTUs. For magpie nestlings, we found a positive relationship between matrices of similarity in RISA profiles and in PHA response (N = 41, Mantel test, $r = 0.18$, $p < 0.0001$). This relationship was not affected by possible confounding factors such as nest of rearing (Mantel test, Partial correlation coefficients: nest of rearing = 0.09, $p = 0.09$; PHA = 0.17, $p < 0.0001$), nest of origin (Mantel test, Partial correlation coefficients: nest of origin = 0.09, $p = 0.01$; PHA = 0.17, $p < 0.0001$) or type of nest (i. e. only with magpies or both species) (Mantel test, Partial correlation coefficients: type of nest = 0.09, $p = 0.06$; PHA partial $r = 0.17$, $p < 0.0001$). However, the relationship between the Dice's coefficient matrix and the physical condition of magpie nestlings did not reach statistical significance (Mantel test, $r = -0.07$, $p =$

0.05). In addition, matrices of differences in PHA response and body condition of magpie nestlings were not significant (Mantel test, r = -0.01, p = 0.62), and thus, we did not detect a correlation between these two variables in magpie nestlings, although it was demonstrated in a previous work with a larger sample size (Soler *et al*. 1999).

In relation to great spotted cuckoos, the results agree with the hypothesis that bacterial assemblage is a good predictor of nestling phenotypic quality, since we found a significant positive relationship between matrices of similarity of bacterial assemblage and physical-condition index (Mantel test, r = 0.16, p = 0.007). This relationship was still statistically significant after correcting for the nest of rearing (Mantel test, Partial correlation coefficients: nest of rearing = 0.10, p = 0.17; physical condition = 0.15, p = 0.007). However, the matrix of similarity among bacterial assemblages did not correlate with that of differences in PHA response (N = 26, Mantel test, r = -0.01, p = 0.88). Finally, the matrices of differences in PHA response and body condition of nestling cuckoos proved significantly related (Mantel test, r = 0.02, p = 0.0005), which did not vary after nest corrections (Mantel test, partial r = 0.02, p = 0.0008).

DISCUSSION

Positive relationships suggest that the intestinal bacterial community both of magpies and of cuckoos is associated with variables related to probability of survival (i.e. phenotypic quality). While magpies having similar gut bacterial communities also demonstrated a similar level of immunocompetence, cuckoos with more similar bacterial structure were those with a more similar physical condition.

Given that bacteria in the intestinal tract are vital for digestion in general, and in the synthesis of some important micronutrients as amino acids and vitamins (e.g. Stevens & Hume 1998) in particular, we predicted a relationship between bacterial community and phenotypic quality of magpie and great spotted cuckoo nestlings. Body condition as well as PHA response are nutritionally conditioned traits (see Introduction) and, thus, we used these variables to test the hypothesis that the bacterial community in the intestinal tract is related to nestling traits that

depend on nutritional condition. In addition, both variables are good predictors of nestling survival and recruitment (see Introduction) and thus the detection of the expected relationship with microbiota characteristics in the gastrointestinal tract would suggest an important role in later nestling survival. Actually, in accordance with the hypothesis, we found a significant relationship between bacterial community in the cloaca with the PHA response of magpie, and with physical condition of great spotted cuckoo nestlings.

However, because cause cannot be deduced from correlations, we cannot rule out the possibility that, rather than bacterial communities being the responsible of nutritional conditioned traits (i.e. PHA response and body condition), variation in bacterial assemblage was the consequence of variation in the individual phenotypic quality. This possibility is likely because it is known that either, anatomy, physiology or local immune system of the digestive tract are key in shaping bacterial gut communities (e.g. Steven & Hume 1998). A third non-exclusive possibility explaining the detected associations is that, because diet is an important factor influencing both bacterial communities (Glunder 2002; Gabriel *et al*. 2005) and phenotypic condition of nestlings, variation in parental quality would be responsible for the relationship between gut microbiota and variables related to nestling phenotypic quality. However, because our results were corrected for variation among nests (i.e. statistically controlled for variation in nest of origin and nest of rearing), this possibility is unlikely. In any case, because of the correlational nature of this study, experiments are necessary to explore the causal explanation of the detected relationships.

The variable related to phenotypic quality of nestlings that was found to be associated with their bacterial assemblage structure at the cloaca differed for great spotted cuckoos and magpies. While the PHA response was the variable most closely related to characteristics of magpie microbiota, residuals of body mass after controlling for tarsus length was the variable that best explained microbiota of cuckoos. Moreover, we did not detect a correlation between these two variables in magpie nestlings, even though this relationship was established in a previous work (Soler *et al.* 1999), which is probably due to a lower sample size in the present study. On the contrary, in cuckoos, the two variables were related. In general, it is well

demonstrated that body mass and/or condition correlate with nestling immunocompetence (i.e. PHA response) (see, Soler *et al*. 1999; Alonso-Alvarez *et al*. 2001; Westneat *et al*. 2004), and that a trade-off exists between growth and immunity (Soler *et al*. 2003; Brommer 2004). Consequently, because natural selection favours the evolution of physiological mechanisms that ensure optimal allocation of limited resources to competing activities (Stearns 1992), our results suggest that optimal allocation differed for the two species studied.

Post-fledging survival is a crucial determinant of future reproductive success in birds (Clutton-Brock 1998). There is strong evidence suggesting that both body condition (e.g. Naef-Daenzer *et al*. 2001) and immunocompetence at fledging (Cichon *et al*. 2005, Moreno *et al*. 2005) are good predictors of post-fledging survival. Body condition predicts the probability of predation (e.g. Naef-Daenzer *et al*. 2001), and body condition and immunocompetence, which indicates the health status of fledglings, predicts the degree of negative effects that parasites and diseases may exert on juveniles (see Møller 1997). Moreover, it has been suggested that predators may preferentially prey upon sick individuals (Temple 1987; Møller & Erritzøe 2000) and thus, health status of fledging is of prime importance for future survival and therefore reproductive success. However, it has been suggested that interspecific brood parasites would suffer less from parasitism than their hosts because parasites can be highly specific (Brooks & Mclennan 1993) and the brood parasitic strategy of reproduction prevents vertical transmission of specific pathogens during the nesting phase of reproduction (Soler *et al*. 1999). Therefore, because development and maintenance of the immune system, as well as immune response, is costly (see, Lochmiller *et al*. 2000; Bonneaud *et al*. 2003), investment in a developing immune system should be adjusted to environmental conditions (i.e. risk of parasitism; see, Sheldon & Verhulst 1996; Szep & Møller 1999). Consequently, because great spotted cuckoo nestlings suffer less from parasites (i.e. blood parasites) than do their magpie foster siblings (Soler *et al* 1999), magpies should invest relatively more in immunity during development. Moreover, great spotted cuckoos are migratory birds and, at least for some other species, it is known that the probability of survival during migration is correlated with the body mass and body condition at fledging of chicks (Reed & Plante 1997; Schmutz & Ely 1999; Menu *et al*. 2005).

Consequently, nestling cuckoos that invest more resources in growth during development would have a higher probability of survival during migration and consequently improve their probability of recruitment.

To our knowledge, a positive relationship between traits related to phenotypic quality of nestlings and intestinal microbiota has rarely been detected in wild birds. On the one hand, Lombardo *et al*. (1996) found a positive relationship between bacteria loads (i.e. total number of bacterial colonies that grew in nine different media) and wing length of tree swallows (*Tachycineta bicolor*) nestlings when 12 days old. On the other hand, Moreno *et al*. (2003) found that the presence of *Enterococus faecium*, a bacterium with a well-known beneficial effect in poultry (Foulquié-Moreno *et al.* 2006), was positively associated with pied flycatcher (*Ficedula hypoleuca*) nestling's growth. This effect was interpreted as the result of physiological benefits for their hosts through competitive exclusion of potentially pathogenic bacteria, enhancing host nutrition and the development of host immunocompetence (see, Foulquié-Moreno *et al.* 2006). In fact, it is known that symbiotic intestinal microbiota provide benefits for hosts, not only by contributing to host nutrition but also by producing compounds that inhibit antagonistic-competing microorganisms (Riley & Wertz 2002) and, therefore, such microbiota may protect hosts against pathogens and parasites due to bacterial interference (Ji, *et al*. 1997). However, our results refer rather than to a single beneficial bacterium, to the bacterial community in the cloaca. Differences in our cloacal samples are assumed to reflect differences in the bacterial community in the intestinal tract (Savage 1977; Vaahtovuo *et al*. 2001) of cuckoos and magpie nestlings, and are associated with variables that are important explaining post-fledging survival, recruitment and, afterwards, host fitness in nature.

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II

HOOPOES KEEP ANTIBIOTIC-PRODUCING BACTERIA THAT PREVENT INFECTIONS

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ABSTRACT

The uropygial gland of female and nestling hoopoes (*Upupa epops*) contains during the nesting period a bacterium, *Enterococcus faecalis*, which produces at least two different kinds of bacteriocin-like inhibitor-substances (BLIS) active against a broad spectrum of pathogenic bacteria. These bacteriocin-producing bacteria would, among other functions, prevent the colonization of eggshells by pathogenic microorganisms and, thus, avert embryo infection. By sampling uropygial gland secretions (UGS) of hoopoes, we found the bacteriocin-producing bacteria in the brown UGS of incubating-or-brooding females and nestlings, but not in the white secretion of males and non-breeding females. Moreover, both UGS and bacteria colonies that grew from these brown secretions demonstrated antimicrobial activity. Finally, eggs from hoopoe nests, but not those from spotless starling (*Sturnus unicolor*), where the action of bacteriocins was experimentally inhibited, had more bacteria and suffered more frequent hatching failure than eggs from control nests. Therefore, the detected link between brown UGS of hoopoes and its antimicrobial properties, as mediated by antibacterial peptides produced by culture of *E. faecalis*, together with our experimental results, provides the first evidence of a symbiotic mutualistic association between a vertebrate host and an epibiotic bacterium producing substances with antimicrobial properties that is internally kept within an special organ different of the digestive tract.

INTRODUCTION

Epibiotic bacteria produce compounds that inhibit antagonistic-competing microorganisms (Riley & Wertz 2002) and, therefore, they may provide hosts with protection against pathogens and parasites due to bacterial interference (Ji, *et al*.1997). Accordingly, there are reports of highly integrated symbiotic associations (sensus, Herre *et al.* 1999;Thompson 2005) between animals and antibiotic-producing bacteria (Gil-Turnes *et al*. 1989;Currie *et al.* 1999;Oliver *et al.* 2003). For instance, epibiotic bacteria are responsible for the resistance of embryos of some species of shrimp and lobster to pathogenic fungi (Gil-Turnes *et al.* 1989;Gil-Turnes & Fenical 1992). Likewise, fungus-growing ants, that are associated with mutualistic bacteria that protect cultivated fungi from pathogenic infections (Currie *et al*. 1999), have special crypts connected to exocrine glands where the symbiotic bacteria grow. These crypts vary among species suggesting an ancient coevolutionary interaction between ants and bacteria (Currie *et al*. 2006).

Although such an integrated relationship has not previously been described in vertebrates, several previous results suggest that a similar kind of association with bacteria that confer hosts with protection against pathogens could occur in hoopoes (*Upupa epops*) for different reasons. In a closely related species, the red-billed woodhoopoe (*Phoeniculus purpureus*) (Feduccia 1975;Krištín 2006), a novel bacterium has recently been isolated from the uropygial gland secretion, which is brown and malodorous (Law-Brown & Meyers 2003). Because most bacteria produces bacteriocin-like inhibitor substances (BLIS) against other bacteria, the well-known antibiotic properties of uropygial gland secretions (UGS) (Jacob & Ziswiler 1982;Shawkey *et al*. 2003b) can be mediated by antimicrobial substances produced by symbiotic bacteria. More importantly, although bacteria from woodhoopoes glands have not been tested for antimicrobial activity yet, recently it has been detected that the culture of the single bacterium (*Enterococcus faecalis*) isolated from the UGS of a nestling of another Coraciform species, the hoopoe (*Upupa epops*) produced at least two different kinds of peptides (i.e. BLIS) with strong antibiotic properties (Martín-Platero *et al*. 2006). Because UGS of nestling and female hoopoes during the breeding season are very similar (brown and malodorous (Cramp 1985), see Study species) to those of woodhoopoes and in both cases bacteria have been isolated

from the secretion, there seems to be an association between the dark secretions of these two coraciform species and symbiotic bacteria living within their uropygial glands, which in the case of hoopoes produce antimicrobial substances. This association may be the first example of a vertebrate with epibiotic bacteria that are kept in special organs (i.e. uropygium) and confer protection against pathogens. Here, we explore this possibility through laboratory and field experiments, attempting to elucidate whether (i) the UGS of hoopoes has antimicrobial properties, (ii) whether brown UGS are linked to presence of symbiotic bacteria, (iii) whether the antimicrobial properties of UGS are, at least partially, mediated by BLIS produced by the bacteria living in the uropygial gland. If symbiotic bacteria were responsible for inhibition of growth of other bacteria, colonies of bacteria isolated from secretions should demonstrate similar inhibition properties as whole secretions. Finally, because we found evidence of antibacterial properties of UGS being mediated by BLIS, (iv) we manipulated the efficiency of these substances by experimental addition of protease to the nests of hoopoes. Because protease destroys peptide bounds of BLIS, this experimental approach allowed us to test whether the production of BLIS by the bacterium provides hoopoes with defenses against pathogens (i.e. fitness advantages), at least in the nest environment. To rule out the possibility that protease, rather than acting on BLIS, directly affects embryos, or that a general antibacterial property of UGS in birds (i.e. non-mediated by bacteria) was inhibited by protease, we performed similar control experiments in nests of another hole-nesting species, the spotless starling (*Sturnus unicolor*) which does not harbor bacteria in its gland.

MATERIAL AND METHODS

Study species

The hoopoe is a coraciiform bird that nests in holes without adding nest material, and it experiences seasonal changes in the properties of the UGS of females and nestlings, but not in that of males (Cramp 1985). While male UGS is invariably white and oily, that of females and nestlings is water soluble, brown, and malodorous during their stay in the nest, but not at other times of the year when UGS of females and males apparently do not differ (Cramp 1985).

Study area

The fieldwork was performed during the breeding seasons 2003-2007 in the Hoya de Guadix (SE Spain). Uropygial gland secretions from adults and nestling were collected in all years, while the protease experiment and quantification of bacteria colonies on the eggshell were performed during 2005 in a population of hoopoes and starlings breeding in next boxes.

Sampling of uropygial gland secretion and bacterial determination

We sampled uropygial secretion of both adults and nestling hoopoes. Adults were caught in mist-nets or within nest boxes while brooding hatchlings, and nestlings were sampled at the age of ringing (19-23 days). We analyzed 23 randomly selected samples including seven nestlings from six different nests, eight females, and eight males. In females, three of the samples were brown (i.e. during the incubation and first 10 days of nestling period) and five were white (i.e. before incubation, or after the nesting phase), while all from nestlings were brown, and all from males were white. Samples were collected with a micropipette directly from within the uropygial gland after feathers around the gland were separated and washed with ethanol to avoid contamination. We obtained up to 100 µl of uropygial gland secretion for brown samples and only up to 10-20 µl for white samples because of the smaller available volume. Samples were introduced in a sterile Eppendorf tube (1.5 ml.) and stored at 4ºC until processed in the laboratory shortly after collection.

We added 100 µl of sterile distilled water to each sample and vigorously mixed by pippeting, and then smeared 5 µl of this solution onto two different culture media. As a general bacterial growth medium, we used tripticase soy broth (TSB). As selective medium for growth of enterococci we used Kenner fecal agar (KFA) supplemented with 0.01% 2-3-5 triphenyl-tetrazolium chloride. All bacteria were incubated aerobically without agitation at 37 ºC for 24-48 h. Five colonies were picked randomly from each plate of each sample for ulterior characterization, isolated in TSA (TSB with 1.5% of agar) tubes and maintained at 4 ºC.

In nine out of ten brown secretions, all five bacterial isolates from each sample were initially assigned to the genus *Enterococcus* according to the classification by Schleifer & Kilpper-

Bälz (1984) and the criteria of Orvin (1986). To type the isolates from each sample, we used RAPD methodology (Williams *et al*. 1990), which allows us to detect genomic DNA changes between samples, even of closely related strains of the same species (Welsh & Mcclelland 1990). Bacterial genomic DNA was extracted from overnight cultures at 37 ^{o}C in 1 ml of BHI using the AquaPure Genomic DNA isolation kit (Bio-Rad). DNA amplification was made using the following primer of arbitrary nucleotide sequence M13: 5´-GAGGGTGGCGGTTCT-3´ at a concentration of 1 µM in the reaction mixture. Amplified fragments were visualized on 1.5% agarose gels containing 1 ug/ml ethidium bromide, using the 1-Kb pair ladder (Biotools, Madrid, Spain) as the molecular weight standard. Isolates of each RAPD type were identified to the species level by API 20 Strep strip (Biomerieux, Lyon, France). Genus and species were designed according to phenotypic characteristics and confirmed by PCR amplification using specific primers. For confirmation of the genus *Enterococcus* we used the oligonucleotides "entero 1" (5´-CCCGGCTCAACCGG-3´) and "entero 2" (5´-CTCTAGAGTGGTCAA-3´), at concentration of 2.5 µl each one in the reaction mixture (50 µl), to amplify a 500 bp fragment for the identification of enterococci. PCR was carried out as previously described by Deasy *et al*. (2000). A multiplex PCR assay based on the specific detection of genes encoding D-alanine:D-alanine ligase (*ddl*) was used to confirm the identification of *E. faecalis* and *E. faecium* (Dutka-Malen *et al*. 1995;Cheng *et al.* 1997). The DNA sequences (5´to 3´) for the primers used in this study and their corresponding specificities were as follows: the pair of 21-mer primers EM1A (5´-TTGAGGCAGACCAGATTGACG-3´) and EM1B (5´-TATGACAGCGACTCCGATTCC-3´) were used to confirm the identification of E. *faecium* (Cheng *et al*. 1997); ddl-E1 (5´-ATCAAGTACAGTTAGTCTT - 3´) and ddl-E2 (5´- ACGATTCAAAGCTAACTG -3´) for *E. faecalis* (Dutka-Malen *et al*. 1995). The specific amplicons were 658 bp and 940 bp, respectively.

Detection of antimicrobial activity

In 2004, 2005, 2006 and 2007 we performed tests of UGS activity against *Bacillus licheniformis* directly in the field. For these tests, we used *B. licheniformis* D13 (Galvez *et al*. 1994). We cultured this strain in 5 ml. brain hearth infusion (BHI) medium and after growth it was mixed with 15

ml BHA and put on a plate. In the field, the activity test was performed with 2-5 µl UGS of males, females and nestlings, directly after extraction of the secretion from the bird. Plates were incubated at room temperature for 24 h and checked for inhibition of growing *B. licheniformis* around the inoculated UGS samples.

For detection of antimicrobial activity by bacteria isolated from UGS we used BHA, dissolved in sodium phosphate buffer 0.1 M, pH 7.2 (BHA-B) as culture medium. All five isolates from each sample, which were positive for the *E. faecalis* test, were replicated by spotting on BHA-B plates. They were incubated at 37 ºC for 24 h and then overlaid with 5 ml of BHI with 0.8% agar inoculated with 0.1 ml of an overnight culture of *E. faecalis* S-47 (from our collection), *Listeria innocua* CECT 4030 or *B. licheniformis*.

Activity of bacteriocin isolates from brown UGS of hoopoes was tested in a similar way, but using a large number of bacterial strains (Martín-Platero *et al*. 2006).

Protease-field experiments

During the breeding season of 2005, before laying commenced, we covered the bottom of nest boxes with a permeable carpet that was permanently punctured with a butterfly needle and connected to the exterior by a plastic tube of 3 mm diameter. Carpets were partially covered by nest material (small pieces of bark that are commercialized for gardens and that were added to the nest boxes when installed), but assuring that eggs were in contact with the carpet. Each occupied nest box was randomly assigned to one of three treatments, namely, (i) protease; (ii) water and (iii) untreated control. After clutch completion, all nests were visited every second day to perfuse the carpet with 50 ml of protease dissolved in distilled water (dilution 5 g / 200 ml) or distilled water respectively, at experimental (protease) and control 1 (water) nests. Previous experience in non-occupied nests allowed us to estimate the appropriate amount of water to assure proper nest moistness (i.e. avoiding carpet inundation or total desiccation) during 48 h. The untreated control nests (natural) were also visited every two days. Because protease may directly act on the embryo and affect egg hatchability, we also performed the experiment on spotless starling as a control species, as there is no evidence of BLIS-producing

bacteria living in the UGS in this species. We directly applied protease, water, or nothing to the eggs on every second day and explored the effects on bacteria living on the eggshell and on hatching success.

Bacteria sampling on eggs of hoopoes and starlings

After clutch completion, all eggs within a nest were sampled for bacterial contamination. Briefly, we cleaned each eggshell with a swab that was subsequently stored in an eppendorf tube with sterilized water (0.6ml). Swabs with the bacterial sample were transported to the lab in the Eppendorf tube with the rest of the sterilized water at 4ºC. In the afternoon, 50 µl of bacterial suspensions (water in the eppendorf tubes where the cottons with bacteria were transported to the lab) were spread on Hektoen plates, a selective medium for Enterobacteria, and incubated during 72h at 37ºC. After incubation, the number of colonies in the Petri dishes was estimated. When there were less than 300 colonies, the absolute number of colonies was used, but when more colonies were detected we used an approximated estimation (i.e. 500, 1000, 2000). This protocol was again used after 9 days, therefore, allowing estimation of the change in number of bacterial colonies in relation to experimental treatment. Before the analyses, bacterial counting was log-transformed, and nest identity was introduced as a random factor to avoid pseudo-replication.

RESULTS

In agreement with an antimicrobial function of brown, but not white UGS, in a field experiment, we found that white secretions from adults (N = 20) did not inhibit growth of *B. licheniformis*, a common feather decomposing bacterium (Burtt & Ichida 1999) (percentage of samples with activity: 0%), while brown secretions did (N = 49; percentage of samples with activity: 93.9%, Fisher exact test, P = 0.0001). Moreover, bacteria grew more often and produced more colonies per standardized volume of UGS when using brown secretions from females or nestlings (prevalence of bacteria: 100%, N = 10; number of colonies per sample > 100) than when using white secretions (prevalence of bacteria: 30.8%; N = 13; Number of colonies < 5;

none of the colonies were enterococci), Fisher exact test, P = 0.0016). Interestingly, from brown UGS (i.e. females or nestlings), we invariably detected a single specific bacterium, *E. faecalis*, (see Material and Methods) as it is demonstrated by the identical RAPD profile of the five isolates from each UGS sample (see Fig. 1). Furthermore, we found a significant association between prevalence of *E. faecalis* and brown UGS of hoopoes (Fisher exact test, P < 0.0001).

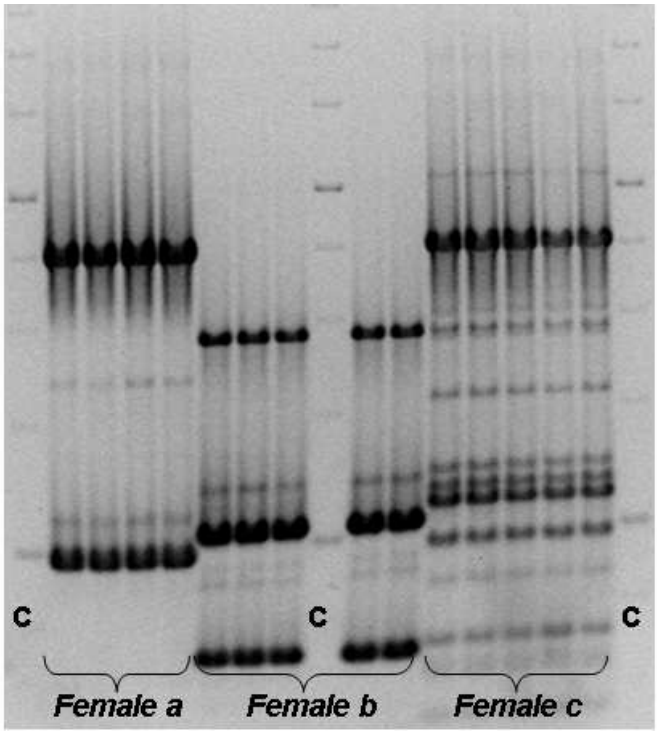

Fig.1. *RAPD patterns for 14 strains isolated from three different females plus three columns with control bands for calibration (C).*

The microscopic study of the uropygial gland of hoopoes, as well as the magnification of the uropygial secretion, clearly showed an exaggerated bacterial density, both in the secretion and inside the papilla of the uropygial gland (Fig. 2). Moreover, dissection of the papilla wall allowed the observation of aggregations of bacteria among epithelial layers that are also detected in the secretions under electronic microscope (Fig. 2).

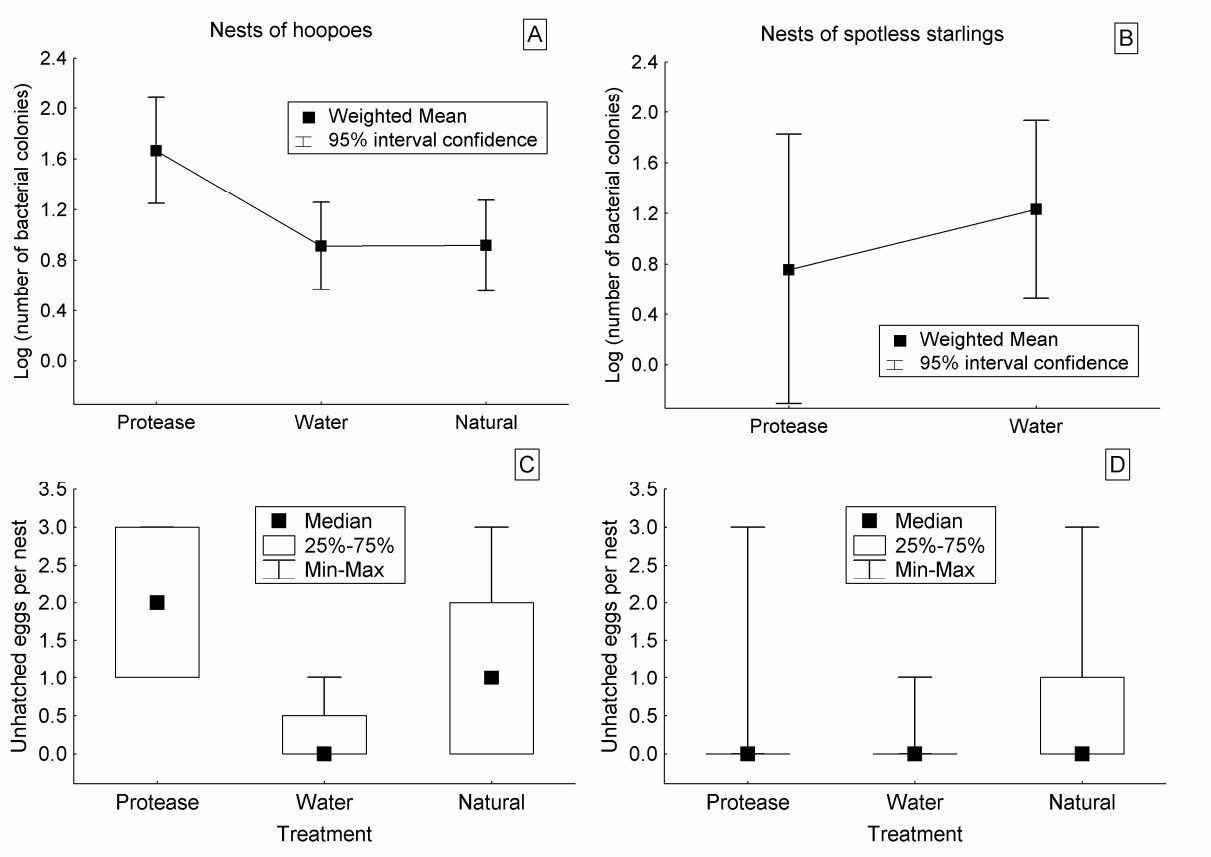

Fig. 2. *Differences in number of bacterial colonies grown from egg samples before and 9 days after treatment with protease or water, and in untreated-control nests of hoopoes (A) and spotless starlings (B). Median, 25%-75% quartile, and minimum-maximum values of number of eggs that failed to hatch in nests of hoopoes (C) and starlings (D) under different experimental treatments are also shown.*

In addition, colonies of *E. faecalis* cultivated from UGS of 3 different females and 7 nestlings from 6 different nests demonstrated clear growth-inhibition activity against *E. faecalis* S-47, *L. inocua* CECT 4030 and *B. licheniformis* D13, which was due to bacteriocins because the antimicrobial effects of such colonies were suppressed by the addition of proteases to the culture media in all cases (N = 20 (two samples per individual). This last result confirms the use of protease as a valid experimental approach for inhibition of BLIS activity in UGS.

