Las bacterias como agentes modeladores de las estrategias vitales en aves

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Estación Experimental de Zonas Áridas

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Las bacterias como agentes moderadores de las estrategias vitales en aves

Memoria presentada por D. Juan Manuel Peralta Sánchez para optar al Grado de Doctor en Ciencias Biológicas por la Universidad de Granada.

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A mi madre y a mi abuela,

a todas las mujeres que como ellas

han sido luchadoras anónimas de la vida,

aunque nunca han recibido

el merecido reconocimiento

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RESUMEN

Los efectos de los diferentes tipos de bacterias en la reproducción y evolución de las aves han sido escasamente investigados. La mayor parte de nuestro conocimiento proviene principalmente de la avicultura, donde las repercusiones económicas y de salud pública son muy importantes. En condiciones naturales, existen algunos indicios de efectos negativos de las bacterias sobre el embrión, como así lo demuestran algunos experimentos que al alterar la carga bacteriana de la superficie de los huevos, afectan a la eclosión de estos. Por ello, cualquier rasgo que disminuya la carga bacteriana podría verse favorecido por selección natural y fijado rápidamente en la población. En este contexto, algunos caracteres de estrategia vital relacionados con el comportamiento y la reproducción de este grupo de animales se han considerado funcionales, reduciendo la probabilidad de infección de huevos o pollos por bacterias oportunistas. El tipo de nido, el uso de plumas y plantas para forrar el nido o el comienzo de la incubación (con la puesta completa o no) podrían al menos parcialmente influir en la carga bacteriana de los nidos de las aves silvestres, y por tanto en la carga bacteriana de sus huevos. En esta tesis doctoral exploramos la asociación interespecífica entre la variación en la carga bacteriana de la superficie de las cáscaras de los huevos entre 24 especies de aves en la comarca de Guadix y las estrategias que estas especies siguen en sus nidos. En concreto, al principio y durante la incubación, estimamos la carga bacteriana y su crecimiento para cuatro tipos de bacterias: bacterias mesófilas totales (como una estima de la carga bacteriana total), y de tres tipos de microorganismos con conocida capacidad para penetrar el interior de los huevos: *Enterococcus*, *Staphylococcus* y *Enterobacteriaceae*. Una vez controlado el análisis por las diferencias significativas encontradas entre los diferentes años de estudio, encontramos, que las variaciones en la carga bacteriana de la superficie de los huevos y su crecimiento fueron menores dentro de la especie que entre especies, validando así el uso de la carga bacteriana y su crecimiento en análisis comparativos entre especies. El efecto significativo del año sugiere también una influencia ambiental en la carga y crecimiento de las bacterias en la superficie de los huevos. La variación interespecífica fue explicada por el tipo de nido, el

material usado para forrarlos (plantas o plumas), y el comportamiento incubador, aunque la importancia de cada uno dependió del año de muestreo y del tipo bacteriano en cuestión.

Una vez controlada estadísticamente la variación interespecífica, la variación intraespecífica en carga de bacterias mesófilas totales y el crecimiento relativo de enterobacterias explicaron significativamente la variación en el éxito de eclosión, apareciendo relacionados negativamente. Sin embargo, el crecimiento de *Enterococcus* a lo largo de la incubación estuvo positivamente relacionado con el éxito de eclosión. A nivel interespecífico, la carga bacteriana o su crecimiento no explicaron las diferencias en eclosión entre especies. Estos resultados sugieren que, aunque a nivel intraespecífico aquellos individuos con una mayor carga bacteriana sufren una mayor probabilidad de fallos de eclosión, cada especie podría estar adaptada a soportar la carga bacteriana típica de sus nidos.

Dado el papel que juega el material del nido en el ambiente bacteriano, exploramos en la golondrina común (*Hirundo rustica*) la relación entre: la carga bacteriana de los huevos, las plumas utilizadas para forrar los nidos y el éxito de eclosión. Existen evidencias que sugieren que el color de las plumas podría influir en la comunidad bacteriana de las mismas, y por ello hemos explorado experimentalmente la importancia de la elección del color de las plumas en el ambiente bacteriano del nido y su influencia en el éxito de eclosión. Encontramos que las golondrinas eligieron fundamentalmente plumas blancas para forrar sus nidos y que, además, los nidos experimentales con más plumas blancas al principio de la incubación experimentaron una menor probabilidad de que ocurrieran fallos en la eclosión. Además, el número de plumas estuvo negativamente relacionado con la carga bacteriana de la superficie de los huevos. Estos resultados sugieren que la preferencia de las golondrinas por plumas blancas para forrar sus nidos puede estar relacionada con un descenso en la carga bacteriana de la superficie de los huevos y un aumento en el éxito de eclosión.