Finally, the experimental addition of protease to nests of hoopoes resulted in a higher eggshell bacterial colonization in comparison with that of eggshells from natural nests or from those with experimental addition of water (Fig. 3a) (GLM, log-transformed differences in number of colonies between the two sample dates as dependent variable; experimental treatment as the fixed factor, and nest identity as random factor; 22 nests, 155 eggs; LSD post-hoc comparisons: protease vs. water, $P < 0.0001$; protease vs non-manipulated, $P < 0.0001$; water

vs non-manipulated, $P > 0.95$). However, the experiment had no effect in nests of spotless starling (Fig. 3b) (GLM, log-transformed differences in number of colonies between the two sample dates as dependent variable; experimental treatment as the fixed factor, and nest identity as random factor; 8 nests, 32 eggs; LSD post-hoc comparisons: protease vs. water, $P = 0.34$). In addition, the effect of the experiment was not due to the water used to perform protease dilution (i.e. increase in nest humidity) because bacteria increased at a similar rate in natural and water treated nests (Fig. 3).

Further, our experiment affected the hatching success of hoopoes but not that of starlings. In hoopoes, while hatching success of control-treatment nests did not differ ($N_{(un-manipulated)} = 5$, $N_{(control-water)} = 5$; Kruskal-Wallis test: $H = 1.91$; $P = 0.17$), the addition of protease to experimental nests significantly increased hatching failure ($N_{(experimental)} = 5$; Kruskal-Wallis test: $H = 3.92$; $P = 0.048$) (Fig. 2c). Again, the experiment had no effect in hatching failure of starlings (protease treatment ($N = 8$; only in one nest occurred hatching failure), vs water treatment ($N = 7$; only in one nest occurred hatching failure), vs natural-control treatment ($N = 38$; some egg(s) from 11 nests failed to hatch); Kruskal-Wallis test: $H = 1.19$; $P = 0.55$) (Fig. 3d). These results were not the consequence of the protease diluent because nests that were treated with water experienced the lowest rate of hatching failure in both hoopoe and starling nests (Fig. 3).

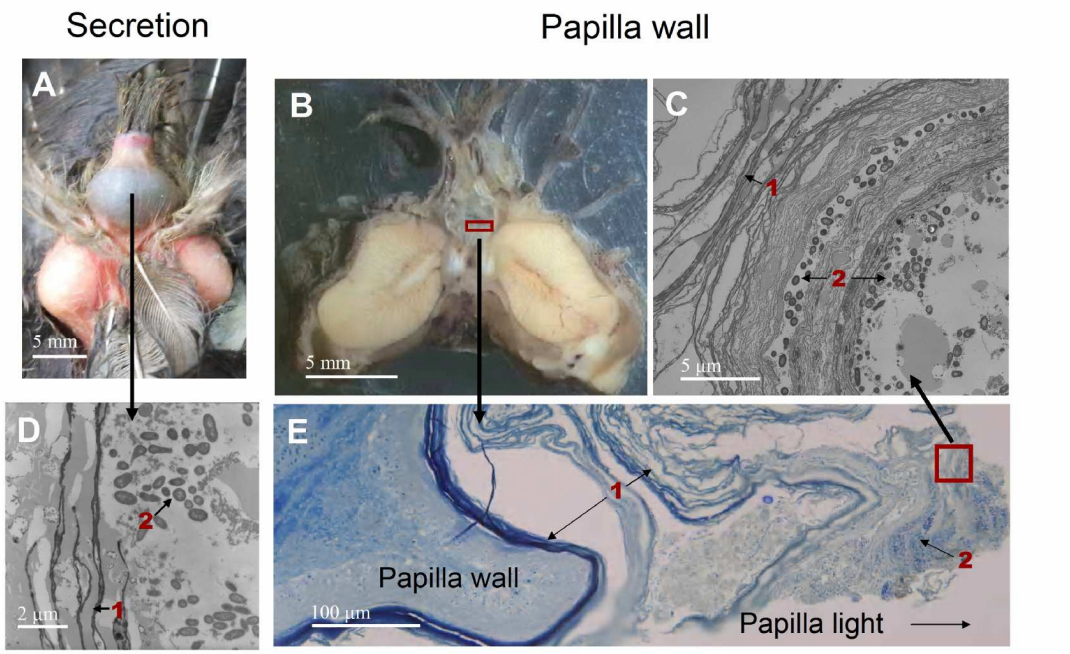

Fig. 3. *Location of bacteria living within hoopoe uropygial glands. (A) Uropygial gland of a female hoopoe with the papilla filled with brown secretion. Visualization of UGS under electronic microscope where bacteria (2), but also epithelial layers occur (1) is showed in picture D. Picture B shows a transversal dissection of the uropygial gland, and the square represents the area of the papilla wall that was serially slashed and prepared for observation under both optic (E) and electronic (C) microscope. In both pictures the aggregation of bacteria (2) among epithelial layers at the limit between the papilla wall and the cavity are patent (E).*

DISCUSSION

The main findings of this study were that (1) a single species of bacterium, *E. faecalis*, grew with high prevalence from brown, but not from white UGS of hoopoes; (2) microscope observation of brown secretions and uropygial glands revealed bacterial aggregations within the papilla of the gland; (3) the UGS, but also isolated colonies of *E. faecalis* from UGS, demonstrated antibiotic properties against pathogenic bacteria; (4) the addition of protease to cultures of *E. faecalis* isolated from the gland inhibited their antibiotic properties suggesting that at least some of the antibacterial activity of the UGS of hoopoes was mediated by BLIS produced by *E. faecalis*; (5) the experimental addition of protease to nests of hoopoes resulted in a relatively higher egg bacterial infection and hatching failures than natural nests or from those with experimental addition of water; (6) the same experiment performed in spotless starlings has no effect on level of egg infection or hatching failures.

We found a significant association between brown UGS of hoopoes and the prevalence of *E. faecalis*. Mechanisms explaining the infection (i.e. colonization) of the UG by the bacteria

could be mediated by a possible immune depression of hoopoes during reproduction. The elevated energetic costs of reproduction and the production of sexual hormones at this stage implies a physiological stress that may have immune suppressive effects (Alonso-Alvarez *et al*. 2004). *Enteroccocus* sp. are abundant in the digestive tract and faeces of birds and, contrary to most hole-nesters, hoopoes do not maintain the nest clean of faeces. Thus, the faecal contamination of nests of hoopoes, together with a possible worsened immune system during the nesting phase, would facilitate the colonization of the UG of hoopoes by *Enterococcus sp*. However, immune responses of females toward injections of the mitogenic phytohaemagglutinin (PHA) were significantly larger during the nesting phase (incubation (N = 3): mean PHA response (SE) = 1.18 (0.16); brooding (N = 6): mean PHA response (SE) = 1.34 (0.11)) than when UGS of females were of white colour (pairing (N = 12): mean PHA response (SE) = 0.43 (0.08); after abandoning the nest for feeding nestlings (N = 19): mean PHA response (SE) = 0.68 (0.06)) (Martin-Vivaldi *et al*. unpublished information), which suggests that females have an enhanced immune system during the nesting phase of reproduction instead of suffering immunosuppression.

The presence of this bacterium in the UGS is not the result of accidental contamination from nest boxes because several reasons. The first one is that, we have detected bacteria aggregations at a high density inside the uropygial gland of a female hoopoe (Fig. 1). Secondly, while accidental contamination of uropygial gland would predict for a low prevalence, we detected this association invariably in all the brown samples collected during the breeding season. In addition, we know from experiments of inter-specific egg exchange that all (N = 10) hoopoe nestlings that hatched in lab conditions and were reared in nests of great tits (*Parus major*) produced uropygial gland secretion with characteristics (colour and odour) similar to those produced by natural nestlings (Martin-Vivaldi *et al*. unpublished data). More importantly, all the UGS of nestlings reared in nests of great tits contained cultures of *E. faecalis*, and the analyses of colonies from one of those nestlings revealed the production of two different kinds of bacteriocins active against a broad spectrum of bacteria (Martín-Platero *et al*. 2006). Therefore, although we do not know the mechanisms explaining bacterium acquisition, our results

indicate that it is not the result of accidental contamination in the nests of hoopoes, and suggest an intimate association between hoopoes and *E. faecalis* leaving in the uropygial gland during the nesting phase of reproduction.

Although it is well known that UGS of birds may contain antibiotic substances (Jacob *et al.* 1982; Shawkey, Pillai, & Hill 2003a), at least some of the antibiotic properties of UGS of hoopoes are likely mediated by the associated bacterium because of the detected antibiotic properties of the BLIS produced by *E. faecalis* isolated from UGS of hoopoes (see, Martín-Platero *et al*. 2006). In agreement with this possibility we found that brown, but not white, secretions from adults inhibited growth of *B. licheniformis*, a common feather decomposing bacterium (Burtt *et al.* 1999). Further, colonies of *E. faecalis* from hoopoes demonstrated clear growth-inhibition activity against different bacteria including *B. licheniformis*. More importantly, in lab conditions, the addition of protease inhibited the antimicrobial activity of *E. faecalis* colonies, which suggests that the antimicrobial properties of brown UGS of hoopoes are mediated by antibacterial peptides produced by symbiotic bacteria. Therefore, although brown UGS of hoopoes may contain antibiotic substances not directly related to the associated enterococci bacterium, the detected antimicrobial properties of bacteria living in UG of hoopoes suggest that at least part of UGS activity is due to the presence of the symbiotic bacteria, and therefore suggest beneficial effects for hoopoes of such association.

The beneficial antimicrobial-effect of enterococci, due to the production of BLIS (i.e. enterocins), are not new and is well-known from research on poultry but also on food conservation (Foulquié Moreno *et al*. 2006). Among the two species of enterococci that have been intensely studied, *E. faecium* and *E. faecalis*, the beneficial effects are clearer for the former than for the later. For instance, although strains of both species have been applied in human and veterinary probiotic supplements, nine different strains of *E. faecium*, but none of *E. faecalis*, are authorized as additive in feeding stuffs in the European Union (see, Foulquié Moreno *et al*. 2006). Moreover, in a wild population of flycatchers, prevalence of both enteroccoci species in the cloaca of nestlings were negatively associated and that of *E. faecium* resulted strongly positive associated with fledging body size and mass (Moreno *et al*. 2003). With respect to *E.*

faecalis, although it is considered opportunistic pathogen responsible of nosocomial infections (Franz, Holzapfel, & Stiles 1999) some clear benefits due to competitive exclusion of pathogenic bacteria have been detected (Wagner, Holland, & Cerniglia 2002). In any case, because the bacteriocins isolated from cultures of *E. faecalis*, but also bacterial colonies of this species (see results), collected from the UGS of hoopoes demonstrated activity against all gram-positive bacteria assayed and also against some gram-negative strains (Martín-Platero *et al*. 2006), it is likely that in hoopoes, the production of these broad-spectrum antibacterial substances by the enterococcal strain living in the uropygial gland was important to the hygiene of the nest and thus to the health of the eggs and chicks.

To test this hypothesis, we used protease to experimentally deactivate BLIS in nests of hoopoes and found that, in comparison with control nests, the experimental addition of protease to nests of hoopoes resulted in a relatively higher egg bacterial infection and hatching failures. These effects were not due to protease directly affecting both bacterial infection and hatching failure because the experiment had no effect in nests of spotless starling, where BLIS have not been detected. The above results suggest a mutualistic association between hoopoes and *E. faecalis* living in the UGS that provide hosts with substances that inhibit growth of pathogenic bacteria. Against this interpretation, it can be argued that starlings and hoopoes differ, not only in the production of BLIS by associated bacteria, but also in nest's environmental conditions and eggshell characteristics, which otherwise may also explain the interspecific differences in the effect of our experiment. For instance, it is known that starling use plants with antibacterial properties for nest building (Gwinner & Berger 2005) and thus, because protease may also act deactivating antibacterial components of plants, starling may not be a good control species. However, in this scenario, we should find that starling eggs treated with protease had higher density of bacteria than control nests, which were far from being the case (see results). Other potential problem is that hoopoes, but also starlings, may have other antimicrobial peptides different from the BLIS produced by the *E. faecalis* living in the uropygial gland of hoopoes and thus, our experiment, not only deactivate BLIS but also another potential antimicrobial peptides. However, in this case, we should find differences between protease and

water treatment in hoopoes, but also between protease and water in starling. However, contrary to this possibility, we found no differences in number of colonies detected on the eggshell of both species (Fig. 3). Therefore, the most likely explanation of our experimental results is that protease deactivated BLIS produced by *E. faecalis* of hoopoes provoking an increased probability of eggshell colonization by pathogenic bacteria that would affect probability of hatching failures.

Recently Cook *et al*. (2005) detected that incubation of eggs reduced microbial growth on eggshells and the opportunity for trans-shell infection. Among other possible explanations, they (Cook *et al*. 2005) suggested the existence of certain protective microbial species living on the incubating parents (i.e. feathers or skin) that prevent eggshell contamination by pathogenic bacteria during incubation. Here, as predicted, by deactivation of BLIS produced by enterococci living in the UGS of hoopoes, we have found evidence of a positive effect of the symbiotic bacteria on hatchability of hoopoe eggs. In addition, because UGS of nestlings also showed identical properties, and nestlings, as well as females, spread UGS containing the symbiotic *E. faecalis* and its BLIS on their body, the symbiotic bacteria may protect hoopoes from bacterial diseases due to the strong activity and broad spectrum of their BLIS. This benefit would be particularly important for hole-nesting species that do not sanitize their nests, given the elevated risk of parasitism (Møller & Erritzøe 1996) and the large number and diversity of bacteria in these environments, which have the potential of becoming opportunistic pathogens (Burtt *et al.* 1999;Pinowski *et al.* 1994).

In contrast with the coevolutionary diffuse association between intestinal flora (including enteroccoci) and their hosts (see, Thompson 2005), here we present evidence of a symbiotic association of a single bacterium that grows in the uropygium of hoopoes and are used by host to prevent infections (i.e. of eggs). Thus, although tight mutualistic relationships between animals and bacteria are well-known in different taxa of invertebrate (see introduction), results presented here are the first evidence of such as kind of symbiosis occurring in vertebrates outside the digestive tract.

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III

SYMBIOTIC BACTERIA ARE RESPONSIBLE OF SOME OF THE SPECIAL PROPERTIES OF HOOPOE (*UPUPA EPOPS*) UROPYGIAL GLAND

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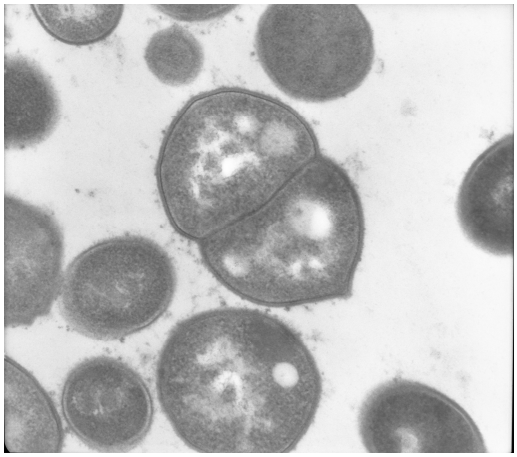


ABSTRACT

Bird uropigial glands serve multiple functions, and there is great interspecific variability in the composition and properties of its secretion. One special case is the secretion of the hoopoes (*Upupa epops*) and woodhoopoes (*Phoeniculus purpureus*), that differing from the most common white and odourless secretion in other groups of birds, is dark and has a pungent odour. Recently, it has been shown that glands of both woodhoopoes and hoopoes shelter symbiotic bacteria, which could be responsible of some of the special properties of glands and secretions of this group of birds. Here, we study this hypothetical relationship by exploring natural seasonal changes and intersexual differences in the properties of hoopoe glands and secretions, and analyse the effect of experimental injection of antibiotics (i.e. clearing bacteria from the gland) on such uropygial gland properties. Male glands did not experience seasonal changes, and their secretions were always white and odourless, very similar to female glands outside the breeding season. However, in comparison with the uropygial gland of non-breeding females, those of nesting females showed a marked increase in size and volume of secretion produced, which moreover became dark and pungent. All these parameters increased until hatching date and, afterwards, decreased returning in the second half of the nestling period to values similar to those detected for the prelaying phase. Nestling glands produced secretions similar to those of females in colour and odour and gland size of both females and nestlings predicted the amount of secretion produced. The experimental clearance of bacteria in gland of nestlings did not affect size of glands but provoked a decreased volume of secretion that was significantly lighter in colour than that of control nestling. In nesting females, the experimental injection of antibiotic affected both gland size and secretion colour. These results suggest that some of the special properties of hoopoe glands are mediated by the presence of the symbiotic bacteria.

INTRODUCTION

Bacteria interact with other organisms at several levels and with different output (McFall-Ngai 2002; Tannock 1999), thereby influencing the evolution of all live beings (Moran 2002). Bacteria may have pathogenic effects, thus, selecting for antibacterial defences by hosts (Clayton & Moore 1997; Dieckmann *et al*. 2002; Haldane 1949; Stearns 1999). However, due to their biosynthetic capabilities, they may also have beneficial effects for other organisms since they produce compounds essential in most environments (e.g. fixation of N_2 by Rhizobium and other species, Long 1989; Vessey *et al*. 2004), are able to process energetic substrates which are not accessible to plants or animals (such as sulfides in hydrothermal vents, Bright & Giere 2005; Tunnicliffe 1991), and provide some vitamins and micronutrients in the digestive tract of animals (see Hooper *et al*. 1998). Given the importance of beneficial bacteria for eucarionts, hosts tolerate, or even promote, strong associations with such bacteria (McFall-Ngai 2002; Moran 2002; Ruby *et al*. 2004). Commensal or mutualistic symbioses of bacteria with animals are quite common at the digestive tract, where mixed communities of bacteria help in proccessing nutrients (Backhed *et al*. 2004; Dillon & Charnley 2002; Dillon & Dillon 2004; Hooper *et al*. 2001; Hooper & Gordon 2001). However, tight mutualistic relationships between animals and particular bacteria in which, for instance, hosts have modified or even evolved special organs for bacteria which, in return, provide hosts protection against pathogens, parasites or predators by mean of substances synthesised by them, are more rarely detected (Barbieri *et al*. 2001; Currie *et al*. 1999; 2006; Currie 2001; 2002; Foster *et al*. 2000; 2002; Foster & McFall-Ngai 1998; Oliver *et al*. 2003; Scarborough *et al*. 2005; Visick *et al*. 2000). These kinds of mutualistic relationships have been described in a variety of invertebrates but never in vertebrates. Vertebrates, however, are good candidates for presenting tight mutualistic relationship with bacteria similar to those detected in some invertebrates as ants (Currie *et al*. 1999, 2006) and aphids (Oliver *et al*. 2003). For instance, exocrine glands involved in the production of substances that are spread on the body surface may also be excellent places for hosting bacteria that synthesise beneficial products. Sebaceus and uropygial glands of mammals and birds produce substances that these animals extend over their body for fur and feathers protection. In addition, several glands are

responsible of secreting the external layers of eggs in fishes, anfibians, reptiles and birds, which may shelter beneficial bacteria. However, very few studies have analysed whether these glands in general (McFall-Ngai 2002), and the uropygial gland in particular, host symbiotic bacteria that produce beneficial substances that, for instance, could explain the antimicrobial properties of uropygial glands (Jacob & Ziswiler 1982; Shawkey *et al*. 2003).

Recently, two closely related families of birds ((Feduccia 1975): woodhoopoes (Fam. Phoeniculidae) and hoopoes (Fam. Upupidae)) have been shown to host bacteria in their uropygial glands (Law-Brown & Meyers 2003; Soler *et al*. unpublished). In both groups, the uropygial gland exhibits special properties that most species of birds lack. The uropygial gland secretions of these groups are dark and pungent, and the glands are of exaggerated size (Cramp 1998; Ligon 2001). In the case of the hoopoe *Upupa epops*, such special properties are present only in females and chicks during their stay within the hole-nest (Cramp 1998). Curiously, eggs of this species change drastically in colour during incubation, from very pale blue at laying to beige or brown soon afterwards (Cramp 1998), apparently due to impregnation of the dark uropygial secretion of females (pers. obs.). Therefore, the antimicrobial properties of the uropygial secretion would confer protection not only to the producer female, but also to her eggs. In addition it is known that secretions of the hoopoe *Upupa epops* have antimicrobial properties, which are likely mediated by bacteriocin like inhibitor substances (BLIS) produced by the symbiotic bacteria living in the uropygial gland (Martín Platero *et al*. 2006; Soler *et al*. unpublished). Therefore, hoopoes and their symbionts are a good system for the study of possible roles of symbiotic bacteria mediating properties of the uropygial gland and its secretion. In hoopoes, only brown secretions produced by the enlarged uropygial gland of nesting individuals demonstrate bacterial-growth inhibition properties and contain symbiotic bacteria (*Enterococcus* sp.) (Soler *et al*. unpublished; Ruiz Rodriguez *et al*. unpublished). The association between special characteristics of the uropygial gland (enlarged) and secretion (brown and pungent) of hoopoes, and the presence of the bacteria, suggests that bacteria may cause such properties. Alternatively, such changes may be directly produced by the bird (e.g. mediated by hormonal changes), while the presence of bacteria may simply be the consequence

of colonizing uropygial gland with such traits (i.e. enlarge glands that produces brown secretion).

Here we provide detailed data on natural variation of hoopoes uropygial gland and secretion and, by experimentally eliminating bacteria from glands of both female and nestling hoopoes using antibiotics and comparing them with control individuals, we study the effects of bacterial clearance on both uropygial gland morphology and characteristics of their secretion.

MATERIAL AND METHODS

Study area, study species and general procedures

The study was performed between 2003-2006, in the Hoya de Guadix (37º18' N, 38º11'W), southern Spain, where hoopoes breed in crops, forests and gullies within nest-boxes placed in trees or buildings (for a more detailed description of the study area see Martín-Vivaldi *et al.* 1999).

Hoopoes are medium sized coraciiformes that nest in a variety of holes that are re-used successfully by the same or different individuals for many years (Cramp 1998). Females lay one or, at most, two clutches along the breeding season. Incubation (17 days) and brooding is performed only by the female, that stays the whole day within the nest until nestling are about eight days old, and during those three weeks, the male provide all the food for both female and nestlings. Afterwards both pair members carry food to the nest until fledging of chicks when they are 24-30 days old (Baldi and Sorace 1996, Cramp 1998, Martín-Vivaldi *et al*. 1999). Incubation usually starts with the first or second egg, producing a complete hatching asynchrony in which eggs hatch at 24 h or even greater intervals (Bussman 1950, Gupta and Ahmad 1993, Baldi and Sorace 1996, Cramp 1998). This generates a marked size hierarchy within the brood that can be used to deduce hatching order (Martin-Vivaldi *et al*. 2006).

Each breeding season the nest-boxes were visited twice per week from mid February to the end of July to record laying dates, clutch size and hatching dates. In 2005-2006, nestlings were measured soon after hatching to deduce their age from the initial weight hierarchy. Nestlings were individually painted with permanent markers on their tarsus every two days

until they were banded with numbered metal rings. When the brood reached 19-21 days old, nestling were measured again.

Adults were caught with miss-nets along the whole breeding season, and with special nets for nest-box entrances during the nestling period. Incubating or brooding females were caught by hand within the nest-box and after manipulation were released again within it to reduce disturbance. For individual recognition, all adults were ringed with a numbered (Spanish Ministerio de Medio Ambiente) and coloured rings. To reduce manipulation time and disturbance of incubating females, minimising risk of nest desertion, they were marked only with the numbered metal ring.

Gland measurements

The uropygial gland of hoopoes comprises two secretor lobes that flow into a papilla where the secretion accumulated. The papilla presents a single orifice to the exterior that is surrounded by a circlet of feathers forming a tuft (Jacob & Ziswiler 1982). The papilla varies greatly in size, which mainly depends on the amount of secretion stored. Walls of the papilla are elastic and thin, which make difficult its measurement once the secretion has been extracted. Thus, trying to avoid unreliable measurements, we characterized the lobe area, which is the responsible of the secretion produced. Briefly, with a digital calliper (Mitutoyo) we took three linear measurements of the lobes to the nearest 0.1 mm: gland width, gland length, and gland height (Fig. 1). A composed measurement of gland volume was also estimated as the product of the three linear measurements.

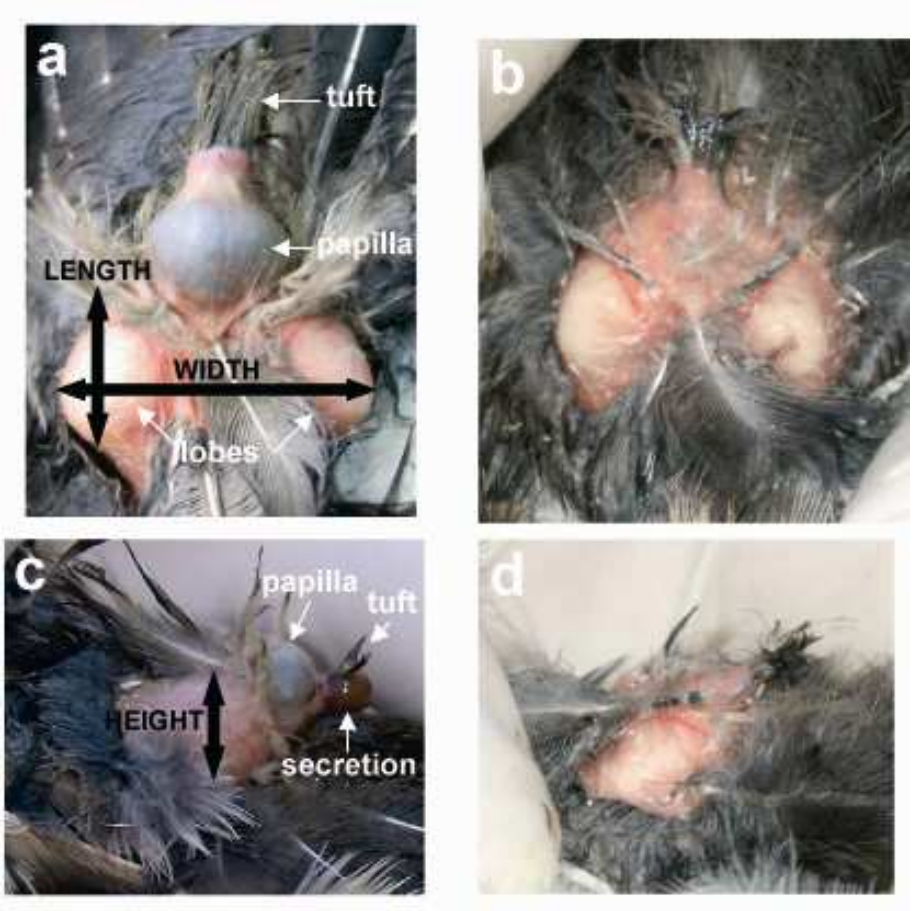


Fig. 1. *Pictures of the uropygial glands of a breeding female hoopoe (a, c) and a male (b, d). (a) Dorsal view of a female gland showing a papilla filled with dark secretion. (b) Dorsal view of a male gland. (c) Lateral view of a female gland showing a papilla filled with dark secretion a part of which is being exuded through the papilla aperture. (d) Lateral view of the male gland. The different parts of the gland and the three linear measurements taken are indicated.*

Adult glands were measured in all captures to describe changes in their size along the breeding season. Along the paper we distinguish the following stages for females: (1) non-reproducing female: before laying, with white and odourless secretion. (2) prelaying females: few days before laying, with odorous secretions turning to brown colouration. And (3) nesting females: laying, incubating or brooding females with brown and odorous secretions. Nestling glands were measured at 11-14 and 19-21 days old ages.

Secretion measurements

Given that hoopoes can exude the secretion from the gland when handled, to estimate the amount of secretion produced and its colour, we extracted all the secretion available in the papilla as soon as possible after capture. We wore latex gloves for the whole process. The

extraction protocol was as follows. First, we washed softly the circlet and surrounding skin with a cotton embebed in ethanol to reduce the risk of contamination of the secretion with bacteria from outside the gland. After evaporation of the alcohol remains, a sterile needle was used to set aside the circlet discovering the papilla entrance where a sterile tip of a 1-10 µl micropipette (Finpipette) was introduced gently inside the papilla. The automatic pipet was calibrated with a known volume that was absorbed several times while the papilla was pressed softly with a finger up to emptying it completely. The extracted secretion was introduced in a sterile eppendorf vial while the number of extractions allowed the estimation of volume of secretion that was present in the papilla of the gland. Immediately after extraction, approximately 5 µl of the secretion were introduced into a capillary tube, the extremes were sealed with plasticine, and kept at about 4ºC in a portable icebox until colour evaluation in the laboratory within the next 10 hours. The colour of secretions was estimated under standardized light conditions by comparison the colour of the secretion (i.e., filled capillary tubes) with colour tables available in Küppers Colour Atlas (Küppers 2002) in the field of orange (combination of black-yellow-magenta coordinates). We recorded the values in the black, yellow and magenta axes coordinates, each ranging from 0 to 99 in 10-units steps, of the colour square better matching the colour of the secretion.

Experimental methods

In 2004 we manipulated bacteria presence in breeding females that were randomly assigned to two treatment groups. During three consecutive days, and after emptying the papilla as explained above, the gland of experimental females was injected with 0.04 ml of amoxiciline (Clamoxyl GlaxoSmithKline S.A.). 0.02 ml of antibiotic were injected through the wall of each of the two secretor lobes, directly into the secretory tissues. We were confident that the antibiotic reached the secretory area because it flew from the lobes into the papilla through the ducts, which assure that the papilla was full of antibiotic. Control females were treated in the same way but we injected sterile saline solution in their gland. The first capture of adult females was performed the third day after hatching of the first egg, while the last fourth capture took place

seven days after the third injection (thirteen days after hatching). Since hatching date is the moment of maximum development of female glands (see Fig. 4 and 5 in Results), the treatment was not intended to affect growing but instead rate of reduction after the gland reach the maximum size. We measured gland size, secretion volume and colour of secretion of females all catching days.

During the breeding seasons of 2005-2006, we performed a similar experiment in nestling hoopoes. Hatchlings were ranked according to their size. Starting with the heaviest or second heaviest hatchling (randomly selected), half of the hatchlings, intermittent according to their size, were assigned the antibiotic treatment. Other chicks in the nests were injected with saline solution as control treatment. The treatments were similar to those used in females (see above) but nestling were injected every second days after the start of the experiment. Nestling glands start to have visible lobes and to produce secretion after the 4th day of life (Cramp 1998) and, thus, we started the experiment when the older nestling in the nest was 11 days old. All nestlings were injected 5 times, but glands were measured and the secretion extracted to estimate volume and colour only twice, before the first and two days after the last injections.

Statistical methods

Descriptive data

Adults

Although some non-experimental individuals were captured and measured several times and/or study years, except when specifically mentioned, only information collected during the first capture was used. The changes along the breeding season in gland size, secretion volume and secretion colour were analysed by mean of quadratic regressions. For between-periods and between-sexes comparisons General Linear Models (GLMs) were used. To control for the allometric effects of gland size and secretion volume, but also for among-year variations, we used residuals after these variables were controlled for tarsus length and year in our analyses. On the other hand, since the three values (i.e. coordenates) of secretion colour (black, magenta

and yellow) did not vary among years (one-way ANOVAs, all $p > 0.15$), we pooled data from different years.

Nestlings

Repeated measures anovas with brood stage (two levels, for 11-14 and 19-21 days old nestling) including only the oldest nestling in each brood, were used to study the changes in gland properties along the nestling period.

Experimental data

Nestlings

We used General Lineal Models with nest identity (nested within year) and year as random factors, and treatment as fixed factor to test the effects of the antibiotic treatment. Several dependent variables were homoscedastic and normally distributed within treatments (gland volume, level of magenta in secretion, level of yellow in secretion; non-significant Levene and Kosmogorov-Smirnov tests). On the other hand, volume of secretion and level of black in secretion had to be Log-transformed, or converted in an ordinal scale from 1 to 6 (by collapsing adjacent classes in the Küppers table), respectively, to reach homoscedascity and approximately normal within-treatment distributions.

Females

To test the influence of the supression of bacteria on seasonal changes in gland properties, we used a Repeated Measures Anova with the measurements obtained in consecutive captures as within-effect and treatment as between-effect. Planned comparisons of Least Square Means were used to test the specific effects of interest: (a) changes of gland properties between consecutive captures within treatments, and (b) interaction between the treatment and the changes between pairs of captures.

All statistical tests used were two-tailed, with α-level established at 0.05. Points and whiskers in graphs represent means and 95% confidence limits of the mean.

RESULTS

Natural variation in gland properties

Adults

During the prelaying period, UG of males were of larger relative volume than those of non-reproducing females after controlling for tarsus length and year (Table 1. Fig. 2a). Such sexual differences were mainly due to differences in the height, but not in width and length of the gland (Table 1, Fig 2b-d). Despite inter-sexual differences in volume of the UG, males and females did not differ in the amount of secretion produced in this phase (GLM with sex as fixed factor, year as random effect and tarsus length as covariable, $F(1,15) = 0.37$, $p = 0.55$).

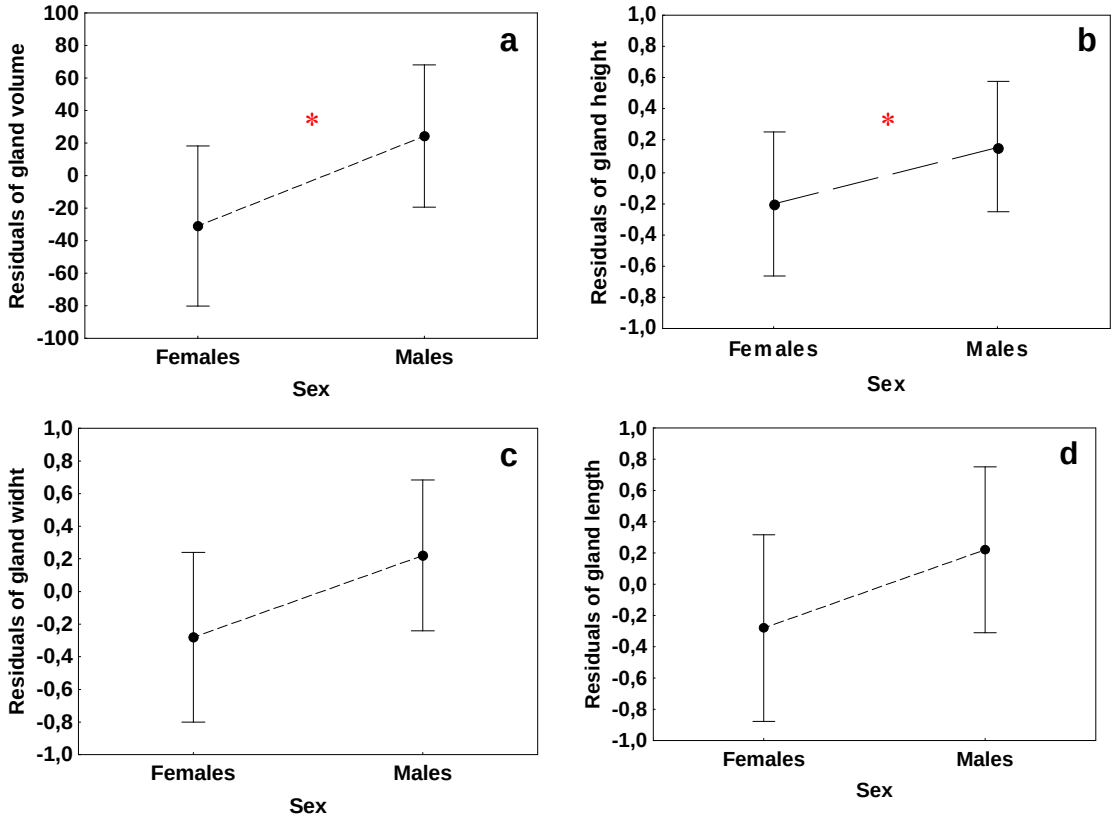


Fig. 2. *Intersexual differences in hoopoe gland properties in the non-reproducing period (individuals with white secretions) after controlling for year and tarsus length in GLMs. Asteriscs mark significant differences at the 0.05 a-level*

		Volume		Width		Length		Height	
	df	F	P	F	P	F	P	F	P
Intercept	1	4.02	0.05	2.14	0.15	1.88	0.18	2.14	0.01
Tarsus	1	2.26	0.14	1.05	0.31	0.04	0.85	5.44	0.03
Year (random)	2	26.09	0.03	10.22	0.18	2.01	0.43	53.46	0.048
Sex	1	8.50	0.01	2.51	0.20	0.46	0.58	6.04	0.044
Year x sex (random)	1	0.03	0.86	0.16	0.69	0.45	0.51	0.08	0.76
Error	28								

Table 1. *Intersexual differences in size of hoopoe uropygial glands in the non-reproducing period, still with white secretions. The table shows the results of General Linear Models with year and nest (nested within year) as random factors, and sex as fixed factor.*

The uropygial gland of pre-laying females increases considerably in volume ($F(1,13) = 36.87$, $p < 0.0001$, Fig. 3a), length ($F(1,13) = 16.24$, $p = 0.001$), and height ($F(1,13) = 14.40$, $p = 0.002$, in all cases including tarsus length as covariable in GLMs). Moreover, larger glands of pre-laying females produced larger volume of secretion than non-reproducing females (GLM with tarsus length as covariable $F(1,13) = 6.99$, $p = 0.03$, Fig. 3b).

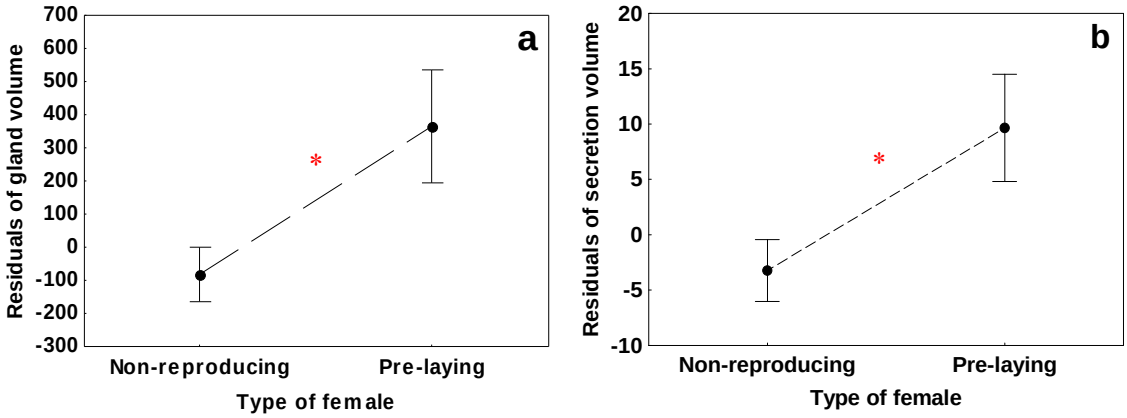
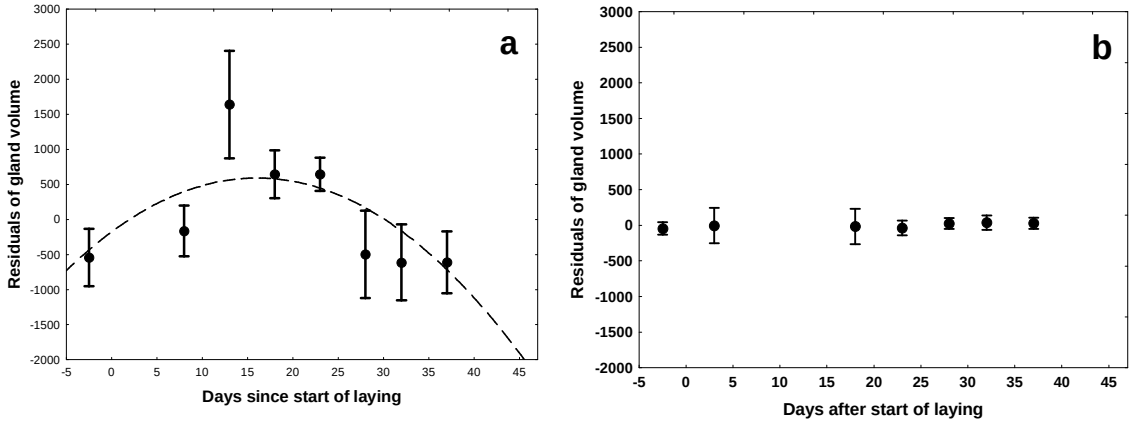


Fig. 3. *Differences in gland volume and volume of secretion between non-reproducing females and prelaying females after controlling for year and tarsus length in GLMs. Means and 95% confidence intervals for the dependent variables are shown. Asteriscs mark significant differences at the 0.05 a-level.*

Once females started to reproduce, their gland changed drastically along the breeding cycle. Gland volume increased after the start of laying up to the hatching time, when gland size reached the maximum values. After hatching time, gland size decreased drastically up to the second half of the nestling period, when gland volume return to values similar to the prelaying

period (Fig. 4a). Thus, variation of both gland volume and volume of secretion of females fitted quadratic regression lines (Fig. 4), and secretion volume was significantly positively correlated with gland volume along the breeding season (GLM, with year (random factor) and tarsus length as covariables, beta(SE) = 0.57(0.10), F(1,65) = 31.01, p = 0.000001). The three linear measurements of gland size were responsible of the parabolic pattern of change in gland volume (significant quadratic regressions, data not shown), and all three biometrical measurements correlated with volume of secretion when analysed separately (GLMs, with year (random factor) and tarsus length as covariables, gland width: beta(SE) = 0.65(0.10), F(1,65) = 45.36, p < 0.000001; gland length: beta(SE) = 0.53(0.11), F(1,65) = 24.80, p = 0.000005; gland heigth: beta(SE) = 0.60(0.10), F(1,65) = 32.67, p < 0.000001). On average, volume of glands of incubating or brooding females (i.e. from five days before to five days after hatching date) was about eight times larger (X ± SE = 1844.7 ± 187.3 mm^3, n = 12, maximum = 3268.3) than that of the glands of non-reproducing females (X ± SE = 239 ± 34 mm^3, n = 13). Similarly, the average amount of secretion produced by glands of females in such reproductive stage was almost 30 times larger (X ± SE = 43.33 ± 12.3 µl, n = 11, maximum = 137.8) than that produced by non-reproducing females (X ± SE = 1.53 ± 1.32 µl, n = 15). None of the seasonal changes reported for female uropygial glands and secretions occurred in glands of males, with volume of both gland and secretion remaining very similar along the whole breeding season (Fig. 4b, 4d).



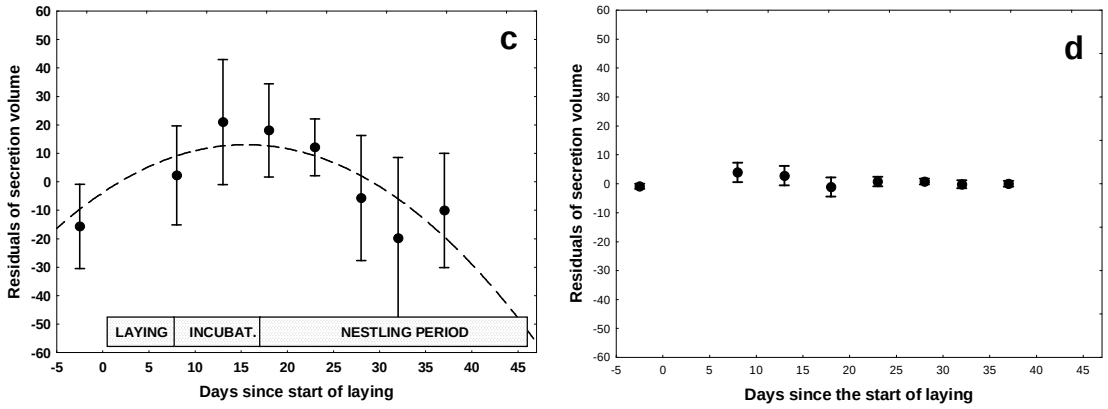
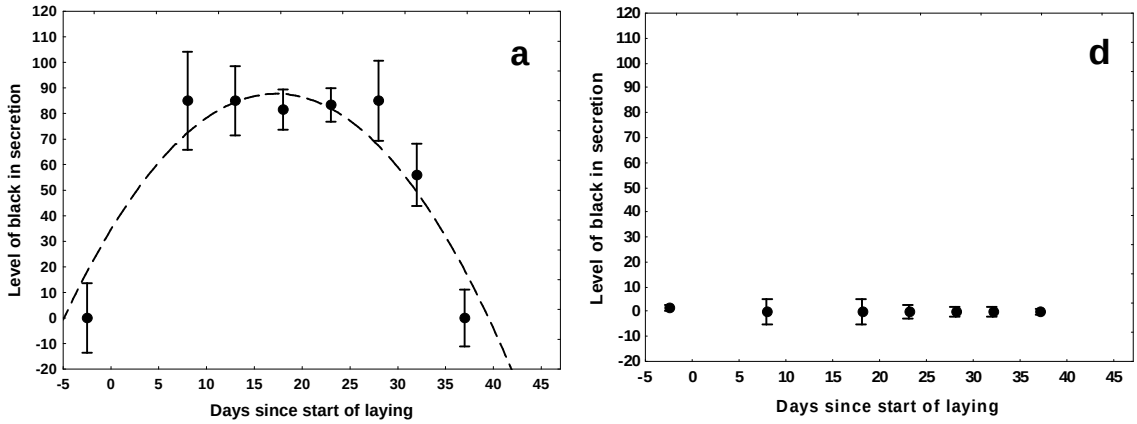


Fig. 4. *Changes in adult hoopoe uropygial gland properties along the breeding cycle. Discontinuous lines are the Quadratic regressions fitting raw data. Means and 95% confidence intervals for the dependent variables are shown for consecutive five days periods. The correspondence with the different phases of the breeding attempt (laying, incubation and nestling period) are indicated in (c). (a) Changes in female gland volume: Quadratic regression, $F(3,59) = 14{,}61$, $p < 0.00001$, $R^2 = 0.40$, $b_0 = -176.3 \pm 162.1$ ($t = -1.09$, $p = 0.28$); $b_1 = 95.7 \pm 16.6$ ($t = 5.76$, $p < 0.00001$); $b_2 = -2.98 \pm 0.47$ ($t = -6.28$, $p < 0.00001$)). (b) Changes in male gland volume: Quadratic regression, $F(3,40) = 1.17$, $p = 0.33$. (c) Changes in the amount of secretion produced by female glands: Quadratic regression, $F(3,68) = 6.26$, $p = 0.0008$, $R^2 = 0.20$, $b_0 = -3.8 \pm 5.2$ ($t = -0.73$, $p = 0.47$); $b_1 = 2.2 \pm 0.5$ ($t = 4.02$, $p = 0.0001$); $b_2 = -0.07 \pm 0.02$ ($t = -4.07$, $p = 0.0001$)). (d) Changes in the amount of secretion produced by male glands: Quadratic regression, $F(3,48) = 1.92$, $p = 0.139$.*

The most drastic change in female gland properties occurs in the characteristics of the secretion. While the colour of the UGS of non-reproducing females is predominantly white, it became dark for the whole period that females stayed within the nest incubating or brooding. Estimated colourations of the UGS, i.e. coordinates of the Black-Yellow-Magenta axes within the field of orange (Küppers 2002, see Material and Methods) varied in different breeding phases. All estimated colour values experienced an increase during the laying period and decreased markedly in the second half of the nestling period (Fig. 5). Again, colour characteristics of male secretions did not change along the whole breeding season (Fig. 5).



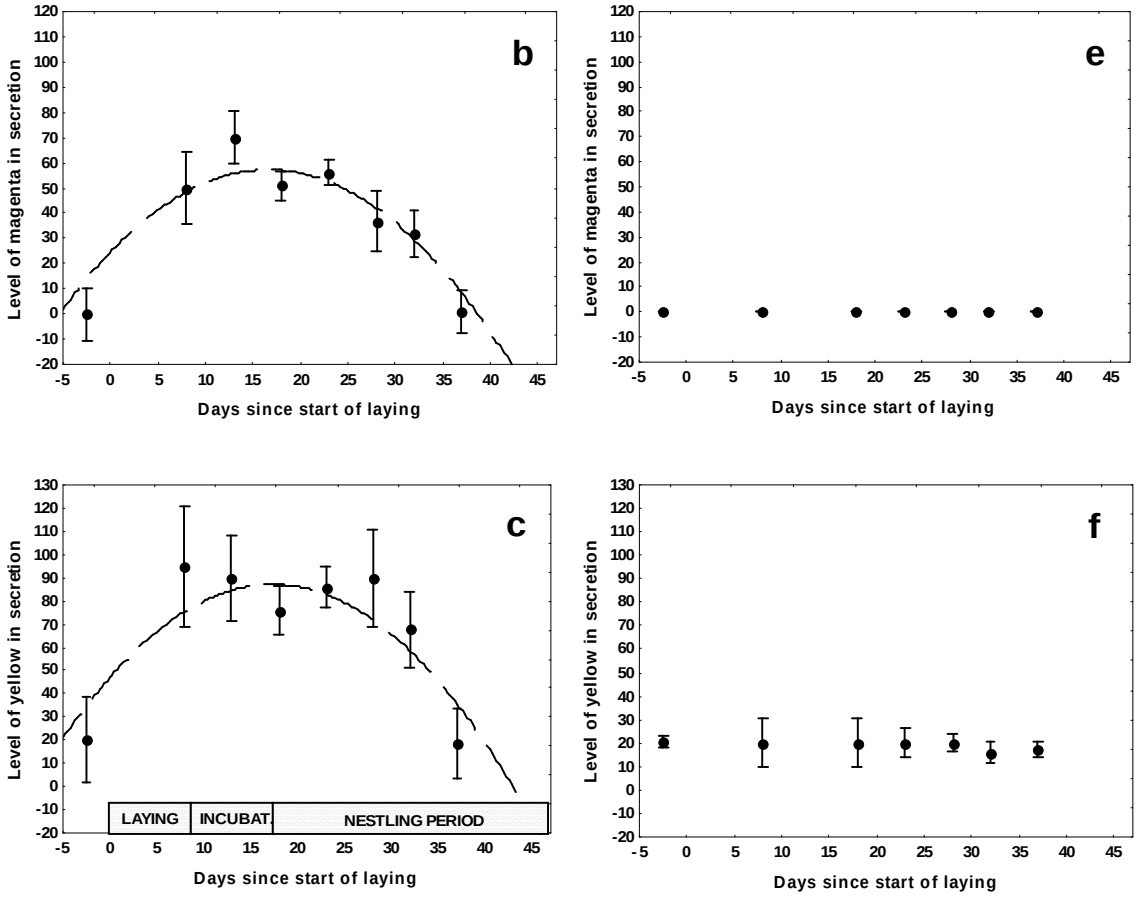
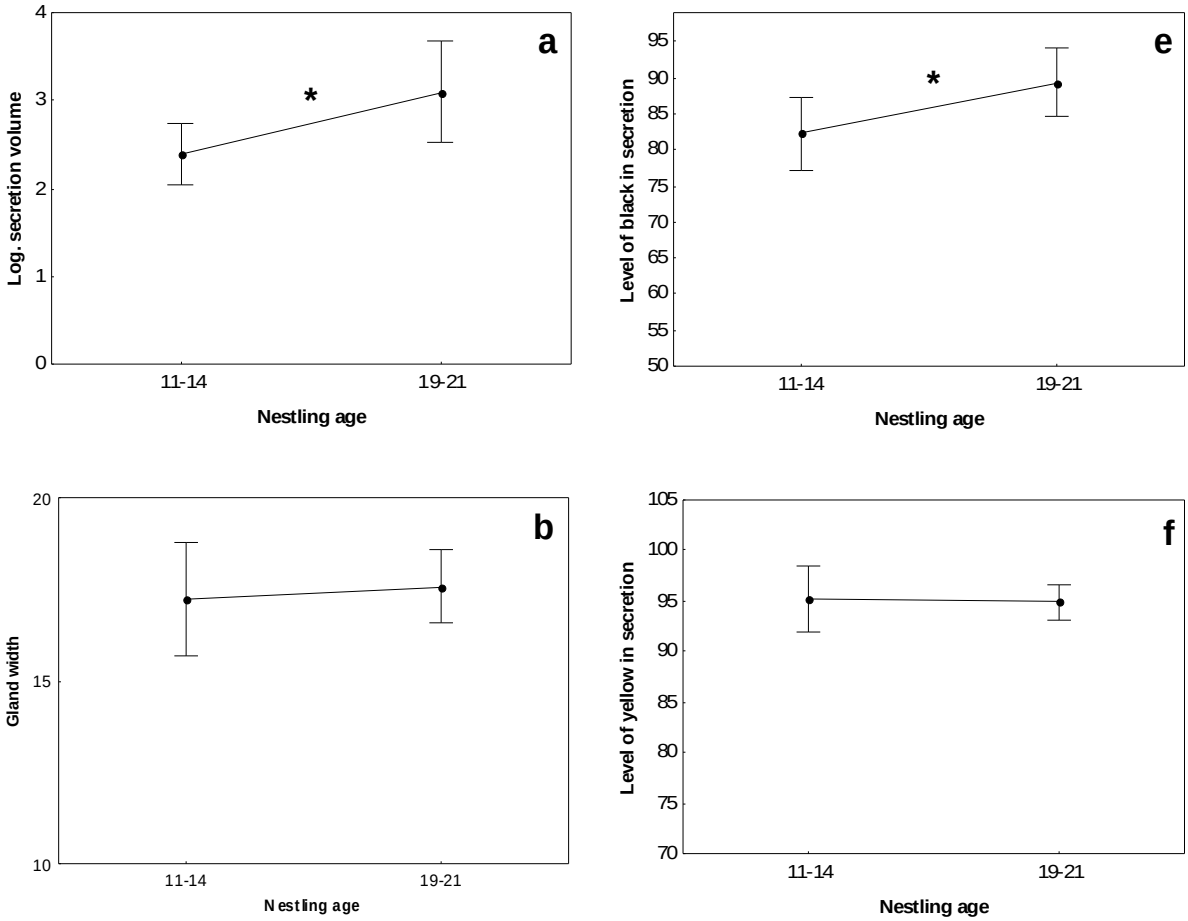


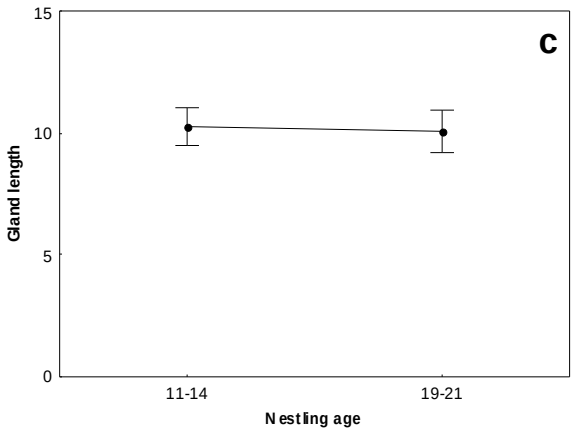
Fig. 5. *Changes in the colour of female and male secretions along the breeding cycle. Discontinuous lines are the Quadratic regressions fitting raw data. Means and 95% confidence intervals for the dependent variables are shown for consecutive five days periods. The correspondence with the different phases of the breeding attempt (laying, incubation and nestling period) are indicated in (c). (a) Changes in the level of black colour in female secretion: Quadratic regression, $F(3,50) = 395.2$, $p < 0.001$, $R^2 = 0.82$, $b_0 = 34.58 \pm 5.25$ ($t = 6.6$, $p < 0.000001$); $b_1 = 6.15 \pm 0.47$ ($t = 13.1$, $p < 0.00001$); $b_2 = -0.18 \pm 0.01$ ($t = -14.4$, $p < 0.00001$)). (b) Changes in the level of magenta colour in female secretion: Quadratic regression, $F(3,50) = 273.4$, $p < 0.001$, $R^2 = 0.77$, $b_0 = 24.54 \pm 4.08$ ($t = 6.0$, $p < 0.000001$); $b_1 = 3.93 \pm 0.37$ ($t = 10.8$, $p < 0.00001$); $b_2 = -0.118 \pm 0.01$ ($t = -12.7$, $p < 0.00001$)). (c) Changes in the level of yellow colour in female secretion: Quadratic regression, $F(3,50) = 235.3$, $p < 0.001$, $R^2 = 0.59$, $b_0 = 47.15 \pm 6.97$ ($t = 6.8$, $p < 0.000001$); $b_1 = 4.61 \pm 0.62$ ($t = 7.39$, $p < 0.00001$); $b_2 = -0.133 \pm 0.02$ ($t = -8.39$, $p < 0.00001$)). d) Changes in the level of black colour in male secretion: Quadratic regression, $F(3,44) = 3.1$, $p < 0.037$, $R^2 = 0.12$, $b_0 = 1.24 \pm 0.53$ ($t = 2.4$, $p < 0.024$); $b_1 = -0.09 \pm 0.07$ ($t = -1.4$, $p = 0.166$); $b_2 = 0.002 \pm 0.002$ ($t = 0.78$, $p = 0.44$)). e) Changes in the level of magenta colour in male secretion: there is no variation, all values are zero. d) Changes in the level of yellow colour in male secretion: Quadratic regression, $F(3,44) = 234.3$, $p < 0.001$, $R^2 = 0.1$, $b_0 = 21.18 \pm 1.12$ ($t = 18.93$, $p < 0.00001$); $b_1 = 0.03 \pm 0.14$ ($t = 0.24$, $p = 0.81$); $b_2 = -0.004 \pm 0.004$ ($t = -0.86$, $p = 0.40$)).*

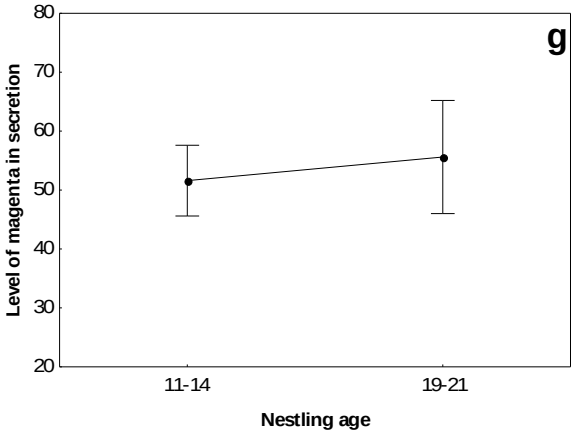
Nestlings

When using single measurement of individual nestlings of different age, gland size explained volume of secretion produced after statistically controlling for the effect of age, year (random factor) and nest identity (random factor nested within year). Gland length, but not gland width or height, was significantly positively correlated with secretion volume when analysed separately (GLMs, gland length: beta (SE) = 0.38 (0.13), $F(1,96) = 7.98$, $p = 0.006$; gland height:

beta (SE) = 0.19 (0.15), F(1,96) = 1.59, p = 0.209; gland width: beta (SE) = 0.28 (0.16), F(1,96) = 2.97, p = 0.088). In addition, the volume of secretion produced by nestlings that were not injected with antibiotic but with saline solution increased with age when comparing UG of 11-14 vs 19-21 days old nestlings (Repeated Measures ANOVA age effect: F(1,13) = 6.75, p = 0.022, Fig. 6-a). None of the biometrical parameters increased with nestling age at this stage (Repeated Measures Anova, age effect, for gland width: F(1,13) = 0.222, p = 0.645; gland length: F(1,12) = 0.151, p = 0.704; gland height: F(1,12) = 0.073, p = 0.792). Regarding the analysis of variation of the colour of secretions produced in nestling glands, we found that values in the black axis, but not those in the magenta or yellow axes, significantly increased with nestling age at this stage (Repeated Measures Anova, effect of age, for black: F(1,8) = 8.08, p = 0.022; yellow: F(1,8) = 0.143, p = 0.715; magenta: F(1,8) = 0.645, p = 0.445), Fig. 6e-g).







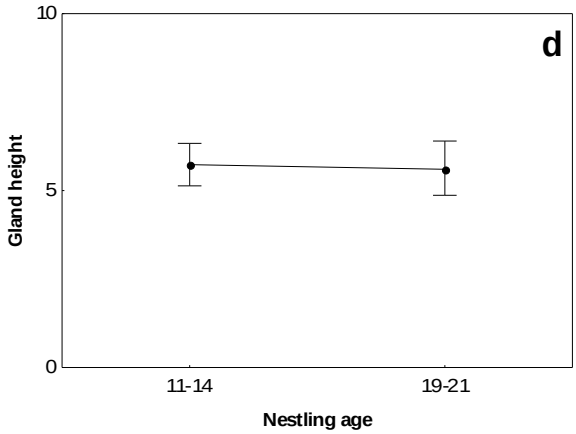


Fig. 6. *Within individual differences in gland properties between the early and late nestling period. Asteriscs mark significant differences at the 0.05 a-level. Means and 95% confidence intervals for the dependent variables are shown.*

Do symbiotic bacteria mediate variations in properties of the uropygial gland and its secretion?

Nestlings

The experimental injection of antibiotic successfully reduced Enteroccocci from the UG since probability of bacterial growth in specific medium for Enterococcus (Kenner Faecal, Scharlau, Barcelona, Spain) was larger for control than for experimental siblings (percentage of nestlings with Enterococci growth: Control nestlings 75% (n = 28), experimental nestlings 21.7 % (n = 23), Fisher exact test, P = 0.0002).

In accordance with the hypothetical role of symbiotic bacteria explaining changes in uropygial glands of hoopoes, we found that the antibiotic treatment affected the volume of secretion stored in the papilla of the gland of nestlings, but did not affect the volume of the uropygial gland (Table 2, Fig 7) or any linear measurements of gland size (all P > 0.05). Further,

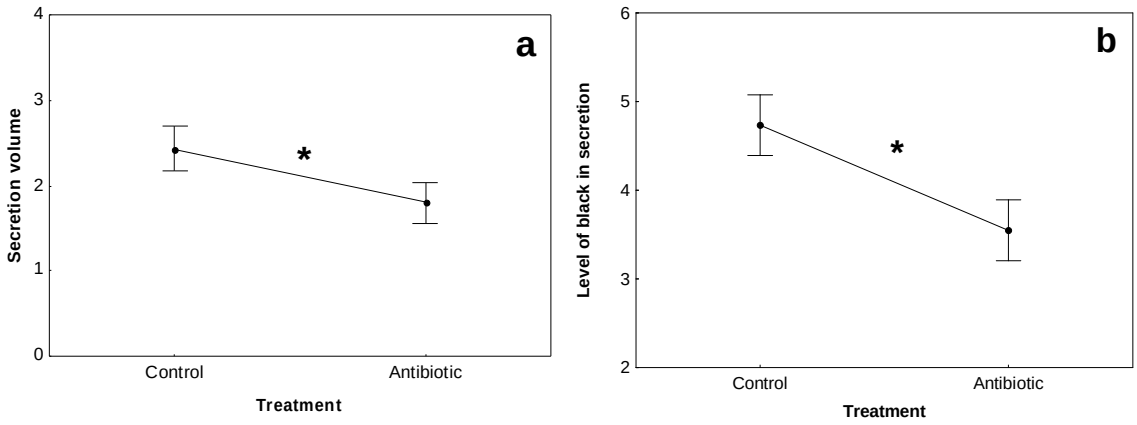
nestlings treated with antibiotic produced a lower amount of secretion (Fig. 5a) which, in comparison with secretions of control nestlings, was paler (i.e. less black, Table 3, Fig. 7b) and more reddish (Table 3, Fig. 7c). On the other hand, the antibiotic injections did not affect the estimated yellow colour of the secretions of nestling hoopoes (Table 2, Fig 7d).

	Log (Secretion volume)			Gland volume		
	Df	F	p	Df	F	p
Intercept	1	39.3	0.10	1	8.0	0.22
Year	1	8.4	0.007	1	46.0	<0.0001
Nest (Year)	26	1.8	0.082	25	3.6	0.0009
Treatment	1	22.7	<0.0001	1	1.7	0.201
Nest(Year)*Treatment	23	1.34	0.184	24	0.8	0.760
Error	56			61		

Table 2. *Effect of the antibiotic treatment on the volume of secretion produced by nestlings (Log secretion volume), and the volume of their gland (Gland volume). The table shows the results of General Linear Models with year and nest (nested within year) as random factors, and treatment as fixed factor.*

	Black			Magenta			Yellow		
	Df	F	p	Df	F	p	Df	F	p
Intercept	1	524.5	<0.0001	1	2067.6	<0.0001	1	25663.0	<0.0001
Nest	11	1.71	0.194	11	0.43	0.910	11	9.72	0.0004
Treatment	1	17.80	0.001	1	8.03	0.014	1	0.00	0.953
Nest*Treatment	11	1.44	0.202	11	1.26	0.289	11	0.22	0.994
Error	33			33			33		

Table 3. *Effect of the antibiotic treatment on the colour (within the three axes system in Küppers 2002: Black, Magenta and Yellow) of the secretion produced by nestlings in 2006. The table shows the results of General Linear Models with nest as random factor and treatment as fixed factor.*



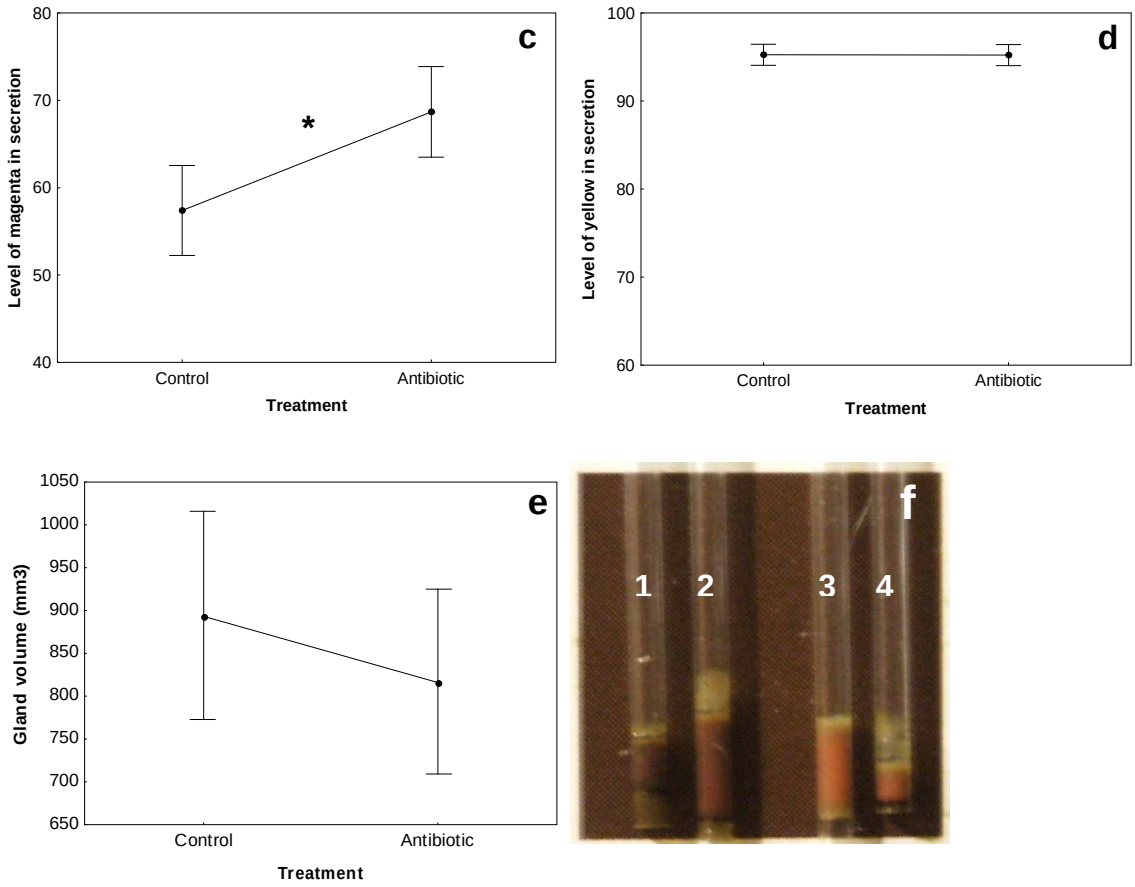
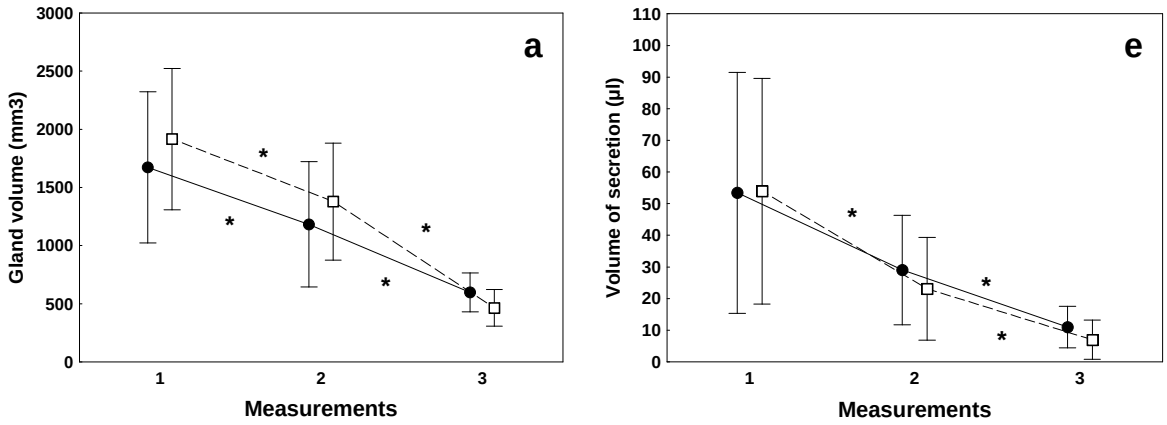


Fig. 7. *Effects of the antibiotic treatment on properties of nestlings glands. Analyses of secretion and gland volumes include data from 2005 and 2006, those of colour of secretions are only from 2006. Means and 95% confidence intervals for the dependent variables are shown. (a) Volume of secretion (Log transformed), (b) Black colour of secretions (in an ordinal scale), (c) Magenta colour of secretions, (d) Yellow colour of secretions, (e) Gland volume. (f) Picture of capillary tubes filled with four secretions of the chicks from one experimental nest, 1 and 2 control nestling, 3 and 4 antibiotic-treated nestling.*

Females

In a repeated measures design, with three measurements per bird (see Material and Methods), we detected a significant reduction in most of the considered parameters of the uropygial gland and its secretion independently of experimental treatment (Fig. 8, Repeated Measures Anovas, Planned Comparisons of LS Means, $F(1,12/13)$, all $p < 0.01$). The experimental injection of the antibiotic did not affect gland volume, gland width, gland length or volume of secretion, but had an effect on gland height and colour of the secretion for two of the three time of measurement (i.e., phases of reproduction) (Fig. 8). Females treated with antibiotic showed a deeper reduction of gland height than control females (Repeated measures Anova, interaction between experimental treatment and phase of reproduction, Planned Comparisons of LS Means

between second and third measurements: F(1,13) = 3.78, p = 0.074; between first and third measurements: F(1,13) = 4.75, p = 0.048; Fig. 8d). The antibiotic treatment also caused a non-significant steeper reduction of the level of black coloration of the secretion along the three measurements (Fig. 8f, Repeated Measures Anova, interactions between Antibiotic treatment and phase of reproduction, Planned Comparisons of LS Means between first and second measurements F(1,12) = 4.58, p = 0.053; between first and third measurements: F(1,12) = 4.31, p = 0.060). On the other hand, colour values of secretions for the magenta channel increased after two days of treatment in experimental but decreased in control females (Fig. 8g, Repeated Measures Anovas, Planned Comparisons of LS Means between first and second measurements, F(1,12) = 8.18, p = 0.014). The variation in yellow colour followed a similar trend, but was not significant (Repeated Measures ANOVAs, Planned Comparisons of LS Means between first and second measurements, F(1,12) = 1.47, p = 0.25; Fig. 8h). Finally, variation in yellow and magenta colour decreased from the second to the third measurements at the same rate in secretions of both control and experimental females (Fig. 8g-h, Repeated Measures Anovas, Planned Comparisons of LS Means between second and third measurements, Magenta: F(1,12) = 0.16, p = 0.699; Yellow: F(1,12) = 0.08, p = 0.78).



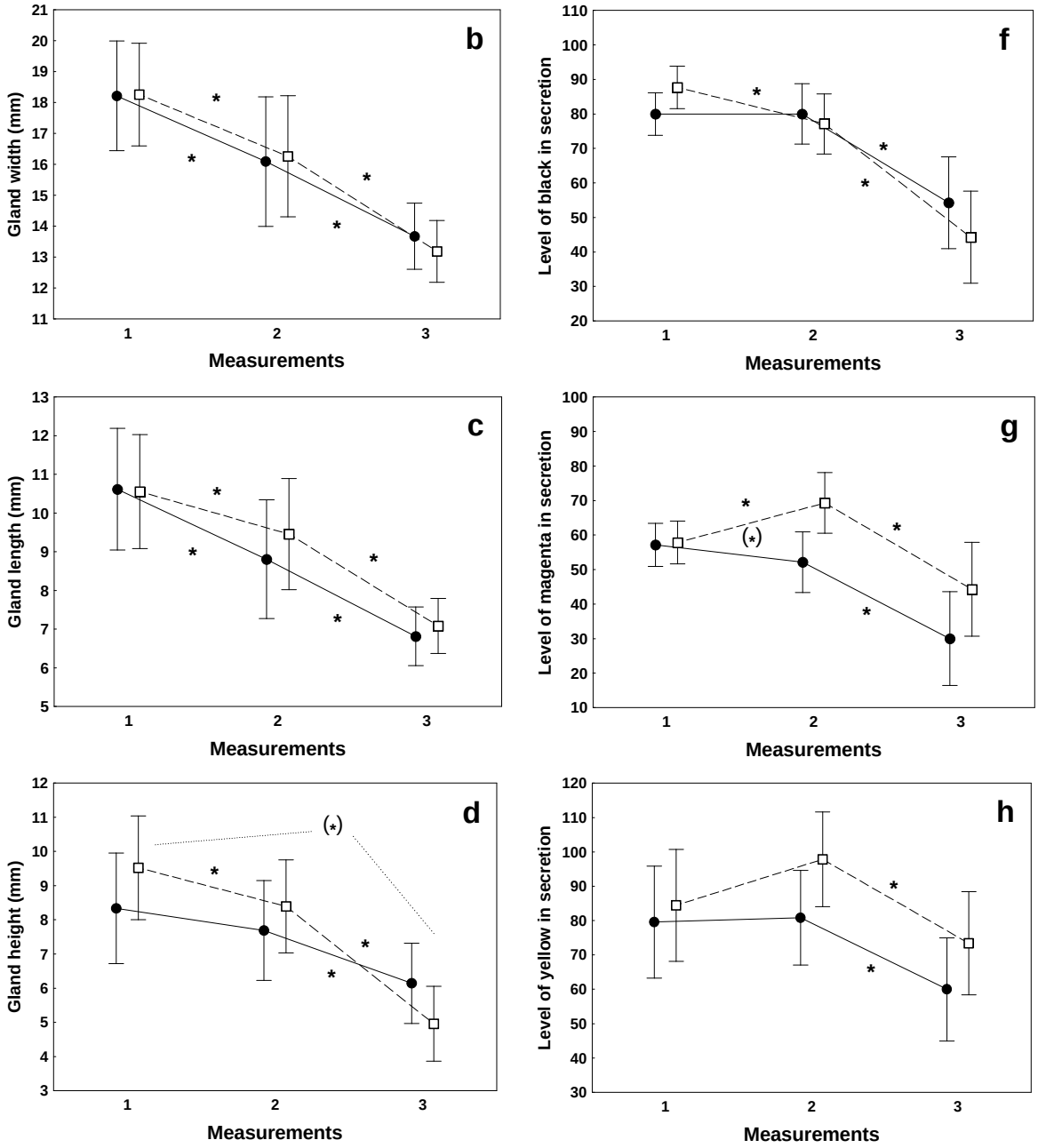


Fig. 8. *Effects of the antibiotic treatment on properties of female glands after hatching date, therefore a period where the values for all variables decrease naturally (see Fig. 3 and 4). Graphs show the results of Repeated Measures Anovas with three consecutive measurements per individual (1 = before injection, 2 = after two injections, 3 = seven days after the third injection) as the within effect, and antibiotic treatment (antibiotic versus saline solution) as the between effect. Asteriscs indicate significant Planned Comparisons of Least Square Means: * indicate significant differences between consecutive measurements within a particular treatment (antibiotic or saline solution); (*) indicate a significant interaction between the antibiotic treatment and the within effect. Points and whiskers are means and 95 % confidence limits. Filled points are values for antibiotic-injected females and white squares values for saline solution-injected females.*

DISCUSSION

The uropygial gland of female hoopoes experiences drastic changes along the year. During the breeding season it increases in size and its secretion becomes dark and foul-smelling (Sutter

1946). We have quantified such changes and shown that they start before egg laying, the maximum size is reached around the hatching date of nestlings and, after the eigth day of the nestling period, decreases in size up to values similar to the prelaying period.

The amount and properties (colour and odour) of secretions produced by female glands showed a similar pattern of change along the reproductive phases than the uropygial gland did. Although it is well established that for many species gland size varies between sexes, among individuals of a particular sex, and seasonally within an individual (Jacob & Ziswiler 1982), the most extreme changes cited are cases of doubling size (referred in Jacob & Ziswiler 1982; e.g. Corvus frugileus, Kennedy 1971). This is far from the changes detected in the UG of hoopoes, which around hatching time was on average 8 times larger, and secreted 30 times the volume produced by gland of hoopoes before laying. Such magnitude of seasonal changes in uropygial-gland size and secretion has no parallel in any other bird species.

Gland size of different females (all measurements of gland size) and nestlings (only gland length) were correlated with the volume of secretion produced. All these results suggest that the increase in gland size involves a proliferation of secretory tissues that is responsible of the production of secretion components. The exaggeration of the gland of females was strictly associated with the period that they stay within the nest, which extends from laying to the eighth day of the nestling period. The morphological change detected in the UG of nesting females is related to the production of a larger amount of secretion in this phase. It is known from other species that some of the lipidic components of bird secretions have antimicrobial activity (Jacob *et al.* 1997; Jacob & Ziswiler 1982; Shawkey *et al.* 2003) and, consequently, it is possible that females used such exaggerated production of secretion to cover feathers or eggs protecting them from pathogens.

Uropygial secretions of nesting females differ from that produced by non-nesting females, not only in the amount produced, but also in some conspicuous properties such as colour and odour. In this phase, secretions were much darker and odorous implying a change in chemical composition. Indeed, brown secretions incorporate more than 40 compounds to the wax esters and fatty acids present in white secretions (Martín-Vivaldi *et al* unpub. data). Several

studies have found that before laying the composition of uropygial secretions in ducks and shorebirds changes from monoester to diester waxes, and switch back again to monoesters after egg hatching, but no change was detected in gland size (Kolattukudy *et al*. 1987; Reneerkens *et al*. 2002; 2006). It has been suggested that these changes in composition of the uropygial gland secretion are the results of producing a female sexual pheromone in the case of mallards (Jacob *et al.* 1979; Kolattukudy *et al.* 1987; Kolattukudy & Rogers 1987), or a way of concealing the incubating female in shorebirds (Reneerkens *et al*. 2005). This last explanation cannot be applied to female hoopoes because their secretions incorporate a great amount of volatile compounds (Martín-Vivaldi *et al* unpubl. data) and, thus, become more odorous during the nesting phase. One likely function of the change in uropygial gland secretion of female hoopoes during reproduction could be related to protection of feathers or eggs against pathogens because it is known that their dark, but not their white secretions, are active against bacteria (Martín-Platero *et al*. 2006; Soler *et al*. unpublished), which are a main cause of hatching failure (Cook *et al*. 2003; 2005a; 2005b) and feather degradation of wild birds (Whitaker *et al*. 2005).

The colour of hoopoe eggs changes drastically along incubation, from pale blue to light brown, a colour similar to that of secretions. Females may impregnate the eggshells directly with the bill after removing secretion from the gland because dispersed thin dark-brown stains recalling bill prints are abundant on the eggshell. Moreover, the preening of their belly feathers that are in contact with eggs would also result in eggshells impregnated with UGS. The covering of eggs with uropygial secretion occurs very soon after laying allowing distinguishing the bluish colour of the last laid eggs from other brownish eggs (Fig. 9). Thus, it is possible that the huge increase in production of uropygial secretion of laying and incubating females allow them to fill completely eggshells with the secretion thereby reducing the risk of bacteria contamination of the embryo.

Fig 9. *A hoopoe clutch during laying showing the difference in egg colour between the last (right, in the middle, the whiter) and previously laid eggs (the rest). Notice the dark stains on the surface of beige eggs, which probably result from female bill impregnated with uropygial secretion.*

Even if preventing infection of eggs was one of the functions of brown secretions, given that hoopoe nestlings also develop similar exaggerated glands with a similar secretion that females, the access to a great amount of secretion could also be useful for chicks. Hoopoe nestlings show antipredator behaviours that involve shooting of faeces against predators while maintaining a drop of the uropygial secretion on the tuft of the gland (see Fig. 1c). These behaviours have been considered evidence that the secretion functions as a defence against predators (Cramp 1998). In accordance with this possibility, it has been shown that some of the compounds found in the very similar secretion of green woodhoopoes may deter predator of selecting food with these malodorous compounds (Burger *et al*. 2004).

Given the antimicrobial properties of brown secretion of hoopoe chicks, it may also function to prevent infections. We have videorecorded that nestlings frequently use their dark secretion to preen plumage (Martín-Vivaldi *et al* unpubl. data) and, therefore, the activity of the secretion against pathogens such as the feather-degrading bacteria *Bacillus licheniformis* (Martin-Platero *et al*. 2006) would prevent feather degradation of nestlings and, thus, allow a proper plumage for the fledging period (Ruiz Rodriguez *et al*. ms).

The very particular trait of brown secretion of hoopoes, but also of secretions of the closed related woodhoopoes, is that it contains bacteria that live in their uropygial gland (see Introduction). Here, by injecting uropygial gland of females and nestlings with antibiotics we successfully reduced or eliminated bacteria within the gland (see results) and, thus, we explored its effects in properties of both glands and secretions. We have found experimental evidence of a role of symbiotic bacteria explaining some of the seasonal changes in gland properties of females and nestlings. The colour of the secretion stored in the papilla of the uropygial gland of both female and nestling were affected by the injection of antibiotic within their glands. These results indicate that some of the compounds present in the dark secretions of hoopoes are mediated or directly synthesised by the bacteria. However, our experimental clearance of bacteria in the uropygial gland of nesting chicks and females did not provoke a complete return to values of colour of secretion similar to the white secretions that hoopoes produce out of the breeding season. Experimental individuals produced lighter and redder, but not white secretions and, thus, bacteria are not responsible of all the particular properties of brown secretions. Working with woodhoopoes, a close relative of hoopoes with very similar gland and secretion characterisitics, Law-Brown (2001, cited in Law-Brown *et al*. 2003) found similar results. Briefly, working in aviaries they observed that the treatment of the gland of three adults of this species with the antibiotic enrofloxacin also modified the colour of secretions that also became less viscous and less malodorous than that of three control birds. We also found that experimental hoopoes produced less malodorous and less viscous secretions that control individuals (pers. obs.) but, because of the difficult quantification of these aspects in field conditions, we were not able to perform statistical comparisons. Therefore, for the two bird species known to harbour bacteria within their uropygial gland and produce malodorous secretions, experimental evidence suggest a role of bacteria explaining such secretion properties.

The experimental clearance of bacteria living in the uropygial gland of hoopoe nestlings also affected the volume of secretion available in the papilla of the gland. This result apparently is not the consequence of a reduced glandular tissue, since, contrary to the result found in

females, in the case of nestling we did not detect an effect of the antibiotic on gland-size measurements. In nestlings we injected the antibiotic when the gland had already developed, as shown by the absence of differences in gland biometric measurements between the start and end of the experiment in control individuals. On the other hand females were treated in a phase of marked change in gland size. Therefore, given that the experimental birds also produced less viscous secretions, a possible alternative explanation for the lower volume of secretion in experimental nestling may be that the more fluid secretion of experimental nestlings more easily leaves the papilla than the viscous secretion of control nestligns. The papilla of the uropygial gland is the place where the symbiotic bacteria has been detected (Martín-Platero *et al* 2006, Soler *et al*. unpublished) and, thus, an exaggerate volume of stored secretion may also allow higher number of beneficial bacteria. Some invertebrates benefit from bacteria that produce defensive products and are housed in special cavities or cells of the hosts. Examples are the cuticle crypts of attine ants (Currie *et al*. 2006), the accesory nidamental glands of female cephalopods (Barbieri *et al*. 2001), light organs of luminiscent cephalopods (Foster *et al.* 2002), or bacteriocytes in aphids (Oliver *et al.* 2003; Scarborough *et al.* 2005). Moreover, in all these examples of symbiosis between bacteria and invertebrates, hosts provide symbionts with growing required substances. To our knowledge, no example of such kind of symbiosis with special organs to harbour symbiotic bacteria has been previously described in vertebrates (see McFall-Ngai 2002 for a review of the absence of specialized symbioses of bacteria with vertebrates). In this sense, properties of both uropygial gland morphology and secretion that were not affected by our antibiotic experiment could be seen as traits that favour bacterial colonization and growth. However, because the antibiotic treatment affected gland height of females, a trait that explain female seasonal variation in secretion volume, a direct role of bacteria explaining the exaggerate volume of brown secretion cannot be disregarded. A direct role of the symbionts in the establishment of the relationship and the development of special organs in host has been described in some of the specialized symbiotic relationships mentioned before (Darveau *et al.* 2003; Foster & McFall-Ngai 1998; Koropatnick *et al.* 2004; McFall-Ngai 2002; McFall-Ngai & Ruby 1991; Visick *et al.* 2000).

We have shown that the uropygial gland of female and nestling hoopoes show seasonal changes in size and secretion properties linked in time with their stay within the nest-hole, and that several of such changes depend on the association with symbiotic bacteria that live in a specially enlarged papilla and produce antibiotic substances. This can be the first case known of a group of vertebrates in which modifications in one organ have evolved to host microorganisms involved in the production of defensive (against pathogens and predators) substances.

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IV

ANTIMICROBIAL ACTIVITY ASSOCIATED TO GENETIC VARIATION IN ENTEROCOCCUS SIMBIONTS FROM HOOPOES UROPYGIAL GLAND

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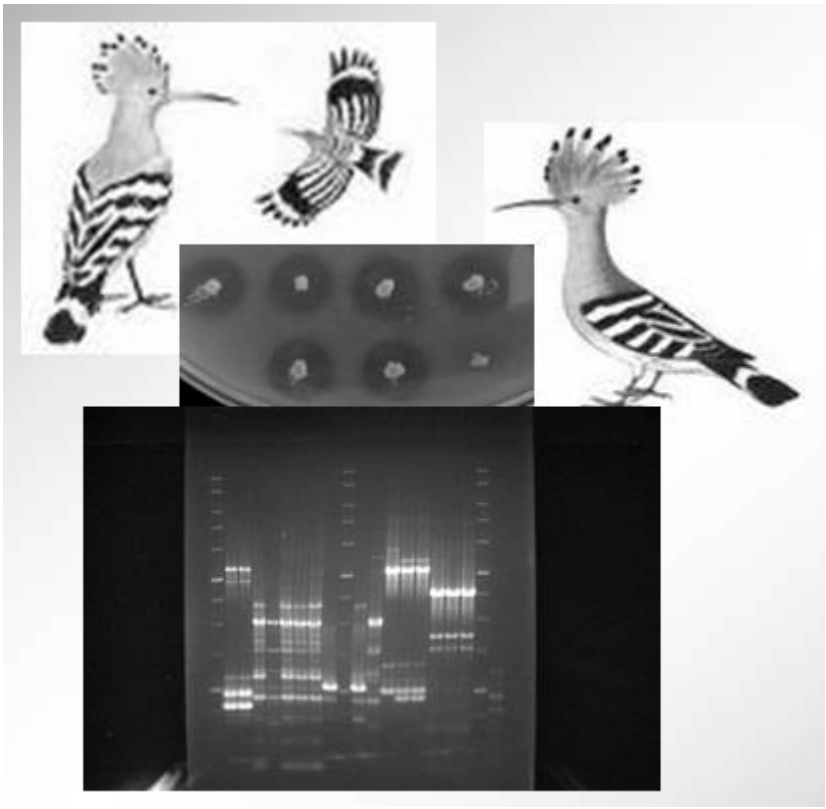

ABSTRACT

Natural selection operates on variable traits, inherited from parent to offspring, which are consistently related with individual fitness. Because symbiotic organisms affect host fitness, but also may be inherited from parents to offspring, or depend on hosts special structures with a genetic bases, natural selection would operate on symbiotic relationships if strain variability and host fitness are related. Therefore, the study of symbiont variability within a host species and its relationships with potential beneficial effects for hosts is of prime importance in coevolution. In this paper, we explore variability of the *Enterococci* symbionts living in the uropygial gland of hoopoes (*Upupa epops*) that synthesises bacteriocins (i.e., antimicrobial peptides that inhibit the growth of potentially competitors, including pathogenic bacteria) in relation to their antimicrobial properties, which presumably reflect differential beneficial effects for hoopoes holding different bacteria strain. By using RAPD-PCR analyses, we determined the genetic profile of the isolates, while antimicrobial properties of different bacteria strains were estimated by performing antagonistic tests against two commonly used strains of bacteria (*Enterococcus faecalis S47* and *Listeria inocua CECT 4030*). We found a hue variation of the antibacterial properties of bacteriocins produced by different *Enterococci* strains isolated from hoopoes. Importantly, strains within the same groups (i.e. similar genetic profiles) demonstrated similar antimicrobial activity. It has been previously suggested that the identity of *Entorococci* strains living in the uropygial gland of nestling hoopoes are, at least partially, inherited or acquired in the nest of hatching, presumably from those living in the mother uropygium. Therefore, bacteriocin production by *Enterococci* symbionts living in the uropygial gland of hoopoes should be under selection, and traits favouring the acquisitions of the most efficient strains should be of selective advantage.

INTRODUCTION

Predation and microbial infections are two of the major causes of natural mortality during early life stages (Giacomello *et al*. 2006) and, therefore, any trait that reduce the effect of parasites would rapidly be selected in host populations (Price 1980). A wide variety of animals and plants bear symbiotic microorganisms which, although mainly transfer nutrients to their hosts (Steinert *et al*. 2000), can also contribute to host defence against predators and parasites (Douglas 1998). There are in the literature abundant examples of production of chemical defences by symbionts that protect hosts against consumers or pathogens, not only in terrestrial plants (Saikkonen *et al*. 1998) but also in some marine invertebrates (Lindquist *et al*. 2005). For instance, through the production of chemical substances, some endophytic fungi confers to plants resistance against herbivores and pathogens, and bacterial films on developing embryos of the shrimp or the lobster produce isatin and tyrosol (Gilturnes *et al*. 1989; Gilturnes & Fenical 1992) that prevent infection by pathogenic marine fungus. Moreover, in some ant species, ecto-symbiotic bacteria (*Actinomycetes* living in their cuticles) play an important role suppressing the growth of potentially devastating pathogens (Currie *et al.* 1999), and also in aphids an obligate association with the bacteria *Buchnera aphidicola* has been shown to contribute directly to aphid fitness by conferring resistance to parasitoid attack (Oliver *et al.* 2003). Therefore, microbial symbionts constitute a route by which some hosts might combat infectious diseases through their shielding effects (Evans & Armstrong 2006).

Dumbacher & Pruett-Jones (1996) proposed that birds could obtain defensive chemicals against microbial infections not only from the environment, but also from symbiotic organisms. In birds, the uropygial gland (UG) may play an important role in the production of these chemical defences, not only because it is their unique external gland, but also because it is known that wax acids in the uropygial gland secretion (UGS) may have antimicrobial properties (i.e., could impede pathogenic growth of bacteria and fungi (Shawkey *et al*. 2003)). In addition, it has been recently detected that *Enterococcus* strains isolated from the uropygial gland of hoopoe (*Upupa epops*) nestlings produced, at least, two bacteriocins (i.e., small peptides with antimicrobial activity towards closely related species, including spoilage or pathogenic,

(Riley & Wertz 2002)), with important antibiotic properties (Martin-Platero *et al*. 2006). The activity of the bacteriocins isolated from hoopoes was intense and of broad spectrum, inhibiting the growing of several bacteria species, some of them potentially pathogenic (*Enterococcus faecalis*, *E. faecium*, *Lactococcus lactis*, *Listeria inocua*, *L. monocytogenes*, *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus licheniformis*, *B. cereus*, and even against a Gram negative *Escherichia coli* strain (Martin-Platero *et al.* 2006)). This capacity to eliminate a wide range of bacteria may confer to nestling hoopoes protection against potential pathogens during their nesting phase.

Given variation in the effects of symbionts on host performance (Douglas 1998; Koch *et al*. 2004), natural selection would favour mechanisms or traits in hosts that allow the establishment of optimal symbiotic relationships. An example of this was provided by Read & Perez-Moreno (2003) who found that associations of plants with fungus capable of retain more efficiently nitrogen and phosphorus, the two limiting resources in poor soils, were selected in some kind of those soils over fungus without this capability.

From previous work with hoopoes, we have pointed out variability within the *Enterococci* strains detected in their UGS (Ruiz-Rodríguez *et al*. *in prep. A*). Moreover, we have found evidence of beneficial effect for hoopoes since the bacteriocins produced by symbiotic bacteria affected hatching success (Soler *et al*. *submitted*) and feather degradation in the laboratory (Ruiz-Rodríguez *et al*. *in prep. B*). The spreading of the uropygial secretion onto their feathers and body skin (Martín-Vivaldi *et al*. *in prep*.), thus, is probably to use the symbiont-derived antimicrobial properties for protection against pathogens. In the present paper, we describe variation in the antimicrobial activity of different strains of the *Enterococcus* colonies living in the UG of nestling hoopoes that were fingerprinted for clustering them in different strains by using RAPD analyses.

MATERIAL AND METHODS

Study area and bacterial sampling

The study was performed during the 2003 and 2005 breeding seasons in the Hoya de Guadix (SE Spain) where, during the last 5 years, more than 500 nest boxes were installed and some of

them used by hoopoes. The UGS was extracted from nestlings at the age of ringing (19-21 days after hatching of the first egg), when they were about to fledge. Samples were collected with a micro-pipet by introducing the tight tip of the pipette within the papilla of the gland, which act as the recipient of the UGS. To avoid contamination, before sampling, feathers around the gland were separated and surrounding of the gland pore washed with ethanol. Samples were introduced in a sterile Eppendorf tube (1.5 ml.) and stored at 4ºC until processed in the laboratory.

Just few hours after sampling, 5 µl of a 1:5 solution of each UG secretion into sterile distilled water was spread onto an agar plate with the general culture medium Trypticase Soy Broth (TSA, Scharlau, Barcelona, Spain). Afterwards, plates were incubated aerobically for 24h at 37º C. Five randomly collected colonies from each plate were isolated by transferring them to a Brain Heart Infusion (BHI, Scharlau, Barcelona, Spain) liquid medium, that was incubated for 24h at 37ºC. These cultures in liquid medium were used to perform two analyses: the typification of the strains, and a test of their antimicrobial activity.

For testing the antimicrobial activity of the colonies, the bacteria from the liquid medium were spread onto a M17 broth medium (Scharlau, Barcelona, Spain) in an agar plate, and incubated for 24h at 37ºC. After growing, the plate with the replicated colonies was covered with a layer containing 6 ml of BHI dissolved in sodium phosphate tampon (0.1M, pH 7), and 0.8% agar, which was previously inoculated with 100 µl of a 24h. liquid BHI culture of the indicator strain (*E. faecalis* or *L. inocua*) and incubated 24h at 37ºC (following the double layer technique proposed by Gratia & Fredericq 1946). The antimicrobial activity of cultures was measured as presence or absence of a halo of growth inhibition in the indicator strain around the colony isolated from the UGS of hoopoes (Fig. 1). As a positive control, we used a well-known bacteriocin-producer strain against the two indicator bacteria (*E. faecalis* AS48).

For the genetic analyses, 1 ml. of the BHI culture was centrifuged (see above), and the cells obtained were frozen at -20ºC until DNA extraction. Bacterial genomic DNA was extracted using the AquaPure Genomic DNA isolation kit as described by the manufacturer (Bio-Rad, Spain). To type the isolates from each sample, the method of Randomly Amplified Polymorphic

DNA (RAPD) was used (Williams *et al.* 1990). DNA amplification was made in a Biorad Gene Cycler (Bio-Rad) using the primer of arbitrary nucleotide sequence **M13** 5´-GAGGGTGGCGGTTCT-3´ at a concentration of 1 µM in the reaction mixture. Amplified DNA restriction fragments were separated electrophoretically and visualized on 1.2% agarose gels, containing 1 ug/ml ethidium bromide, and using the 1-Kb pair ladder (Biotools, Madrid, Spain) as the molecular weight standard. Amplification reactions were as follows: One cycle of 94ºC for 1 minute, one cycle of 20ºC during 20 seconds, and one more at 72ºC during 80 seconds. The three cycles were repeated 35 times, and were followed by one cycle of 5 minutes at 75ºC. The RAPD-PCR technique is used as a rapid and reliable method for intra and interspecific differentiation of bacterial strains and is an efficient method of typing large numbers of isolates to species and strain levels (e.g. Fitzsimons *et al*. 2001). Although RAPD fingerprints may vary to some extent, reproducibility could be achieved under controlled conditions (Rossetti & Giraffa 2005).

The resulting fingerprints were analysed with the Fingerprinting II Informatix Software 2000 (Bio-Rad, Hercules, CA). Similarity between pairs of fingerprints was calculated by using the Pearson product moment correlation coefficient, with clustering by the Unweighted Pair Group Method with arithmetic mean (UPGMA) algorithm. After repeating RAPD and cluster analyses with some samples chosen randomly, samples that resulted in exactly the same RAPD profile or those in clusters with similarities > 80% were considered to belong to the same strain (hereafter, group). This RAPD-PCR analysis, as well as defining strains as groups with similarities higher than 80%, have been broadly used for grouping samples by genetic similarity in bacteria, with variable similarity degree within groups depending on the results (e. g. (Rossetti & Giraffa 2005; Psoni *et al*. 2006; Morandi *et al*. 2006).

To analyse whether different groups differ in their antimicrobial activity, we used the Mantel test, as implemented in FSTAT software (Goudet 1995), which allows the estimation of correlation coefficients among matrices. For this analysis, binary matrices of similarities in RAPD grouping and antimicrobial activity were prepared. First, for matrices of similarities in RAPD grouping, the value 1 was assigned to pairs of samples belonging to the same RAPD

group, and the value 0 to pairs of samples belonging to different groups. For matrices of similarity in antimicrobial activity 1 represented two samples with the same activity (i.e., if both produced bacteriocins or did not), and 0 if one produced but the other did not. One matrix was made for activity against *E. faecalis*, and another for activity against *L. inocua*. Finally, because more than one colony was isolated from the UGS of each single individual nestling, another binary matrix was built with the information of a couple of colonies being from the same (1) or from different (0) individuals. Statistical significances were estimated by Monte Carlo procedure after 10.000 permutations.

The study includes information on clustering and antimicrobial activity of 80 colonies, isolated from 20 nestlings, growing in 16 nests.

RESULTS

The colonies from the same individual appeared within the same group of RAPD more frequently than colonies from different individuals (RAPD group as dependent variable and individual as independent, $r = 0.36$, $p < 0.0001$). The total number of colonies that presented antimicrobial activity against the tested strain of *E. faecalis* was 36 (45%) from 8 individuals, while antagonism was not detected in 44 colonies from 13 individuals (55%); only in 1 nestling there were colonies with different responses. With respect to antimicrobial activity detected against the *L. inocua* tested strain, it was detected for 35 (43.75%) colonies from 7 individuals. Absence of activity against *L. inocua* was found in 45 colonies (56.25%) from 13 individuals. There were no cases of variation in activity against *Listeria* among samples from the same nestling. All but one of the samples with activity against *E. faecalis* showed antagonism against *Listeria* as well. Statistical analyses showed that colonies within the same RAPD group showed similar activity against *E. faecalis* and *L. inocua* (Mantel test: $r = 0.07$, $p < 0.0001$ for both). From the 3 nests for which we have data for more than one nestling, we isolated 36 colonies belonging to 8 nestlings. In 2 nests, colonies from siblings had identical antagonist activity, while in the third nest with 4 nestlings, colonies from 3 of them demonstrated similar activity.

DISCUSSION

Bacteria colonies isolated from the UGS of the same individual hoopoe were more frequently included as members of the same strains than those from different individual nestling. This result shows that there exist interindividual differences in the community of symbiotic bacteria hosted by hoopoe nestlings within their gland. We also found that strains differed in their ability to inhibit growing of the indicator bacteria. Both results together indicate that there exists variation in the benefits (in terms of antimicrobial protection) that different hoopoe nestlings can obtain from their symbionts.

We found that about 50% of colonies inhibited growth of the indicator species. Since we have previously shown that a strain of these bacteria is producer of a potent bacteriocin (Martín-Platero *et al* 2006), the inhibition halos found around the colonies tested in this study were probably also caused by bacteriocins. We expected a higher percentage of strains producing bacteriocins, given that in previous tests using whole UGS we found antimicrobial activity in more than 90% of individual hoopoes with the bacteria in their UGS (Soler *et al. submitted*). Those differences between the detected activity of isolated colonies and the whole secretions from the UG could be due to the medium where the producer bacteria are growing up, given that the medium where we performed the tests is different than that of the GU. Bacteriocin activity have been shown to be influenced by the medium in which the producer strain growth, for instance, they can vary their activity depending on the pH (e.g. Topisirovic *et al*. 2006), temperature, glucose concentration (Lejeune *et al*. 1998), or availability of other nutrients (Todorov & Dicks 2006). The chemical composition of the hoopoes uropygial gland secretion is quite complex (Martín-Vivaldi, unpublished data), and very different from the media that we used to test bacteriocin production. Indeed, in one strain isolated from hoopoes UGS that was studied in more detail, we found that its inhibitory activity varied according to the culture media (Martín-Platero *et al*. 2006). This could cause the differences found in the frequency of bacteriocin-producers between bacteria growing in whole secretions and those isolated and grew in standard laboratory conditions. Alternatively, part of the antimicrobial activity of whole secretions may be due to substances other than bacteriocins. We have found

that the properties (colour and viscosity) of the brown secretions of nestlings change when symbionts are eliminated experimentally by mean of antibiotics (Martín-Vivaldi *et al* in prep.). This suggests that symbiotic bacteria may be responsible of the presence of other substances, different of the bacteriocin, in hoopoe glands. Such substances could also be involved in defence against pathogens, thus, explaining the higher percentage of samples with activity in the case of whole secretions. Before we compare the chemical composition of secretions with and without symbionts, however, we cannot rule out the possibility that active compounds are secreted directly by the bird.

It is well known that lactic-acid bacteria, group that include the genus *Enterococcus*, are particularly prolific in bacteriocin production (Riley & Wertz 2002). However, their antagonistic spectrum (i.e. the number of bacterial strains against which they are active) is highly variable not only among producer species, but also among strains (Gardner *et al*. 2004), independently of the origin of the strain (de Vuyst et al. 2003). The objective of testing activity against two different strains in our study was to explore the spectrum of strains sensible to the bacteriocin of the symbiotic bacteria. Most bacteriocins have been selected to outcompete closely related species (e.g. Riley & Wertz 2002). However, we found that in our samples, the bacteriocin synthesised by producer strains was effective also against *Listeria*, indicating a wide spectrum. Our results agree with those found for a previously studied strain, which showed inhibition of a broad variety of bacterial species (Martín-Platero *et al*. 2006). All these results show that bacteriocins produced by the strains isolated from hoopoes UG present a spectrum of action wider than usual. Due to this wide range of inhibition, we can also expect that, at least some of the potential pathogenic bacteria growing in the nests should be inhibited by the bacteriocins. Nevertheless, further analyses should consider the inhibitory activity against pathogenic strains, trying to recreate the natural conditions and confronting the symbiotic bacteria with their natural competitors.

Here, we have detected that different bacteria strains involved in the symbiosis with hoopoes may have different antimicrobial properties, indicating individual variability in the benefits that hoopoes could receive from the *Enteroccocci* living in their UG. Differences between

symbionts in their effectiveness as antimicrobial tools are subjected to selective pressures, as have been demonstrated in other symbiotic interactions (see examples in Thompson 2005).

We have previously detected a significant heritable component explaining the *Enterococcus* strain living in the UG of hoopoes. In addition, we found that the genetic profiles of symbionts are quite similar between genetic siblings, even for those reared or hatched in foster nests (Ruiz-Rodríguez *et al. in prep. A*). Therefore, because of the possibility of an heritable component, strains of symbionts that live in the UG of adult hoopoes would have fitness effects, not only in adults, but also in their offspring.

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V

SYMBIOTIC BACTERIA LIVING IN HOOPOES UROPYGIAL GLAND PREVENT FROM FEATHER DEGRADATION

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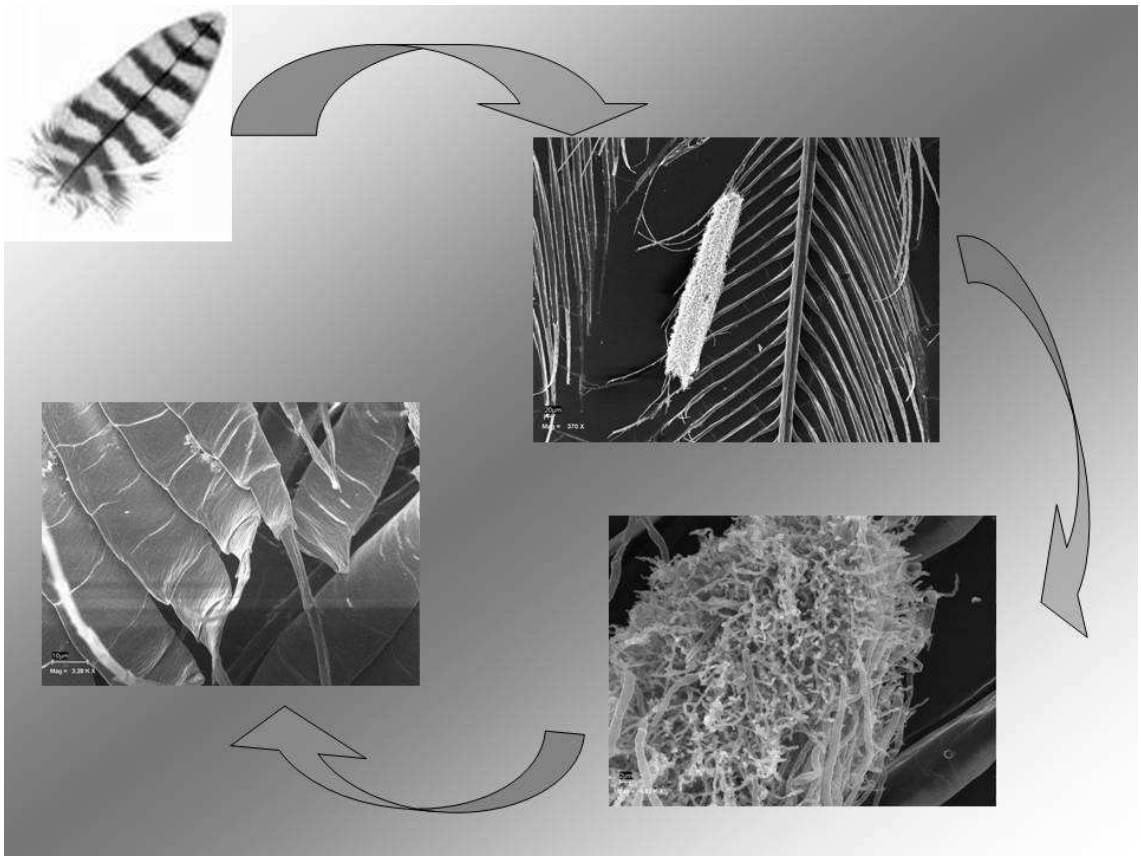

ABSTRACT

Feathers are essential for birds, conferring not only flight capability but also insulation, protection and water proofing, and are involved in communication events such as status and sexual signalling. Therefore, their maintenance is of prime interest for bird life. Among the potential factors damaging feathers there are certain microorganisms that through the secretion of enzymes are capable of digesting the structural protein of the feathers, the keratin. One of these microorganisms is the bacterium *Bacillus licheniformis,* broadly distributed in wild bird species, that has been shown to present keratinolytic activity in several experiments.

In previous works, *Enterococcus* strains isolated from the uropygial gland of nestling hoopoes (*Upupa epops*) showed antagonistic effects against *B. licheniformis* mediated by the production of bacteriocins (antimicrobial ribosomically synthesized peptides of bacterial origin). In the present study, we investigated if, in the presence of a well-characterized *E. faecalis* strain and its bacteriocin, the keratinolytic activity of *B. licheniformis* is reduced. Experiments carried out with both feathers and pure keratin showed a significant decrease of the degradation by the bacillus in the presence of the symbiotic bacteria or its bacteriocin, which imply that hoopoes benefit from this association as a weapon against feather degradation.

INTRODUCTION

Selection pressures from parasites have selected for a high variety of defence mechanisms in their hosts, including the immune system, or behavioural traits that reduces the probability of infection and/or the negative effects of parasites (e.g. Price 1980; Wakelin 1996; Poulin 1998), such as the use of natural insecticides produced by plants (e.g. Clark and Mason 1988; Lozano 1998), anting (Ehrlich *et al*. 1986), or preening (Jacob & Ziswiler 1982).

Some of those pathogens act on epidermal structures like feathers, which are fundamental to the biology of living birds and, therefore, their maintenance in good conditions is of prime importance for them. Feathers not only confer birds with flight capabilities (Sttenheim 1976), but also with thermal insulation and heat transfer, allowing endothermic lifestyle (Ruben & Jones 2000). They also protect birds from ultraviolet radiations (Wolf & Walsberg 2000) and are important in communication in different social contexts including individual recognition (Whitfield 1987), status signalling (see for example Senar and Camerino 1998), sound production as in nightjars *Caprimulgus sp.* (Sttenheim 1976), and many sexual selection events (reviewed in Bennett *et al.* 1994). Finally, feathers may also confer birds with camouflage (i.e. crypsis, Savalli 1995), or even with the ability of transporting water (i.e. in diving birds, Gremillet *et al*. 1998). Thus, due to the great importance of a proper maintenance structure, colour, and other properties of feathers, mechanisms that reduce or impede feather decomposition (including parasites attacks) are of prime importance and, therefore, would rapidly spread in bird populations.

Plumage can be damaged not only by abiotic factors, such as mechanical abrasion or photomechanical processes, but also by ectoparasites (Clayton 1990) such as lice (Clayton 1991; Kose & Møller 1999), or microorganisms such as fungi (Decostere *et al*. 2003) and bacteria (Muza *et al*. 2000). Feather composition is 90% keratin, a protein that confers high mechanical stability and resistance to decomposition, given that molecules are tightly packed and cross-linked ones to another by cysteine bridges (see Sangali & Brandelli 2000). Therefore, organisms exploiting feathers should be able to digest the keratin; this keratinolytic activity is mainly mediated by microorganisms, which otherwise may directly grow on feathers (Lucas *et al*. 2003; Grande *et al*.

2004). Those organisms represent therefore a threat against plumage integrity and functionality. For instance, holes produced by feather lice may increase feather breakage, and thus cause indirect flight-energetic costs (Barbosa *et al*. 2002), affect thermoregulation (Booth *et al*. 1993; Kose & Møller 1999), or mate choice (e.g. Kose & Møller 1999). Consequently, feather degradation may greatly reduce reproductive success of birds (Pap *et al*. 2005).

Feather-degrading microorganisms, through the secretion of keratinases, harm feathers by breaking down their structure integrity (Muza *et al*. 2000). From poultry studies on feather-degrading microorganisms, it is known that *Bacillus licheniformis*, *Streptomyces sp*., *Kocuria rosea* or *Microbacterium sp*. (recently reviewed in Gupta & Ramnani 2006), have strong keratinolytic activity. Precisely, one of the most frequent microorganisms isolated from feathers in the wild is *Bacillus licheniformis* (Lucas *et al*. 2005, Whitaker *et al*. 2005). This bacterium secretes a keratinase that hydrolyzes the keratin matrix of the feather, while bacterial cells grow closely adhered to the barbules and, in optimal lab conditions, they could completely degrade them in 24 hours (Ramnani *et al*. 2005). Therefore, *Bacillus licheniformis* is a good candidate for studying the effects of microorganisms on plumage quality, but also for studying efficiency of possible defensive mechanisms of birds that prevent infection and growth of feather-degrading bacteria in natural conditions.

Several studies have shown that birds defend themselves against feather parasites by using ants chemical secretion, which have bactericidal and fungicidal properties (i.e. anting, Ehrlich *et al*. 1986), by sunning and sand baths (Clayton 1999), or directly by removing louses with the bills (Clayton & Walther 2001). Another solution is self cleaning by using the secretion of their uropygial gland (UG) (i.e. preening, Jacob and Ziswiler 1982), which includes active substances against the growth of some Gram-positive bacteria (Jacob *et al*. 1997, Shawkey *et al*. 2003). Up today, antibacterial properties of the uropygial secretion (UGS) have been considered to be associated with the chemicals directly secreted by the bird gland. However, the antimicrobial property of UGS may be mediated by other bacteria living in the UG, producing bacteriocin-like inhibitor substances (BLIS), which are antagonistic substances produced by some bacteria to inhibit the growing of competitors, and therefore, may inhibit growth of

feather-degrading bacteria. This is likely the case of the hoopoe (*Upupa epops*) and the red-billed woodhoopoe (*Phoeniculus purpureus*), given that two recent studies have isolated in the UGS of these species two bacterial species of the genus *Enterococcus* (Law-Brown & Meyers 2003; Martin-Platero *et al*. 2006). In addition, the bacterium *E. faecalis* MRR-10.3 found in the UGS of a nestling hoopoe produces at least two different bacteriocins with a broad antimicrobial activity (Martin-Platero *et al*. 2006), although in the red-billed woodhoopoe those BLIS have not been investigated. In hoopoes, however, the relationship with the symbiotic bacteria appeared only during the nesting phase of females and offspring, which suggest that BLIS produced by symbiotic bacteria prevent nestlings and incubating or brooding females from infections (Soler *et al*. *submitted*).

Here, by exploring possible fitness benefits that this bacterium may confer to hoopoes, we further explore the hypothesis that these bacteriocin-producing bacteria maintain a mutualistic relationship with their hoopoe host during the breeding season. In particular, we explored the effects of both bacteria and bacteriocin in preventing the degradation of feathers by microorganisms. Briefly, in laboratory conditions we measured feathers degradation after incubation with different treatments: the *B. licheniformis* D-13 strain alone, this bacterium mixed with a bacterium isolated from the UGS of a nestling hoopoe (*E. faecalis* MRR 10-3), and the bacterium plus the bacteriocin. Given that feather's properties others than those controlled in the lab could affect degradation (i.e. fat or wax presence in the feather, or degradation level at the beginning of the experiment), we performed the same experiment with pure keratin to measure the degradation effect over the structure of the protein itself. According to the hypothesized beneficial effects of *E. faecalis*, we predicted a heavier degradation of feather and keratin in treatments with only *B. licheniformis* than in treatments in which symbiotic bacteria or bacteriocin isolated from the hoopoes UG were added.

Evidence of the hypothesis tested would strongly suggest that preening behaviour of nestling hoopoes is an example of self-medication in nature because they would use the organic substances produced by their symbionts (i.e., bacteriocins) to defend themselves against pathogens (Clayton & Wolfe 1993).

MATERIAL AND METHODS

Experimental procedure

We performed two experiments. First, we measured feather degradation after incubating them in different conditions (see below), and second, we made the same experiment with pure keratin instead of feathers, to measure the degradation effect over the structure of the protein itself, and to avoid possible bias due to other feather compounds. Those laboratory experiments consisted on culturing bacteria in substrates of feathers or keratin, in a 0.9 % saline solution (NaCl in distilled water). This solution was distributed in tubes, and then, autoclaved during 15 min at 121 ºC. Briefly, (**treatment 1, T1**) to estimate the feather degradation of the keratinolytic bacterium on feathers or keratin, we added to the saline solution a culture of *B. licheniformis* (see below), and estimated the degradation after selected incubation times (see below). In addition, (**treatment 2, T2**) to estimate the hypothetical effect of the *E. faecalis* on the degradation of keratin and feathers by *B. licheniformis*, we also added a culture of *E. faecalis* MRR 10-3 to that with *B. licheniformis*. Further, (**treatment 3, T3**) we also performed an experimental treatment similar to the T2 but adding purified bacteriocin instead of culture of *E. faecalis*. Therefore, differences in feathers or keratin degradation between treatments (1) and (2), and between (1) and (3), would indicate the effect of the bacterium living in the UG of hoopoes, or that of their produced bacteriocin, on the degradation of feathers and keratin by the widespread feather degrading bacteria *B. licheniformis*. However, because the degree of degradation in treatments (2) and (3) could be due to the direct effect of *E. faecalis* or purified bacteriocin on the keratin or feathers, we also added to the medium a culture of *E. faecalis* (**control 1, C1**) or purified bacteriocin (**control 2, C2**) in the absence of *B. licheniformis*, and estimated the degree of feather or keratin degradation after the incubation time. Finally, because feather or keratin may also degrade with time, as a third control group (**control 3, C3**), we incubated the medium without bacteria or bacteriocin (i.e., only saline solution) and estimated the feather or keratin degradation level. When analysing the feather experiment, we also considered another control group (**control 4, C4**) with feathers that had not been incubated (see below). Each treatment of each experiment was replicated three times. Briefly, under sterile conditions, the substrate

(feather or keratin) were introduced in the experimental solutions and tubes were vigorously agitated before incubation of experimental tubes at 28 ºC in constant agitation at 180 revolutions per minute. Although the optimum temperature for the keratinolytic activity in *B. licheniformis* is around 40 ºC (see Suntornsuk and Suntornsuk 2003), we incubated them at a lower temperature to simulate natural conditions on bird feathers inside the hole nest.

After previous tests to decide the optimum time of the incubations that allows to detect feather degradation by *B. licheniformis* in the laboratory conditions exposed above, feather experiments lasted for 7 days, while the keratin experiments lasted 16 days, given that it was the time when the degradation became evident (i.e., the medium became opaque). Nevertheless, in the keratin experiments we also took samples at 2 and 5 days after the beginning of the experiment, to check previous non-visible degradation.

Feathers and keratin samples

Feathers

Feathers were obtained from hoopoes captured in the Hoya de Guadix, in South-eastern Spain, around 1000 m above sea level, where three adults (one male and two females) were captured in mist-nets at the beginning of the breeding season of 2006. Eight breast feathers from each individual were removed. To avoid differences in degradation due to melanin (Goldstein *et al*. 2004) we selected feathers of approximately similar size and colour (pale brown) that to our eye were not broken or degraded. Breast has been described as one of the parts that support most preening activity (e.g. Van Liere *et al*. 1991) and therefore are natural scenarios of the hypothetical battle between *B. licheniformis* and symbionts of hoopoe glands.

After collection, feathers were stored in Petri dishes and maintained in the laboratory at 4 ºC and dark conditions until the analyses. All of them were collected the same day, and the experiments were performed one week later, in the laboratory of Microbiology at the University of Granada. Before the experiments, feathers were sterilized by exposition of both sides to UV radiation for twenty minutes. All experimental tubes received a final volume of 3 ml. This volume assured that the whole feather was covered and, therefore, in contact with the entire

medium. Consecuently, C3 feathers were immersed in 3 ml of 0.9 % NaCl saline solution, but T1, C1 and C2 feathers were, in 2.9 ml of NaCl plus 0.1 ml of *B. licheniformis*, *E. faecalis* or bacteriocin respectively. In T2 and T3 treatments 2.8ml of NaCl were added of 0.1 ml of *B. licheniformis* plus 0.1 ml of *E. faecalis* or bacteriocin respectively. Finally, to reject the possible effect of the agitation in the liquid medium, we used one additional control (C4), which was not incubated and kept in the fridge (4 ºC) after sterilization (at the same time as the rest) until the end of the experiment.

Keratin

Keratin-azure (Sigma-Aldrich, Madrid, Spain) was used as a substrate for the experiment. Here, instead of feathers, 24 mg of keratin kept in sterile eppendorf tubes was added with the different mixtures associated to different treatments (see above) under sterile conditions. In these cases, however, tubes from each treatment received a total volume of 6 ml, which allow the spectrometric estimation of keratin degradation at three different times. Those 6 ml were the quantity of saline solution that received the C3 tubes, but T1, C1 and C2 were prepared with 5.8 ml of saline solution with 0.2 ml of *B. licheniformis*, *E. faecalis* or bacteriocin respectively, while in T2 and T3 5.6 ml saline solution with 0.2 ml of *B. licheniformis* were added, plus 0.2 ml of *E. faecalis* or bacteriocin respectively.

Bacterial cultures and bacteriocin preparations

For cultures of the feather-degrading bacteria *B. licheniformis* (e.g. Burtt & Ichida 1999a with wild birds, and Suntornsuk & Suntornsuk 2003 with laboratory experiments), we used the strain D13 from our laboratory collection. Moreover, for cultures of bacteriocinogenic *E. faecalis* living in hoopoes, we used the strain MRR10-3, which was isolated from the UGS of a nestling hoopoe in 2003, and whose antagonistic activity against *B. licheniformis* and other bacteria species were previously tested (Martin-Platero *et al*. 2006). Briefly, we spread separately the strains (D13 and MRR10-3) onto agar plates (TSA, Scharlau) and incubated at 37 ºC during 24 h. From these plates, to avoid bacterial contamination in the two cultures, only one colony from

plates of each strain was inoculated in the liquid medium TSB (Trypticaseine, Soja Broth, Scharlau, Barcelona, Spain) and incubated for12 hours before the experiments. With these cultures we performed the experimental treatments exposed before and, while *E. faecalis* culture was incubated at 37 ºC, that of *B. licheniformis* was at 28 ºC in agitation to prevent the lumpy growing typical in *B. licheniformis*. From these liquid cultures, we took the amounts above referred for each experimental treatment.

On the other hand, bacteriocins produced by *E. faecalis* MRR10-3 were purified from *E. faecalis* MRR10-3 cultures in complex medium broth (Galvez *et al*. 1986) by cation-exchange chromatography on carboxymethyl-sephadex CM-25 (see Martin-Platero *et al*. 2006 for more details).

Degradation measurements

Degradation of feathers

After the experiment, feathers were washed with sterile distillate water to remove the bacteria and the medium and, immediately prepared (including C4) for the observation under scanning electron microscope (SEM). Feathers were fixed with 2.5% glutaraldehide in 0.1 M sodium cacodilate buffer, pH7.4 during 2h at 4 ºC. Any remaining fixative was eliminated by washing with the same buffer and then postfixed with 1% osmium tetroxide for 1h at room temperature. After washing with distilled water, samples were dehydrated by immersion in a graded series of ethanol followed by desiccation in a dryer Polaron CPD 7501. After drying, the specimens were coated with a layer of gold by perpendicular ionic bombing in a Polaron Unit SEM Caoting E5000 and then observed by SEM in a microscope Gemini 1530 (Leo, Overkogen, Germany) at the Centro de Instrumentación Científica de la Universidad de Granada. The level of degradation was visually estimated separately by 5 people not involved in the experiments, over the pictures at 50 magnifications, following a degradation index. This index was based on the progressive narrowing of the barbules that the addition of the parasitic bacteria produces in feathers (see Fig. 1a). Degradation starts from the distal part and proceeded reducing them until the complete degradation of the barbules. The index comprised 6 different levels (from 0 to 5

values (Fig. 1b)) defined as follows: **0** if barbules are intact (no degradation); **1** when just the tips of the barbules are degraded; **2** when approximately half of the barbules are degraded; **3** if more than half were degraded; **4** if there are still some vestigial barbules; and **5** if they are disappeared or are close to. For each feather, although an average of three pictures was taken from three different parts, observers were unaware of feather identity from which the picture came from or experimental treatments.

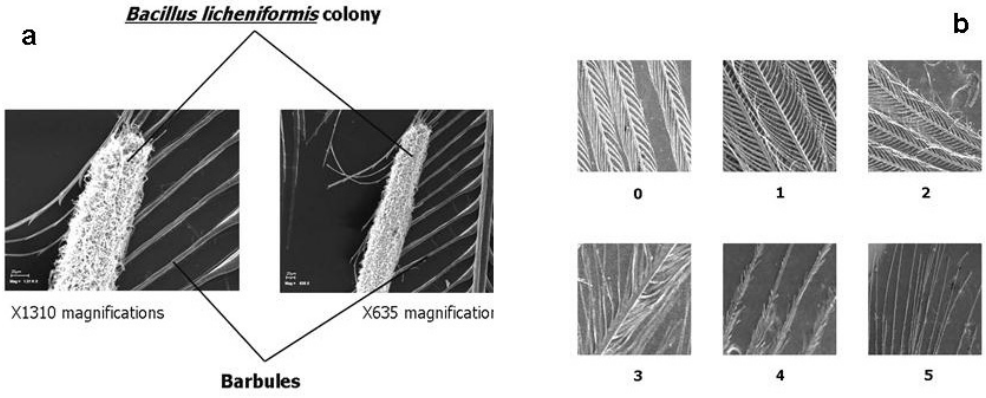

Fig. 1. *Scanning electron microscopy pictures where it can be observed (**a**) the narrowing effect of the Bacillus licheniformis colony over the barbules, and (**b**) the different degradation levels used for estimation of feather degradation after the experiment.*

The repeatability (rc) of estimations was calculated following Senar (1999) and, by using the One-Way ANOVA, we calculated p-values associated to different repeatability indexes. We found significant values of repeatability for different observers ($F = 7.9$; d.f. = 71, 285; $p < 0.001$; rc = 77%) and feathers ($F = 8.82$; d. f. = 21, 335; $p < 0.001$; rc = 85%). These moderated high repeatability values validated our estimations.

Degradation of keratin

Keratin degradation was estimated with a spectrophotometer by estimating absorbance at 595nm (Santos *et al*. 1996), where the pick of reflectance of keratin occurs. Before measurements, tubes containing samples were centrifuged at 13000 rpm during 5 min to separate the pellet containing cells. Peptides remaining in the supernatant were used to measured the absorbance,

which is directly related to the percentage of keratin degraded (e.g. Santos *et al*. 1996). Three measurements were performed of the degradation of samples after 2, 5 and 16 days of incubation.

Statistical procedure

All the analyses were performed by using Statistica 6.1 (StatSoft 2001) Software, and differences between treatments were explored by using GLM, error type VI, where the dependent variable was the degradation level for both substrates.

In the case of feathers, the treatment and the observer were the predictor variables explaining degradation. A non-significant interaction between treatment and observer would indicate that the effect of treatment does not depend of the observer identity. For the analyses of the effect of treatment (between effect) on the keratin degradation along the time of incubation, estimations at three different times (three estimations per sample at 2, 5 and 16 days) were considered as within factor in a Repeated Measures ANOVA. Post-hoc comparisons (i.e. Tukey HSD test) were used to check for statistically significant differences between different treatment groups.

RESULTS

Experimental treatments significantly affected degree of feather degradation (effect of treatment, $F = 10.41$; d.f. = 6, 302; $p < 0.001$), which appeared independently of the observer identity (i.e. interaction between observer and treatment, $F = 0.81$; d.f. = 24, 302; $p = 0.71$). The detected significant differences associated to experimental treatments were due to the high values of degradation estimated for feathers with *B. licheniformis* (T1 in Fig 3). In accordance, post-hoc comparisons revealed that values of degradation estimated for feathers with *B. licheniformis* were significantly higher than that estimated for other groups (Tukey HSD post-hoc comparisons, $p < 0.0001$; Fig. 3) and no differences were detected between the rest of the groups ($p > 0.4$ between all of them). Moreover, although with no quantification, the visual observation of experimental feathers with the bacilli alone, or together with the antagonistic

(enterococci or bacteriocins) after the incubation time revealed the existence of strongly fixed bacterial colonies in the first, but not in the later groups (MRR, pers. obs.).

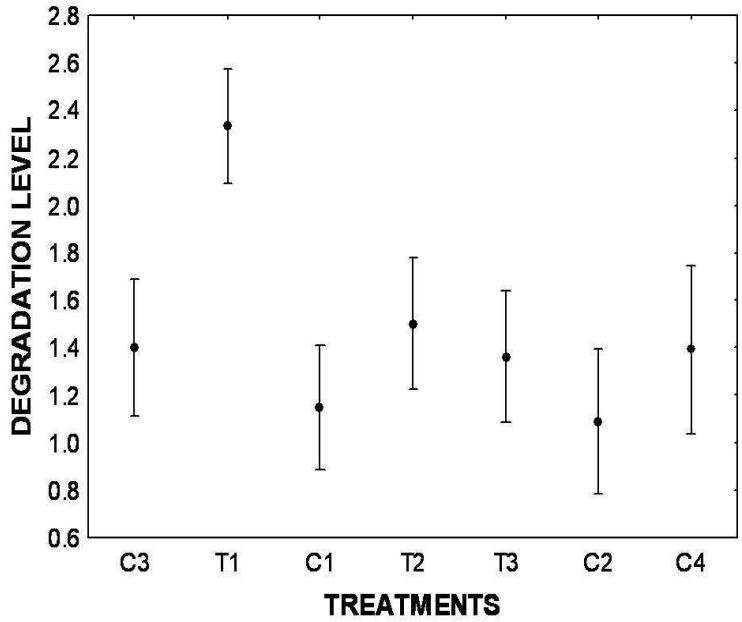

Fig. 3. *Representation of mean values of degradation of feathers under different treatments with the SE intervals. Legend: T1: BL; T2: BL+EF; T3: BL+NC, C1: EF; C2: BC; C3: NaCl control; C4: control feathers without incubation (BL: Bacillus licheniformis, EF: Enterococcus faecalis, BC: Bacteriocin).*

In experiments with purified keratin, the highest degradation index occurred again in the group with *B. licheniformis*, but differences were appreciable only after sixteen days of incubation (Repeated measures ANOVA: effect of treatment, $F = 128.24$, d.f. = (5,6), $p < 0.001$; effect of time, $F = 593.3$, d.f. = (2, 12), $p <$ p 0.001; interaction between R1 and treatment, $F = 108.06$, d. f. = (10, 12), $p < 0.001$, see fig. 4). Degradation estimated for the treatment with *Bacillus* were significantly different from those treatments with the *Bacillus* and the *Enterococcus* (Tukey HSD Post-hoc comparisons $p = 0.04$), or with the *Bacillus* and the bacteriocin ($p < 0.001$), but also with the saline solution, bacteriocin or *Enterococcus* controls ($p < 0.001$ for all of them). Further, treatment where only *E. faecalis* (C1) was added produced highest keratin degradation than other control groups ((C1)-(C2) $p= 0.002$, (C1)-(C3) $p= 0.01$). Therefore, the detected differences in keratin degradation of cultures with only *B. licheniformis* with those that we also added the *Enteroccocci* living in the UG of hoopoes or its bacteriocin suggest that the bacteria from hoopoes at least partially inhibit the degradation of keratin by *B. licheniformis*, which otherwise is completely inhibited by the preformed bacteriocin.

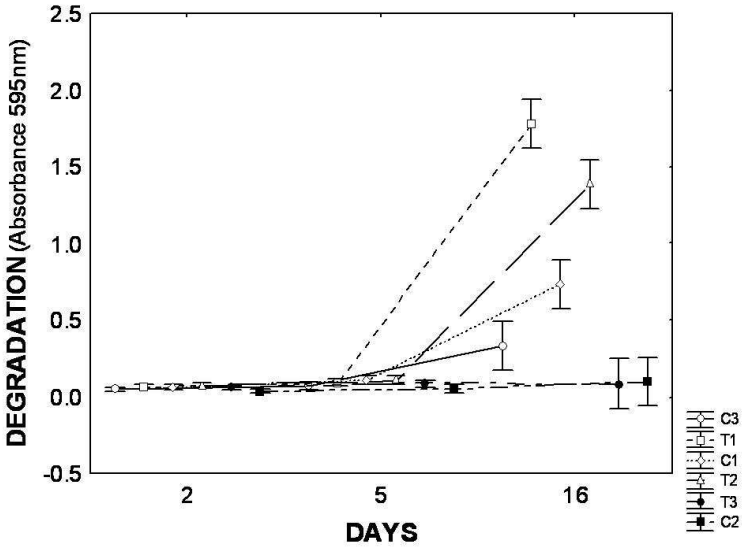

Fig. 4. *Representation of mean values of estimated absorbance in keratin solutions under different experimental treatments, with the SE intervals. Legend: T1: BL; T2: BL+EF; T3: BL+NC, C1: EF; C2: BC; C3: NaCl control (BL: Bacillus licheniformis, EF: Enterococcus faecalis, BC: Bacteriocin).*

To sum up, results suggest that the degradation activity of the keratinolytic *B. licheniformis* over the keratin is partially inhibited or annulled in the presence of the bacteria isolated from the UG of hoopoes, *E. faecalis* MRR-10.3, and especially in the presence of the bacteriocin that they produced.

DISCUSSION

Our results indicate an important antagonistic activity of a bacterial species living in the UG of hoopoes against a generalist parasitic-pathogenic (keratin degrader) bacterium of birds. The antagonist effect on pathogenic bacteria by the symbiotic *E. faecalis* is likely mediated by its production of bacteriocins, given that previous work has shown that this strain produces a bacteriocin active against this and other potential pathogens (Martin-Platero *et al*. 2006). In agreement with this possibility, we found that the addition of such bacteriocin to experimental cultures impeded the feather degradation by the pathogenic microorganism, probably by inactivating the bacillus. Therefore, the two experiments performed highlighted the decrease of the keratinolytic activity in *B. licheniformis* in the presence of either, the symbiont bacteria isolated from hoopoes UG, or the produced bacteriocin. This interference with keratinolysis is

exerted through the bactericidal effect of bacteriocin MR10 on *B. licheniformis* rather than a direct inhibitory effect on the keratinase.

B. licheniformis naturally occurs in the plumage of birds (Whitaker *et al*. 2005) and, by digesting the beta-keratin in avian plumage, would decrease host fitness (Whitaker *et al*. 2005). As a consequence, *B. licheniformis* can be seen as a selective force favouring the evolution of some traits, such as moult, or behaviours that reduce the negative effects of microbial infection of plumage (Burtt & Ichida 1999a). It is known that keratinase production and feather degradation activity of bacilli can be inhibited by some chemical compounds (e.g. detergents, glucose and methanol, Suntornsuk & Suntornsuk 2003) and antibiotics that may exist in the UGS (e.g. Jacob *et al*. 1997, Bandyopadhyay & Bhattacharyya 1999). In this way, preening has been proposed several times to have a beneficial effect on plumage maintenance by reducing the ectoparasites load (Møller 1991; Rozsa 1993), but also bacterial growth (Burtt & Ichida 1999a). However, to our knowledge, this is the first evidence of an inhibition of feather degradation mediated by a bacterium (or their produced bacteriocins) living in the UG of a wild bird. Thus, antimicrobial properties of the UGS of hoopoes protecting feathers are, at least partially, mediated by the *E. faecalis* living in the uropigium.

E. faecalis is present only in brown UGS of hoopoes (Soler *et al*. *submitted*), which are produced by females and nestlings during the nesting period (Martín-Vivaldi *et al*. *submitted*). Thus, because female hoopoes do not abandon the nest during the period of incubation and brooding, preening with that brown secretion may protect their feathers from pathogenic bacteria. For growing nestlings the antagonistic effects of the symbiotic bacteria against feather decomposers would be of prime importance for developing a plumage in good condition, which will determine their fly capability (Machmer *et al*. 1992, Barbosa *et al*. 2003). Indeed, filming inside the nests revealed that nestlings spread UGS onto their plumage (Martín-Vivaldi *et al*. *unpubl. data*).

Traits that reduce the probability of feather degradation would be of selective advantage. This benefit would be particularly important for birds that, as hoopoes, are hole-nesting species that do not sanitize their nests, given the elevated risk of parasitism (Møller &

Erritzøe 1996) and the large number and diversity of bacteria in these environments, which have the potential of becoming opportunistic pathogens (Pinowski *et al*. 1994; Burtt & Ichida 1999b). In addition, it is known that the keratinolytic activity of bacilli largely increases with high temperatures and humidity (Burtt & Ichida 1999a; Cristol *et al*. 2005), conditions present in hole-nests during the incubation and nestling period. Hoopoes do not clean up the nest even from unhatched eggs, eggshells from hatched or broken eggs, died chicks, faeces, nor remains of food (pers. obs.). This organic material accumulated within the nest could theoretically be a focus of nestling and female infection during their nesting period and it is likely that, in addition to *B. licheniformis*, the antimicrobial properties of the UG symbiotic bacteria through its bacteriocin (with a wide antimicrobial spectrum, see Martín-Platero *et al*. 2006) provide hoopoes with protection against a wide range of potentially pathogenic bacteria. Those findings point out that this bacterium and its bacteriocin could be useful for females and nestling hoopoes in their fighting against parasitic microorganisms during the nesting phase of reproduction.

In this paper we have found evidences suggesting that the potential benefits obtained by hoopoes from the symbiotic bacteria *E. faecalis* living in their UG refer, not only to the previously suggested protection of developing embryos against pathogenic bacteria (see Soler *et al*. *submitted*), but also to the reduction of feather degradation by bacteria during the nesting phase. Ability to suppress pathogens through self-medication, defined as the defence against parasites by one species using substance produced by another, has great associated advantages (Newton 1991; Clayton & Wolfe 1993). Hoopoes would thus be another example of self medication in nature (see for instance Lozano 1998; Philippe *et al*. 2003; Christe *et al*. 2003; Revis & Waller 2004) because they use the bacteriocin production of other organisms against potential pathogens.

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VI

INTESTINAL MICROBIOTA AS A POTENTIAL FACTOR FOR COEVOLUTION BETWEEN AVIAN BROOD PARASITES AND THEIR HOSTS

M. Ruiz-Rodríguez, F. S. Lucas, P. Heeb, and J. J. Soler

ABSTRACT

It is known that intestinal microbiota influences the effectiveness of digestion in vertebrates and, although it is phylogenetically constrained, is related to specific characteristics of diets. Avian brood-parasitic nestlings of the family *Cuculinae* develop in nests of phylogenetically distant passerines and are fed with the host diets. If the shaping of bacterial communities is dominated by phylogenetic constraints and, therefore, microbiota of parasitic nestlings differs from that of the host nestlings, energy and micronutrients that parasites and hosts nestlings obtain from a similar amount of food would be different. In this case, bacterial communities of parasitic and host nestlings would have important consequences in the coevolutionary process between them. By experimentally creating mixed broods of magpies (*Pica pica*) and great spotted cuckoos (*Clamator glandarius*), we investigated their cloacal microbiota by using ribosomal intergenic spacer analysis, and although we found significant differences, none of the phylotypes were specific of neither great spotted cuckoo nor magpie nestlings. Cuckoos presented more complex communities, which could be an adaptation to the brood parasitic life-style allowing digesting the food provided by different potential hosts.

INTRODUCTION

Animals depend for their growth on external sources of biomass that, through the digestive processes, are degraded into simple molecules that can be used for cellular metabolism. Apart from the variety of physical and chemical adaptations in the digestive tract that are involved in the digestion, there exist important bacterial assemblages that are essential for hosts. They allow further degradation of large molecules as cellulose, they are also responsible for the synthesis of some vitamins as K, B_{12} and riboflavin among others, ferment sugar, produce lactic acid (controlling thus the intestinal pH), and may impede intestinal colonization by pathogenic bacteria (see Hooper *et al*. 1998).

Bacterial communities in the intestine vary depending on environmental factors, such as diet or habitat, as well as on properties of the digestive tract, including anatomy, pH and retention time of digesta (Stevens & Hume 1998). Most of these factors are species-specific characters and, thus, bacterial communities are relatively stable and vary between species (e.g. Dubs 1966 (cited by Stevens & Hume 1998)). In fact, across species anatomical comparisons reveal that the digestive tract is the most diverse organ system (Klasing 1999). Moreover, gastrointestinal microbiota has a strong phylogenetic component as pointed out by Hackstein and vanAlen (1996) studying the presence of methanogenic Archaea in 253 vertebrate species. Thus, host phylogeny may constrain the evolution of optimal bacterial community for a given diet (Hackstein *et al*. 1996) because of the phylogenetic constraints of gut morphology. On the other hand, there are numerous experimental evidences on the effect of the diet on the microbial composition of the digestive tract allowing an optimal use of nutrients from food intake (e.g., Xu & Gordon 2003; Glunder 2002; Engberg *et al*. 2002). Therefore, bacterial communities of different species should be related to diet, but may be also phylogenetically constrained, which may have important consequences in the evolution of the digestive process. For instance, it could affect the evolution of fermenting structures (i.e. rumina, caeca, etc.) in the digestive tracts allowing alloenzymatic digestion (Langer 2003), which, at the same time, may influence specific life history traits (see, Langer 2002, 2003). Moreover, these constraints referred to gastrointestinal bacteria may also affect inter-species interactions, because species with

bacterial communities which exploit the most abundant resource may have an advantage when competing with species with a phylogenetically constrained microbiota. This mechanism could be particularly important for phylogenetically distant species that exploit exactly the same resource.

Interspecific brood parasitic birds lay their eggs in nests of other species, the hosts, which incubate the eggs and feed the parasitic offspring. Most parasitic species are cuckoos (subFam *Cuculinae*) and are phylogenetically distant from their common hosts, which are mainly Passeriformes (see Davies 2000). Moreover, although most cuckoos are insectivorous, some of them parasitize species that feed their offspring with vegetal material (e.g., Krüger & Davies 2002) and, thus, the diet of these cuckoo species differs greatly between the nesting and the adult stage. This is the case of great spotted cuckoos (*Clamator glandarius*) that in Europe mainly parasitize magpies (*Pica pica*) (Cramp 1985). While adult great spotted cuckoos feed exclusively on caterpillars (Cramp 1985) and are specialists in noxious insects that most birds avoid (Del Hoyo *et al*. 1997), magpies feed their offspring with a great variety of insects, but also with vegetal materials including grains of cereals (Martínez *et al*. 1992). When parasitized, adult magpies feed preferentially cuckoo nestlings, but the diet of cuckoo and magpie nestlings do not differ significantly (Soler *et al*. 1995). Avian species possess a variety of adaptations for digestive processing of their diets (Duke *et al*. 1997) including specific anatomic plan of the digestive tract depending on birds typical diet (Klasing 1999), but also on their phylogenetic history (see above). Therefore, it is likely that gastrointestinal tract of cuckoos and magpies differ. In any case, because the morphology of the gut may plastically accommodate to changes in nutritional needs during the life cycle (Diamond 1991), we cannot completely reject the possibility of cuckoos and magpies showing similar gut morphology and allowing the colonization of similar microbiota. In this coevolutionary scenario, (i) gastrointestinal microbiota of cuckoo nestlings may be adapted to exploit magpie-nestling diet, due to the importance of the environmental component determining cloacal bacterial communities of wild birds (Lucas & Heeb 2005). As an alternative, (ii) gastrointestinal bacterial communities of cuckoos can be phylogenetically constrained, not only because of differences in adult diets, but

also because the two species are phylogenetically distant and thereby with a likely different digestive morphology. Thus, although cuckoo and magpie nestlings are feed with exactly the same diet at the nest, the second-alternative scenario predicts between-species differences in gut microbiota. This possibility may have profound consequences on the coevolutionary process that great spotted cuckoos and magpies are involved (see Soler & Soler 2000).

It can be assumed that magpie nestlings hold a gastrointestinal microbiota allowing an optimal digestion of food carried by parents (Robbins 1983) and, if cuckoo digestion of such diet is suboptimal, cuckoo nestlings would need more food than their foster siblings for getting a similar amount of energy and nutrients. Therefore, selection pressure favouring an increase on begging behaviour and, thus, on feeding effort of foster parents, would be higher for cuckoos than for host nestlings. In fact, previous works revealed that, although body mass of fledgling cuckoos was in average lower than that of fledging magpies (Soler & Soler 1991), cuckoo nestlings need higher amounts of food than their magpie siblings (Soler *et al*. 1995). The higher level of need of cuckoo nestlings may be explained by cuckoos having a suboptimal bacterial community to process magpie diet.

Here, for the first time, we compare the structure of cloacal bacterial community of two wild species that share exactly the same environment, and discuss possible evolutionary and ecological explanations and consequences of microbiota dissimilarities. Between species differences in microbial communities of the cloaca will reflect differences at the intestinal level (Savage 1977; Vaahtovuo *et al*. 2001), which allows the interpretation of results as differences in the bacterial community living in the digestive tract of magpies and great spotted cuckoos. Briefly, we performed the study in a brood parasite – host system by using Ribosomal Intergenic Spacer Analysis (RISA) (García-Martínez *et al*. 1999). Moreover, as a control bacterial community, we also explored the incidence of generic pathogenic bacteria in great spotted cuckoo and magpie nestlings within the same nests, which allows discussing possible interspecific differences in the structure of cloacal bacterial community. To do this, we experimentally moved both magpie and great spotted cuckoo nestlings from parasitized to non-parasitized and vice-versa to created experimental nests with two magpie and two great spotted

cuckoo nestlings. Thus, our experimental approach avoids possible bias due to cuckoo host selection or to local adaptation to nests of hatching of both species. In addition, we dissected one magpie and one cuckoo nestlings of similar age to detect differences in the digestive tracts morphology that confirmed the assumption of interspecific differences in intestine morphs.

MATERIAL AND METHODS

The study area was the Hoya de Guadix (37°18'N, 3°11'W), southern Spain, a cereal-producing plain (especially barley) at approximately 1000 m above sea level. The vegetation is sparse, with some holm oaks (*Quercus ilex*) and many groves of almond trees (*Prunus dulcis*) in which magpies nest at a high density (for a description of the study area, see Soler 1990). Parasitism of magpies by the great spotted cuckoos is quite common in the area and some evidences on an ongoing coevolutionary process between both species have been detected in recent years (see Soler & Soler 2000).

Field work and experimental procedure

At the beginning of the breeding season of 2003, we looked for magpie nests, which were visited twice a week to determine laying date, the start of incubation, and parasitism by the great spotted cuckoo. Incubation period of great spotted cuckoo eggs is in average 4 days shorter than that for magpie eggs and, thus, in most parasitized nests great spotted cuckoos hatched ahead of magpie eggs (Soler 1990). Moreover, magpies preferentially feed the larger nestling in the nest and, therefore, most magpie nestlings died by starvation in parasitized nests (Soler & Soler 1991; Soler *et al*. 1995). We were interested in comparing cloacal bacterial communities of cuckoo and magpie nestling that shared the same nest during growing. Thus, in order to maximize number of nests with nestlings of the two species, soon after hatching, we manipulated magpie broods by exchanging cuckoo or magpie nestlings up to having brood sizes of two cuckoos and two magpie nestlings of the same age per experimental nest. Briefly, in non-parasitized nests we introduced two cuckoo nestlings from other nests, matching them for age with the magpie nestlings, and removed all except two randomly selected magpie nestlings.

In parasitized nests, if necessary, we introduced two magpie and one cuckoo nestling up to complete a brood of two magpie and two cuckoo nestlings of the same age. All experimental broods were performed soon, when nestlings were 1-3 days old. At this age, the original-nest effect on bacterial communities is not supposed to be a problem since the gut microbiota is highly influenced by the environment of the experimental nest of rearing (see Lucas & Heeb 2005). Cross-fostering experiments have been broadly used to distinguish between genetic and environment components of numerous traits (e. g. Brinkhoff *et al*. 1999; Soler *et al*. 2003; Lucas & Heeb 2005) and, thus, this experimental approach is also useful for detecting the influence of nest of origin and nest of rearing explaining gut microbiota.

We performed a total of 23 experiments. After losses due to predation and nestling starvation, we analyzed samples collected from 41 magpies and 26 great-spotted cuckoo nestlings from 27 broods. 19 nests were experimental broods, while 8 were natural, non-parasitized magpie nests. From the 19 experimental nests, we collected data for the two species in 11 (16 magpies and 17 cuckoos), while samples from a single species (i.e., experimental nests in which we have the data only for one species) were collected in the resting 8 experimental nests (9 cuckoos and 2 magpies). In total, 18 great spotted cuckoos and 14 magpies were moved from one nest to another to create experimental broods of nestlings of similar age. All nestlings were included in the analyses because in all experimental nests magpies and great spotted cuckoos were sharing the nests during most of the nestling period.

Bacterial sampling and lab analyses

Bacteria in the cloaca were sampled before fledging, when nestlings were 16-18 days old. Bacterial sampling and DNA manipulation, was carried out in exactly the same way for every individual of the two species. Bacteria were collected by injecting and repipetting 500 µl of sterile phosphate buffer (Na_2HPO_4 0.1 M and NaH_2PO_4 0.1 M, pH 7.4) in the cloaca using sterile tips and an automatic pipett. After collection, we immediately lysed the bacterial cells by adding 500 µl. of lysis buffer (5% Tris HCl 50mM, 5% SDS, 2% EDTA 2mM, 3.3% NaCl 100mM)

and samples were kept in ice. Later in the lab, samples were stored at -20 ºC until molecular analyses.

DNA was extracted from 200 µl of each sample. Samples were thermically shocked to further lyse the cells. We extracted DNA following the protocol proposed by Orsini & Romano-Spica (2001). Shortly, after adding 400 µl of a buffer prewarmed at 65ºC (1% of 10 mM Tris HCl, 0.2% of 1 mM EDTA, 15% of 0.3 mM sodium acetate, and 1.2% of Polyvinylpyrrolidone), the DNA was purified with phenol-chloroform procedure. Finally the DNA was precipitated with isopropanol overnight at -20 ºC. After washing 3 times with 80% ethanol, the DNA was re-suspended in TE buffer pH 8 (10mM Tris HCl and 1mM EDTA).

To study the diversity of the cloacal community, we used the Ribosomal Intergenic Spacer Analysis (here after, RISA) method, which amplify the spacer region between the 16S and 23S rRNA genes in the ribosomal operon. This fragment is extremely variable in both sequence and length for the different prokaryotic species, due to the presence of several functional units within them such as tRNA genes (García-Martínez *et al*. 1999). The primers used were S-D-Bact-1522-b-S-20 and L-D-Bact-132-1-A-18 (Ranjard *et al*. 2000). The Polymerase Chain Reaction was performed in 50 µl., with 100 ng of DNA, 1 x PCR buffer (QIAGEN), 2 mM $MgCl_2$, 0.1 mg/ml BSA, 0.5 µM of each primer, 150 µM of each dNTP and 1 U Taq polymerase (QIAGEN). The amplification reaction was performed using an initial denaturation at 94ºC for 3 minutes, followed by 25 cycles at 94ºC for 1 min., 55ºC for 30 sec., 72ºC for 1 min., and a final extension at 72ºC for 5 min. (Ranjard *et al*. 2000). PCR products were subsequently quantified with a fluorimeter DynaQuant (Hoefler) after staining with Hoechst Dye 1/10000. To separate the PCR products (200 ng), we used a 2% Metaphor agarose (FMC Bioproducts) gel electrophoresis, during 4 hours at 150 V. Each band in the gel corresponds to one "Operative Taxonomic Unit" (OTU), also called phylotype, which is assumed to be one bacterial species (Atlas & Bartha 1997).

RISA is a broadly used method for the study of bacterial communities. It has for instance been used with samples of soil (e.g., Patra *et al*. 2005; Mohamed *et al*. 2005), water (Graham *et al*. 2004; Kent *et al*. 2004), tissues of organisms (Lucas *et al*. 2005), but also to

characterize bacterial community of the gastrointestinal tract (Larue *et al*. 2005; Lucas & Heeb 2005) as it is here the case. RISA, in comparison with culture-based techniques, has the advantage of estimating bacterial diversity by directly analysing the samples, thus avoiding cultural biases, and detecting different phylotypes or OTUs, giving a good approximation of different species of bacteria present in the sample (Stach *et al*. 2003).

Finally, one cuckoo and one magpie nestling of the same age (6 days) and similar size, that for other purposes were sacrificed five year ago and kept in alcohol (76%), were dissected and their gastrointestinal tracts isolated for comparisons of their morphologies (Fig. 3).

Pathogens

We checked for the presence of three generic bacterial genera considered as part of the normal gut microbiota, but opportunistically pathogens. A set of 77 nestlings in total were analysed from 18 nests with the two species, 41 of them were great spotted cuckoos and 36 were magpies. The extraction method was the same as described above, and DNA from cloacal samples were checked from presence of different DNA fragments, specific from *Salmonella* spp., (199 bp salmonella-specific fragment using primers derived from a cloned fragment of *Salmonella weltevreden* genome, Jitrapakdee *et al*. 1995), *Campylobacter* spp., (16 rRNA region of thermotolerant *Campylobacter* species, Moreno *et al*. 2003), and enteropathogenic *Escherichia coli* (multiplex PCR: genes for shiga toxins stx_1 and stx_2, intimine *eaeA*, and adhesine *hlyA*, generating amplification products of 180, 255, 384 and 534 bp respectively, Paton & Paton 1998). Results from PCRs were observed in a 2% agar gel and presence of the specific band indicates presence of particular pathogenic bacteria.

Statistical analyses

The gels were analyzed with GEL COMPARE software (Applied Maths, Kortrijk, Belgium). This program estimates degree of similarity between pairs of individuals giving rise to a similarity matrix that summarizes pairwise similarities among samples (i.e. individuals). This program uses the Dice's binary coefficient: $2a/(2a+b+c)$, where a is the number of OTUs in

common for the two samples, b is the number of OTUs present only in the first sample, and c is the number of OTUs present only in the second sample. We also prepared three binary matrices for comparisons of bacterial communities, one with the two species, another one with the nest of rearing identity, and a third one with the nest of origin identity. Value 1 referred to the same and 0 to different values for individual within a pair (i.e. if a pairs of individuals are of the same species, nest of origin, and nest of rearing, the assigned value in all three matrices would be 1).

These three matrices were correlated with the bacterial community similarity matrix using Mantel tests as implemented in FSTAT software (Goudet 1995). These tests estimate the relationships between the matrices and provide partial autocorrelation coefficients and associated p-values. Statistical significances were estimated by Monte Carlo procedure after 10,000 permutations. Matrices of bacterial assemblage similarity were used as the dependent variable, while those of species identity, nest of rearing, and nest of origin were independent matrices (Lucas *et al*. 2005). To control one variable for another, we performed partial autocorrelations between bacterial community and species identity while keeping in the model matrices related to nest of origin and nest of rearing.

Given that the number of OTUs approximately followed a normal distribution (Shapiro-Wilk normality test $W= 0.969$, $p > 0.9$), a t-test was performed to analyze differences in richness between the two species, using Statistica 6.0 (Statsoft 2001) Software.

Differences in prevalence of pathogenic bacteria were studied by comparing probability of infection of cuckoos and magpies within the same nest and, thus, using paired-statistical tests. We used Wilcoxon test to compare prevalence of *Campylobacter* and *Escherichia coli*. Only 6.5% of nestling demonstrated infection by *Salmonella*, we thus used a Sign Test. Analyses were performed with Statistica 6.0 (Statsoft 2001) Software.

RESULTS

Most of the detected bands occurred in both species, but they appeared more frequently in great spotted cuckoo samples than in those from magpie nestlings (mean of frequencies: magpies 0.12 ± 0.14, cuckoos 0.19 ± 0.18; paired t-test: $N = 45$, $t (1,64) = 2.55$, $p = 0.014$, see Fig.1).

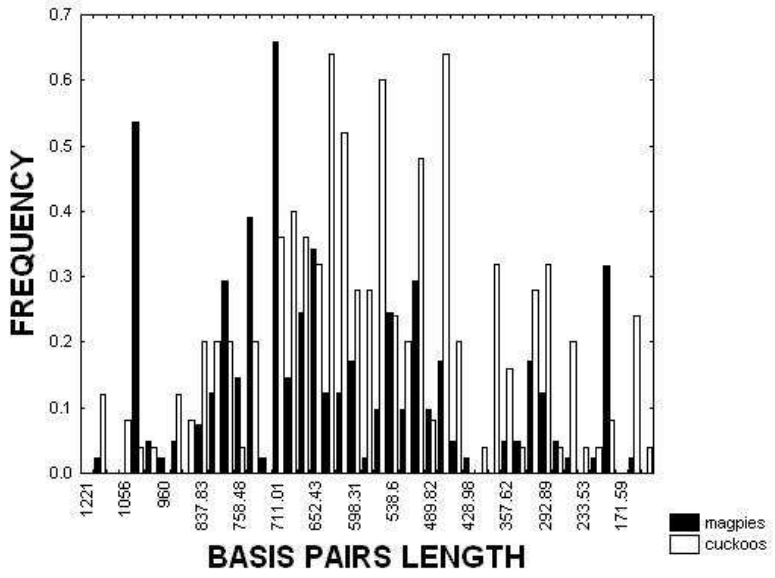

Fig. 1. *Prevalence of different OTUs (phylotypes) in samples of great spotted cuckoo (white, N = 26) and magpies (black, N = 41).*

Within broods variation in bacterial communities was mainly explained by species identity, either when using all the nests together (i.e., non- and experimental nests), or when using only experimentally parasitized nests (see Table 1). Thus, great spotted cuckoo and magpie nestlings sharing the same environment (i.e. nest) differed in their cloacal bacterial communities (Fig 1). In addition, after statistically controlling for species differences, we found that nest of rearing explained a significant proportion of variance of bacterial community, which indicates an important role of the environment determining the cloacal bacterial composition of both great spotted cuckoos and magpies (Table 1). This result did not vary when only experimental nests were used (Table 1).

	All nests (n=27)		**Experimental nests (n=19)**	
Independent variables	**R**	**P**	**R**	**P**
Species identity	0.1643	0.0001	0.2777	0.0001
Nest of Origin	0.1224	0.53	0.0205	0.0001
Nest of rearing	0.1205	0.0003	0.1245	0.0001
Species identity (controlling for nest of origin)	0.1446	0.0001	0.2936	0.0001
Species identity (controlling for nest of rearing)	0.1602	0.0001	0.2864	0.0001

Table 1. *Relationships between matrix of bacterial similarity and matrix of host species identity (i.e. same or different host species). Apart from correlation coefficients and P-value associated to each independent variable, partial correlation coefficients for species identity after controlling for nest of origin and nest or rearing are shown. Sample size refers to number of nests.*

Interestingly, in spite of between-species differences in their bacterial community at the cloaca, no specific OTU for any of the two species were detected (Fig. 1). This is important because it implies that the between-species differences in their bacterial community at the cloaca were due to great spotted cuckoos having a higher diversity of OTUs than magpie nestlings, i.e., great spotted cuckoo nestlings presented a more diverse microbiota than magpie nestlings, since average number of OTUs per sample (± 95% confidence intervals) was higher for the former (N = 25, 8.76 ± 3.96) than for the latter species (N = 41, 5.46 ± 2.61) (t-test: t-value = -4.074, p = 0.00013, see Fig. 2), even after controlling for nest of rearing (GLM, nest identity as random factor and species as fixed factor; effect of species: F (1,28) = 15.57; p = 0.002, effect of nest identity, F (26,28) = 1.25; p > 0.25). Finally, differences in OTU richness between great spotted cuckoos and magpie nestlings did not vary in relation to nest identity because the interaction between nest and species identities was far from statistical significance (F (10,28) = 1.20; p > 0.25).

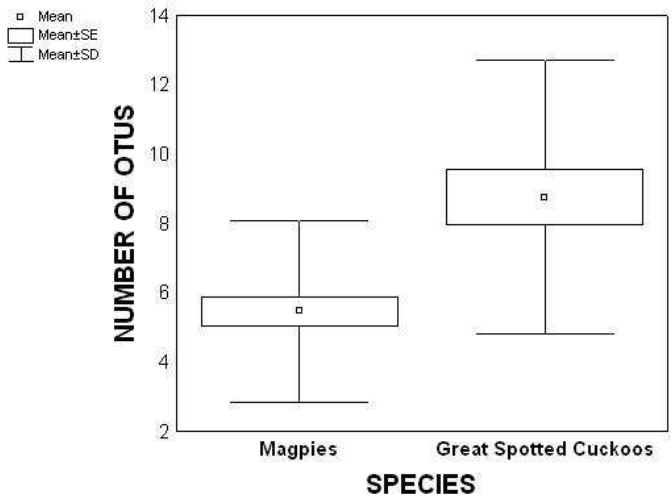

Fig. 2. *Differences in number of phylotypes detected in cloacal samples of great spotted cuckoo and magpie nestlings.*

With respect to the generalist pathogenic bacteria, except for *E. coli*, prevalence of *Salmonella sp.* and *Campylobacter sp.* were quite low in our experimental nests. In any case, prevalence of the three studied bacteria estimated for magpie nestlings did not differ significantly from that estimated for great spotted cuckoo nestlings reared in the same nest (Table 2).

Species	*Salmonella*	*Campylobacter*	*E. coli*
Magpies	2.7% (n = 36)	8.33% (n = 36)	47.22% (n = 36)
Great spotted cuckoos	9.75% (n = 41)	9.75% (n = 41)	31.7% (n = 41)
Comparisons	Sign test, Z =-0.7, p = 0.8.	Wilcoxon test, Z = 0.674, p = 0.5.	Wilcoxon test, Z = 1.4, p = 0.16.

Table 2. *Prevalence of Salmonella, Campylobacter and Echericha coli in cloacal samples of great spotted cuckoo and magpies nestlings. Interspecific comparisons for different bacteria species are also shown.*

The detected interspecific differences in bacterial community may be explaining by differences in the intestinal tract morphology of cuckoo and magpie nestlings as can be observed in Fig. 3. Magpies, as predicted for omnivorous species (Barnes & Thomas 1987), present a larger intestine than cuckoos with the liver lobules separated by a piece of gut that does not occur in cuckoos, which have the two liver lobules very close to each other. Finally, while the intestine of the great-spotted cuckoos present two large and patent caeca sacs at the end, this is not apparent in magpies, according to the usually small or vestigial caecum observed in Passeriformes (Clench & Mathias 1995).

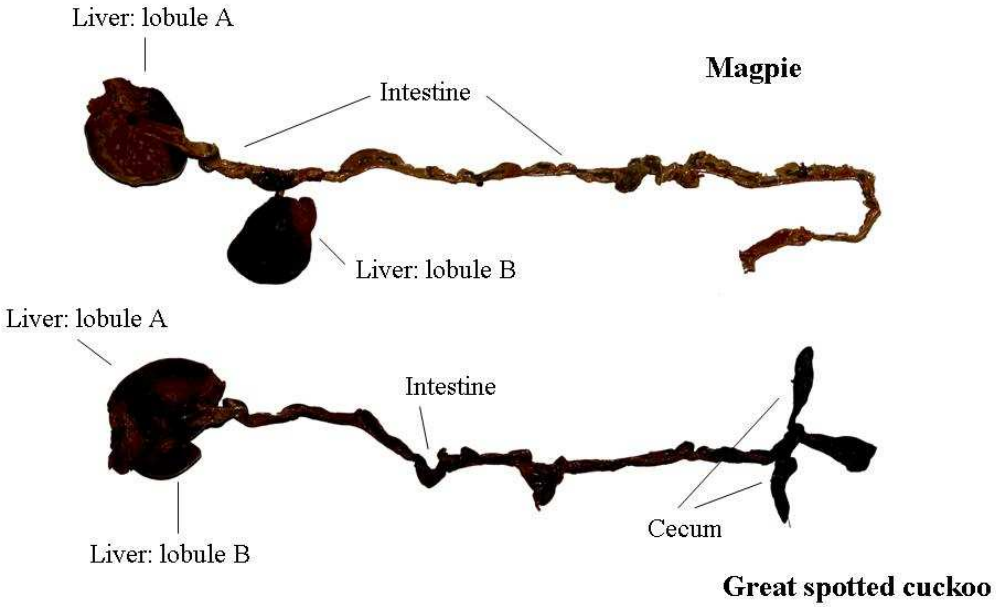

Fig. 3. *Intestine morphology of a magpie (top) and a great spotted cuckoo nestling (down).*

DISCUSSION

We characterized the bacterial community of cuckoo and magpie nestlings by using RISA methodology which, rather than detecting bacteria species, identified phylotypes or OTUs in our samples that allow comparisons of bacteria communities in the digestive tracks of different animals (e.g. Larue *et al*. 2005; Lucas & Heeb 2005). We did not detect host species-specific phylotypes but brood parasitic nestlings showed a richer microbiota than magpie nestlings and none of the detected OTUs appeared exclusive of great spotted cuckoo or magpie nestlings (Fig. 1). Except for two phylotypes (Fig 1) that appeared only in 1% of the magpie samples, all bacteria phylotypes detected in magpies were also detected in cuckoos. This result is in accordance with the importance of the environmental conditions determining bacterial community (Lucas & Heeb 2005) because great spotted cuckoo and magpie nestlings experience the same potential colonisation due, for instance, to similar diet, or to the bacterial transmission from adult hosts when feeding (Kyle & Kyle 1993).

Although we did not find evidence of bacteria specificity in any of the two bird species, our results showed that nestlings of great spotted cuckoos and magpies differ in their cloacal microbiota. These between-species differences were not due to nest environment because in most nests great spotted cuckoo and magpie nestlings were reared together and analyses were corrected for differences due to nest of origin and nest of rearing (see results). One possibility explaining the detected interspecific differences is that the digestive tracts of great spotted cuckoo and magpie nestlings differ in the chemical environment of their intestine, which would allow the establishment of different microbiota. In other words, it is possible that the chemical (i.e. pH) environment of digestive tract of great spotted cuckoos may allow the colonization by a larger number of bacteria species than that of magpies. However, although we have no data of the chemical environment of the digestive tract of magpies and cuckoos, this hypothetical variation in chemical environment did not affect the establishment of three different opportunistic pathogenic bacteria because its prevalence did not differ between great spotted cuckoo and magpie nestlings (see Table 2).

Cuckoos and magpies, however, differ in the morphology of their digestive tracts (see Fig. 3) and it is known that morphology of the digestive track affects the establishment of different bacteria (e.g. Stevens & Hume 1998). Moreover, a more intricate digestive tract allows colonization by a more diverse digestive microbiota in the intestine (e.g. Langer 2002), which may explain the interspecific differences detected here. The digestive track of cuckoos, in contrast to that of magpies, presents a well developed caecum that at least in chicken plays an important role in the digestion of carbohydrates by enzymes of microorganism (e.g., Lan *et al*. 2005). Thus, it is possible that, among other anatomical differences in the intestine of magpies and cuckoos (see Fig. 3), the caecum of the great spotted cuckoos allowed a more diverse microbiota than magpies, and that the presence of caecum could imply differences between digestive strategy and metabolism capability.

Finally, another non-excluding possibility explaining our results is related to between–species differences in the establishment of alliances between the gastrointestinal epithelium, immune cells, and resident microbiota (see McCracken & Lorenz 2001). For instance, it is known that a more relaxed immune response (e.g. McCracken & Lorenz 2001) and a more complex intestinal epithelia morphology (i.e. geographical barrier that reduces bacterial competitive interference) (Ji *et al*. 1997) allow a more diverse flora. But also differences in maternal antibodies transferred to eggs, due to differences in bacterial exposure by cuckoo or magpies females, could have an effect on the type of bacteria that would initially colonize the gut. However, deeper comparative studies of interspecific differences in chemical and immune environments of cuckoos and magpies guts are necessary to further explore these possibilities.

An alternative non-functional explanation is that cuckoos, acquiring more quantity of food than their foster siblings in the nest (Soler *et al*. 1995), would be in contact with more bacteria, which provide more chances of microbial colonisation, and this leads to cuckoos having a richer community than magpies. However, because diet of nestlings of both species were almost the same (Soler *et al*. 1995), this explanation is unlikely.

Independently of the underlying mechanisms explaining the higher bacterial richness of cuckoos, specific characteristics of the bacterial community of the parasitic nestlings may be

an adaptation to exploit specific diets not only during the nestling, but also during the adult phases. While adult magpies are omnivorous (cereals, fruits, carrion, insects, etc.), adults of great spotted cuckoos eat almost exclusively caterpillars (Cramp 1985). In addition, because great spotted cuckoos and magpies are phylogenically distant species, it is likely that, together with the differences in morphology, environmental conditions in their intestinal tract also exist. These interspecific differences would allow an optimal digestion of the diet of each species as well as the establishment of specific bacteria as has been described for most studied species (e.g. Langer 2002).

In addition, because at the nestling stage it can be assumed that intestinal microbiota of magpie nestlings should be close to the optimal for exploiting their diet, the detected differences between these two species could be interpreted as microbiota of cuckoos being suboptimally adapted to the magpie diet. If that was the case, great spotted cuckoo nestlings would need of a larger amount of food than the magpie nestlings for acquiring a similar amount of energy and/or micronutrients. However, natural selection is in general stronger during the juvenile than during the adult phase (Endler 1986) and, thus, adaptations allowing an optimal development of cuckoo nestlings using food items that differ from adult diet is expected. In this scenario, because a diverse microbiota may allow a better degradation of organic matter due to niche complementarities between bacteria species (Loreau 2001), the detected larger bacterial diversity of cuckoo nestlings may be interpreted as a trait that favours digestion of magpie diet. Another adaptive scenario is that the richer bacterial community detected in great spotted cuckoos would allow cuckoos the exploitation of host species, others than magpies, with different diets. In this sense, although we did not detect specific OTUs for cuckoos, it is possible that the ability of cuckoo nestlings of having a more rich bacterial community in their intestinal tract allow the colonization of important digestive bacteria in different environment (i.e. nests of different species). However, studies on digestibility of different kinds of food by great spotted cuckoo and magpies and comparisons with other hosts species are needed to corroborate our suggestion.

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VII

ACQUISITION BY HOOPOE NESTLINGS OF SYMBIOTIC BACTERIA LIVING IN THEIR UROPYGIAL GLAND

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ABSTRACT

Symbiosis between prokaryotic and eukaryotic organisms broadly occurs in nature, and the ways in which hosts acquire symbiont microorganisms vary depending on the species involved, and on the consequences of the relationship for the two parts implicated. Microorganisms can reach hosts by horizontal transmission if the host acquires the microorganisms from the environment or from some other non-related individuals in the population. Vertical transmission, however, would occur when offspring receive the symbionts directly from parents, being mainly the mother who transfers them to offspring. Characteristics of acquisition of symbiont by hosts may affect the strength the coevolutionary relationships between micro-organisms and their hosts. Here, we study how nestling hoopoes (*Upupa epops*) acquire symbiotic bacteria that live in their uropygial gland and that also live in the gland of females during their nesting period. Briefly, from cultures of the uropygial gland secretions of nestlings, we isolated an average of 5 different bacterial colonies and, by using RAPD-PCR analyses, typified their genetic profiles. We compared colonies identities (RAPD-group) within and between individuals of the same and different nests. In addition, by performing cross-fostering experiments before the development of the uropygial gland of nestlings was completed, we tried to detect the effect of common environment (i.e. horizontal transmission) by studying the relative importance of nest of rearing and nest of origin explaining microbiota of the uropygial gland of nestlings just before fledging.

We found that the strains of symbiotic bacteria harboured by nestlings were significantly explained by nest of origin, but also by nest of rearing. The effect of nest of origin shows that the symbiotic bacteria are acquired by nestlings soon, either before (through the egg-shell or oviduct of females), or after hatching. The effect of nest of rearing however demonstrates the importance of common environment explaining the identity of strains that colonize the uropygial gland of developing nestlings.

INTRODUCTION

Symbiosis between two organisms is widespread in nature. This type of association deeply affects the diversity of life, by providing expanded ecological and evolutionary opportunities of speciation and specializations to both hosts and symbionts (Douglas 1994). Until now, the vast majority of described symbioses includes microorganisms (Lindquist, Barber, & Weisz 2005), with the symbiotic relationships between bacteria and multicellular organisms being a prominent feature of life on Earth (Xu & Gordon 2003). Interactions of eukaryotes with environmental microbes have many times resulted in the coordinate evolution of complex symbiosis, which varies in their degree of association, from mutualistic to parasitic (e.g., Moran & Wernegreen 2000). Thus, the bacteria superkingdom should be considered as an important selective force shaping the evolution of eukaryotes (McFall-Ngai 2002).

Environmental factors explaining probability of a host being colonized by bacteria is therefore of prime importance in the evolution of eukaryotes, not only because of the risk of costly pathogenic infections, but also because an optimal development of a variety of animals and plants require of microbial associates that may, for instance, contribute to nutrition and defences of their hosts against predators or parasites (Moran 2002). In squids for example, females include bacteria that live in their accessory nidamental gland as components of the extraembryonic layers of eggs where bacteria profoundly affect the cell biology of the eggs (McFall-Ngai 2002) and protect embryos from potential pathogens (Barbieri *et al.* 2001).

It is particularly important how symbionts transmit from one to another host (Xu *et al.* 2003). The acquisition of symbionts can be horizontal, if they come from non-related host individuals or, simply, from the surrounding environment after embryogenesis (Nyholm *et al.* 2000). On the other hand, vertical transmission occurs from parents to offspring, often through infection of eggs and/or embryos in the mother body (Moran *et al.* 2000; Darby & Douglas 2003). In some animal species, symbionts can use the two forms of transmission, as it occurs in the aphid symbiont *Buchnera aphidicola* (Oliver *et al.* 2003), or in marine sponges (Oren, Steindler, & Ilan 2005). The mode of bacterial acquisition by hosts (i.e. transmission) is highly relevant because it may affect the coevolutionary relationship between hosts and symbionts. For

example, vertical transmission leads to a higher within-host relatedness of symbionts and, thus, to a better alignment of interests between symbionts and hosts, which makes the fitness of the former dependent of that of the later (e.g. Herre 1993; Frank 1996; Douglas 1998; Herre *et al.* 1999; Poulsen *et al.* 2003). Moreover, vertical transmissions also favours intimates coevolutionary process within family lineages, as occurs in the fungus-growing ants, which rear antibiotic-producing bacteria in elaborate cuticular crypts (Currie *et al.* 2005). Horizontally transmitted bacteria however would in general lead to a more relaxed coevolutionary process, as it is the case of most of the interactions between insects and their non-pathogenic bacteria (Dillon & Dillon 2004).

Recently, for two related species of birds, a special symbiotic relationship with some bacteria living in their uropygial glands has been suggested. First, a novel bacterium species, *Enterococcus phoeniculicola*, living in the uropygial glands (UG) of the Green Whoodhoopoe (*Phoenicula purpurea*) has been described (Law-Brown & Meyers 2003). Secondly, it has been detected that the *Enterococcus* sp. living in the UG of the Eurasian Hoopoe (*Upupa epops*) produce substances with antibiotic properties (Martin-Platero *et al.* 2006). In the two species of birds, both of which use holes for reproduction, their UG produce malodorous secretions (UGS). However, in hoopoes malodorous secretions with symbiotic bacteria only occurs during the nesting phase of females and nestlings, but never happen in the UG of male that does not incubate or brood the offspring (Soler et al. unpublished). Therefore, nestling acquisition of the mutualistic bacteria occurs in the nest, either from those present in the material filling the bottom (soil, pieces of wood, or faeces, wood, and food remains from previous nesting attempts), or from the female, whose UG hold mutualistic bacteria since laying to half nestling period (Martin-Vivaldi et al. unpublished).

Here, we study the influence of nest environment explaining the acquisition of symbiotic bacteria by hoopoe nestlings. Briefly, by using molecular analyses, we classified the different types of bacterial strains living in their UG in accordance with some phenotypic characters of the Randomly Amplified Polymorphic DNA (RAPD). Nestlings may acquire the symbiotic bacteria directly from their mothers, or from nest environment. By performing cross-

fostering experiments of hatchlings, and after all bacterial strains were typified, we compared the bacterial strains within individuals and nests, and checked whether bacteria strains were better explained by nest of origin, nest of rearing, or both. If we find that, after controlling for the effect of nest of rearing, nest of origin explains bacterial strains of nestlings, it would suggest that symbiotic *Enterococci* colonized them before crossfostering, either from their mothers, or after hatching from nest materials or other nest characteristics that all siblings had in common. Otherwise, if we found a significant influence of nest of rearing it would imply the possibility of bacterial acquisition later along the nesting phase by horizontal transmission of the symbiotic *Enterococcus*, from those bacteria living in the nest, the foster siblings, or even from the foster mother. Finally, a significant effect of both nest of origin and nest of rearing would indicate an important role of the environment where nestlings develop because it would imply that crossfostered nestlings may acquire the bacteria from different environment if available. Some hoopoe nestlings that hatched in the lab, and that were reared by great tits (i.e., they were never in contact with adults hoopoes) were used to study bacterial associated to their UGS and, thus, to discuss results from crossfostering experiments.

MATERIAL AND METHODS

Field work and bacterial sampling from the UGS

The study was performed during the 2003, 2005 and 2006 breeding seasons in the Hoya de Guadix (SE Spain), where there are many nest boxes frequently used by Hoopoes, that allow us to follow their whole reproduction.

The UG is a holocrine secretory gland of birds (Jacob & Ziswiler 1982) located dorsally in the integument of birds, and is regulated by adrenal steroids (Asnani & Ramachandran 1993; Montalti *et al.* 2005). The UG produces a secretion with a variety of hydrocarbons, fatty acids and esters (Jacob 1978), which may play a role in waterproofing, production of pheromones, prevention of growing of skin pathogenic microorganisms, preservation of physical structure of the plumage, and excretion of pollutants (Montalti *et al.* 2005).

The UGS of nestling hoopoes were sampled at the age of ringing (19-23 days), and was extracted with a micro-pipet directly from the UG of nestlings. To avoid contamination, feathers and the skin around the UG was washed with alcohol (96%), which were air dried before sampling. Samples of UGS were introduced in a sterile eppendorf tube (1.5 ml.) and stored at 4ºC until processed in the laboratory. In 2006, we carried out a cross-fostering experiment where two experimental nestlings with 3-4 days old (with no secretion and the UG starting development) were exchanged with other two nestlings of the same age from another nest. We performed the crossfostering experiments in 23 nests, however after natural deaths, predation events, or samples that failed in the lab, we obtained samples at age of ringing only in 17 nests. We isolated 88 colonies from 17 crossfostered nestlings, and 99 colonies from 26 nestlings that were not moved from their original nest. From nests that were not involved in crossfostering experiment we obtained data for 170 colonies, belonging to 39 nestlings in 22 nests.

Additionally to the cross-fostering experiment, in 2006, we collected 7 eggs from 3 different hoopoe nests that were incubated in the lab, thereby preventing that hatchling became in contact with their mother. Soon after hatching (within the three first hours of life), they were introduced in great tits (*Parus major*) nests where they were fed by tits until fledging. We observed that introduction of hoopoe nestlings on those nests did not affect development and survival of great tit nestlings.

Laboratory analyses

Just few hours after sampling, UG secretion was diluted in sterilize distillate water (usually 1:5, i.e., 1 part of secretion plus 5 parts of water), and 5µl. of the dilution was spread onto two agar plates: one with the general culture medium Trypticase Soy Broth (TSA, Scharlau, Barcelona, Spain), to detect all aerobic bacteria, and other with a specific medium for *Enterococcus* bacteria, Kenner Faecal (KF, Scharlau, Barcelona, Spain) that were incubated aerobically at 37º C for 24 (TSA plates) or 72h (KF plates).

After growing, five randomly colonies from KF were isolated from each sample, transferred to a Brain Heart Infusion (BHI, Scharlau, Barcelona, Spain) liquid medium, and

incubated for 24h at 37º C. Then, 1 ml. of the culture was centrifuged, and cells were kept at -20ºC until processed for DNA extraction. Bacterial genomic DNA was extracted by using the AquaPure Genomic DNA isolation kit, as described by the manufacturer (Bio-Rad, Spain). To type the isolates from each sample, the method of Randomly Amplified Polymorphic DNA (RAPD) was used (Williams *et al*. 1990). DNA amplification was made in a Biorad Gene Cycler (Bio-Rad) using the primer of arbitrary nucleotide sequence **M13** 5´-GAGGGTGGCGGTTCT-3´ at a concentration of 1 µM in the reaction mixture. Amplified DNA restriction fragments were separated electrophoretically and visualized on 1.2% agarose gels, containing 1 ug/ml ethidium bromide, and using the 1-Kb pair ladder (Biotools, Madrid, Spain) as the molecular weight standard. Amplification reactions were as follows: One cycle of 94 ºC for 1 minute, one cycle of 20 ºC during 20 seconds, and one more at 72 ºC during 80 seconds. Those three cycles were repeated 35 times, and were followed by one cycle of 5 minutes at 75 ºC. The RAPD-PCR technique is used as a rapid and reliable method for intra and interspecific differentiation of bacterial RAPD groups. This has been found to be an efficient method of typing large numbers of isolated colonies to species and strain levels (e.g. Fitzsimons *et al.* 2001). Although there is some variability in the fingerprints obtained by RAPD technique, good reproducibility can be achieved under controlled lab conditions (Rossetti & Giraffa 2005). Our molecular analyses were all performed under identical lab conditions and, to check for variation due to different RAPD gels, we repeated 34 randomly chosen samples.

Statistical analyses

Resultant fingerprinting patterns were analysed with the Fingerprinting II Informatix Software 2000 (Bio-Rad, Hercules, CA). Similarity between pairs of strains was calculated by using the Pearson product moment correlation coefficient, with clustering by the Unweighted Pair Group Method with arithmetic mean (UPGMA) algorithm. Analyses were performed separately for experimental (samples belonging to individuals implicated in cross-fostering experiments, i.e. sampled in donors or receiving nests) and non-experimental nestlings. For comparisons of strains detected in different samples, repeat RAPD and cluster analysis of a number of colonies

indicated that more than 80% of similarity was indicative of the isolates being members of the same strain, thus this was the limit to divide the groups used to estimate binary matrices (1 if the two compared samples belong to the same strain with more than 80% of similarity, and 0 if not), which was considered as dependent variable in our analyses. This way of choosing groups of strains have been broadly used, and also shown that is correlated with strains or species identity (e. g. Rossetti & Giraffa 2005, Morandi 2006, Psoni et al. 2006). Additionally, we also built binary matrices for individuals and nests. Further, to analyse the effects of our cross-fostering experiments in the identity of strains detected in the UGS of hoopoe nestlings, we prepared binary matrices for individual identity, and identities of the nest of origin and that of nests of rearing. In all those binary matrices, the value 1 referred to the same and 0 to different identities (i.e. if a pair of strains belong to the same nest of origin, nest of rearing, and same individual, the assigned value in all three matrices would be 1). Multiple autocorrelation analyses were performed with the resultant matrices by using the Mantel test, as implemented in FSTAT software (Goudet 1995). These analyses allow the study of the relationships between the matrices and provide partial autocorrelation coefficients and associated p-values to each of the independent variables, which by definition are controlled by the effects of other independent variables in the analyses (for a similar approach see Lucas & Heeb 2005). Statistical significances were estimated by Monte Carlo procedure after 10.000 permutations.

RESULTS

In non-experimental nestlings, i.e., those nestlings living in nests that were not involved in cross-fostering experiments, we obtained 32 RAPD groups in total, and found that isolates belonging to the same individual commonly grouped as the same strain (i.e. RAPD group) (Mantel test, individual: partial $R = 0.08$, $p < 0.0001$) .In 34.28% of nestlings with more than 1 colony isolated, there was only one strain (frequencies shown in Table 1).

		Nº RAPD groups per nestling					
		1	**2**	**3**	**4**	**5**	**Total**
No-CF nest		12	13	9	1	0	35
CF nest	**CF nestling**	5	8	1	2	0	16
	No-CF nestling	10	4	4	1	1	20
	Total	15	12	5	3	1	36
Great-tit nests		4	2	1	0	0	7

Table 1. *Frequencies of RAPD groups number in nestlings belonging to nests with cross-fostering events (CF nest) or without (No-CF nest), and in nestlings from crossfostered nest which have been changed (CF nestling) or not (No-CF neslting).*

Moreover, when using data from nests with more than one nestling sampled (102 strains from 22 nestlings in 7 nests), nest identity significantly explained variation in RAPD group identities (Mantel test, $R = 0.38$, $p < 0.0001$). This result did not change after correcting by the individual identity (Mantel test, individual: partial $R = 0.43$, $p < 0.0001$; nest: partial $R = 0.2$, $p < 0.0001$) and thus nestlings within the same nest, share bacterial strains.

When analysing samples of nestlings that were reared in nests implicated in the cross-fostering experiment, we obtained 56 RAPD groups, and results demonstrated significant effects of individual identity, nest of rearing, and nest of origin explaining variation in the aggregation of isolates in such RAPD groups (Mantel test, individual: partial $R = 0.33$, $p < 0.0001$; origin nest: partial $R = 0.06$, $p = 0.0004$; rearing nest: partial $R = 0.03$, $p = 0.0002$). To check if the results were influenced by an effect of the experiment itself, we also analysed if there were significant differences between experimental and control nestlings (i. e., exchanged or not among crossfostered nests), however, experimental treatment received by each nestling, did not explain the variation in strains detected in hoopoe nestlings (Mantel test, experimental versus control nestlings: partial $R = -0.004$, $p = 0.38$).

In nestlings that were reared in nests involved in cross-fostering experiments, 41.66% have only one RAPD group (see all the frequencies in Table 1). Therefore, we did not detect larger number of bacterial strains in nests where nestlings from different nests were reared together. Further, exchanged nestlings shared strains either with biological or foster siblings. In 9 moved nestlings for which we know the strains of their biological siblings, we found that 25.5% ($N = 51$) of the isolated colonies were of the same RAPD groups that the colonies isolated from their biological siblings. In 11 nestlings for which we knew their strains and those of their

foster sibling, we found that 36.84% (N = 57) of the colonies were in the RAPD groups also detected in their foster siblings, while 49.15% (N = 49) of the colonies appeared within RAPD groups detected in biological sibling.

Finally, for the 7 nestlings that were never in contact with hoopoes (i.e., reared in 4 great tits nests), we found that the UGS was developed with the same aspect and odour than in those nestlings in hoopoe nests. Interestingly, all of them presented *Enterococcus* living in their UG, thus their UG was also colonized by bacteria in spite of not having contact with hoopoe nests. In these nestlings there was also a significant effect of individual identity (N = 33 strains, Mantel test, $R = 0.8$, $p < 0.0001$).

DISCUSSION

After analysing bacterial diversity in colonies isolated from the UGS of hoopoe nestling, we found that strains ascribed for colonies isolated from the same individual, but also from individuals within the same nest, were usually very similar (i.e. belonged to the same RAPD group), indicating in general a high relatedness among symbionts at both individual and nest level. Most nestlings have only 1 or 2 strains, even in cases of crossfostered individuals that, theoretically, have been in contact with a higher number of strains (i.e., have lived in two different environments). In parasitic relationships, it is known that infections of highly related bacterial strains may be beneficial for hosts since it would reduce competition between symbionts that otherwise may increase negative effect in hosts (Frank 1996). In mutualistic associations, a higher within-host relatedness of symbionts would lead to a better alignment of interests between symbionts and hosts (Herre 1999). The reduced variability in the mutualistic bacteria detected here could result from hoopoes acquiring a single or few bacterial strains in their UG, but also from selection processes within individual hosts after colonization by a varied pool.

To point out the importance of common environment during development explaining the very low variation in the UGS microbiota of siblings, we performed crossfostering experiments. We found that the environment has a strong influence in the composition of

bacterial communities in the UG of nestlings since, after controlling for the effect of nest of origin, nest of rearing had a significant effect in the similarity of colonies. In other words, the results show that crossfostered hoopoe nestlings share bacterial strains with their foster siblings more frequently than with the rest of nestlings in our population. The detected influence of the environment therefore suggests that *Enterococci* bacteria living in foster nests may colonize the UG of crossfostered nestlings, and therefore that in general, hoopoe glands may acquire their symbiotic bacteria from the environment during development. The influence of environment was also revealed in the cloaca of nestlings of two tit species sharing nests and parents (Lucas & Heeb 2005), being thus the environment an important force in shaping bacterial communities hosted by nestling birds. This would suggest that horizontal transmission is an important factor explaining the acquisition of symbiotic bacteria by hoopoe nestlings.

However, the ability of hoopoe glands to harvest symbiotic bacteria from the environment, in a natural situation could result in vertical transmission instead. Incubating or brooding hoopoe females also have *Enteroccoci* in their UGS, secretion that is used for preening but also for besmearing to the eggs (Soler et al unpublished). Thus, bacteria strains from females are available inside the nest and on egg surface when nestling hatch and develop, and therefore have a great probability of reaching nestling glands. If symbionts are beneficial to hosts, the host that harbours the symbionts in its body and transfer them to its offspring would be favoured by natural selection (Genkai-Kato & Yamamura 1999), and therefore some life history variables of breeding hoopoes such as egg besmearing may have been selected for a better transmission of symbionts from females to nestling glands. Our results that nest of origin significantly affected the similarity of colonies in crossfostered nestlings may be an indication of effective vertical transmission from mother to offspring. However, given that females are not the only source of bacteria in nests, but symbionts may have been acquired from nest material before the crossfostering, we cannot rule out the possibility that such similarity could be explained by horizontal transmission. In such case, all biological siblings would have acquired the same strains from bacteria living in the original nest before egg laying. A comparison of female and nestling strains, and an experiment designed to distinguish whether symbionts are

acquired from the nest material or from other hoopoes is needed to determine whether vertical transmission is a common mode of acquisition of hoopoe UG symbionts, and therefore whether a specialization between the two components of the mutualism may be expected. This seems to have been the case in bacteria living in the uropygial gland of green woodhoopoes, where a novel species of enteroccocus apparently specialized in this environment has been found (Law-Brown and Meyers 2003). In this bird, communal roosting of families in holes and maintenance of gland properties, both along the whole year, ensure permanent intimate contact between host and symbiont, and predict association of lineages between them. In the case of the european hoopoe, however, glands host symbionts only during the nesting phase each year. Prebreeding females therefore may not have symbionts in their glands and should acquire them from the environment annually. In this scenario, horizontal transmission and a main role of previously used nests as possible sources of beneficial bacteria may be predicted.

Given the proved influence of the environment in the composition of bacterial communities in hoopoe UG (see above), we would expect to find higher diversities in nestlings having lived in more environments (i.e. crosfosterred nestlings). On the contrary, there were not differences in the frequency with which crossfostered and control nestlings harboured more than one strain (Table 1). It is possible that interactions at a gland level controlled by the bird will not allow the establishment of every strain they become in contact with, as is shown to occur at a gastrointestinal level to impede pathogens adhesions (e. g. Hooper et al. 1998). Alternatively, competition ability among strains may determine which persists after multiple colonization.

We have also shown that nestlings that were never in contact with hoopoes harboured *Enterococcus* symbionts in their UG. Therefore, although we cannot rule out the possibility that the bacteria pass over the eggshell and infect the embryo before egg removal from hoopoes nests, this results open the possibility that *Enterococci*, which otherwise are quite abundant in nature (e. g. Chen et al. (2005) isolated them from different type of soils, Marcinek et al. (1998) from water, and Müller et al. (2001) from plants), may colonize UG of nestlings independently of whether nestlings became in contact with hoopoes related environments. Colonization may

for instance being favoured by specific changes in the uropygial gland of nestling hoopoes that favours colonization of the gland by this and not others bacteria.

There are in nature many examples of mutualistic relationships where microorganisms are acquired only from the environment, such as mycorrhizal symbionts, or cases in with both mode of acquisition (vertical and horizontal) coexist, as in bivalves (Sipe, Wilbur, & Cary 2000), or in some *Gammaproteobacteria* from Aphids (Russell *et al.* 2005)). In hoopoes, because of the significant effect of nest of rearing in crossfosterings, we can assure that they can acquire their UG bacteria from the nest environment. However, we found also indications of an early acquisition of symbionts by crossfostered nestlings at the nest of origin, when they still did not develop the uropygial gland, which could be interpreted as evidences of vertical transmission, although alternative explanations cannot be completely disregarded. Therefore, further investigations are necessary to clarify the role of hoopoe females in the acquisition of symbiotic bacteria by their offspring.

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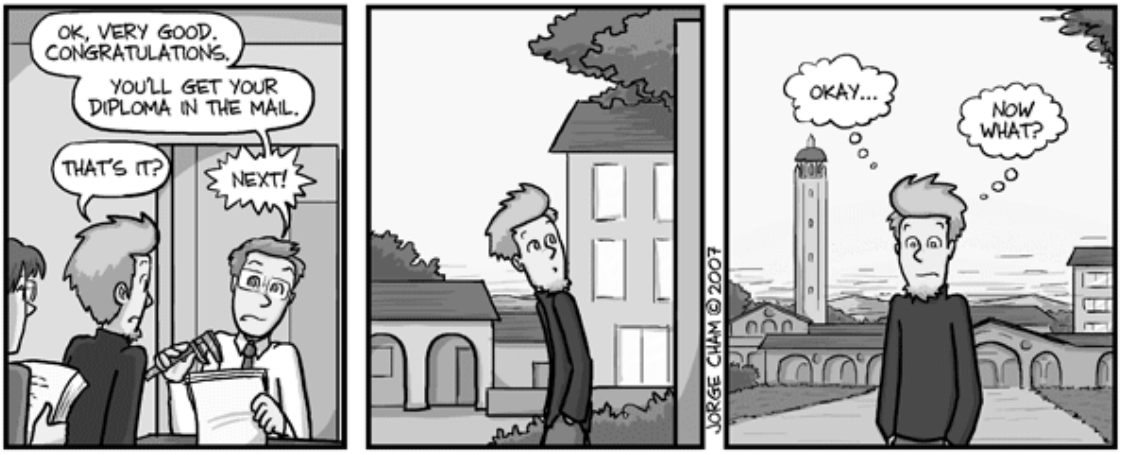

THIS MARKS THE END OF THE THIRD CHAPTER IN THE PHD SAGA..!