Los visitantes del nido tales como los parásitos pueden actuar como vectores de bacterias, afectar las condiciones higiénicas de los nidos y, por tanto, influir en el ambiente bacteriano de estos. Nosotros comprobamos este escenario hipotético en el sistema de parasitismo de cría críalo (*Clamator glandarius*) –

urraca (*Pica pica*). El críalo visita los nidos de su hospedador, la urraca, y frecuentemente daña o rompe alguno de los huevos del hospedador durante la puesta o visitas posteriores, lo cual podría favorecer la colonización de bacterias y su posterior crecimiento. De acuerdo con esta hipótesis, encontramos que la carga bacteriana de la superficie de los huevos de urraca fue mayor en nidos parasitados. Debido a que encontramos evidencias apoyando la asunción de que la carga bacteriana de los huevos de urraca está relacionada con la probabilidad de infección del embrión, estos resultados sugieren que el parasitismo de cría incrementa la probabilidad de infección bacteriana de los huevos de urraca. Además, comparaciones entre la carga bacteriana de huevos de urraca y críalo revelaron que i) la cáscara de los huevos de críalo adquirieron menor densidad bacteriana que los de su hospedador al urraca, y que ii) la prevalencia de bacterias en huevos no eclosionados fue mayor para la urraca que para los críalos. Estas diferencias se pueden explicar porque los huevos parásitos, pero no los del hospedador, siempre experimentan el ambiente bacteriano de nidos parasitados. Por tanto, nuestros resultados sugieren que los huevos parásitos estarían mejor adaptados a ambientes con alto riesgo de contaminación bacteriana que los de su hospedador la urraca.

Nuestro estudio de la comunidad bacteriana en huevos de aves está limitado a técnicas dependientes de cultivo lo que implica algunas limitaciones a la hora de caracterizar las comunidades, ya que sólo una parte de las bacterias son cultivables. El uso de técnicas moleculares tiene grandes ventajas en este aspecto, pero se necesitan protocolos de laboratorio que dependen de las características del material muestreado. La identificación molecular de las bacterias de nuestras muestras de la superficie de huevos de aves silvestres necesita de protocolos apropiados para muestras con escasa carga bacteriana o con propiedades que dificultan ésta. Por ello, en esta tesis hemos puesto a punto protocolos de muestreo, extracción y purificación de DNA para este tipo de muestras. Presentamos una mejora del método de extracción de DNA mediante *Chelex100* para muestras bacterianas obtenidas de la superficie de los huevos. Con las modificaciones propuestas el éxito de amplificación ha pasado de un 20% a un 100% de los métodos tradicionales o kits comerciales frente a casi el 100% que se

obtuvieron con este nuevo método. El rendimiento de este método abre nuevas vías como método independiente de cultivo para el estudio de la comunidad bacteriana y su efecto en la reproducción de las aves.

En la presente tesis presentamos resultados indicadores de la influencia de los ambientes bacterianos sobre la reproducción de las aves, y sobre la evolución de estrategias vitales que de alguna forma permiten controlarlos y así reducir la probabilidad de contaminación bacteriana de los embriones. Serán necesarias nuevas investigaciones que permitan establecer relaciones causa-efecto en las relaciones que hemos encontrado, lo que sin duda abrirá nuevas y fascinantes cuestiones en biología evolutiva.

ABSTRACT

The effects of different types of bacteria in reproduction and evolution of birds have been scarcely investigated in natural conditions and most of our knowledge comes from poultry where economic and public health implications are very important. Under natural conditions, there are some evidences suggesting negative effects of bacteria on egg hatchability (i.e. embryo infection) since experiments that affected eggshell bacterial loads also affected hatching success. Thus, any trait that reduces bacterial load will be favoured by natural selection and rapidly fixed in the population. In this context, some reproductive and behavioural features of bird species have been interpreted as minimizing the probability of egg and offspring infection by opportunistic pathogens. Nest site selection (i.e. nest type), the use of feathers and plants as nest lining material, or the onset of the incubation (before or after clutch completion) may at least partially influence the bacterial environment of nests of wild bird nests and, thus, the bacterial load on their eggshells. We explore the interspecific associations between average values describing eggshell bacterial community of 24 species of birds sampled in Guadix County (Spain) and their life history traits in a comparative approach. In particular, we estimated density at the beginning of the incubation and its growth of four groups of bacteria on the eggshell: total mesophilic bacteria (as an estimated total bacterial load), and three specific bacterial types that are able to penetrate the eggshells: *Enterococcus*, *Staphylococcus* and *Enterobacteriaceae*. We found that inter-specific variation in density and relative growth of bacteria on the eggshells is much higher than the intra-specific variation, even after controlling for a significant effect of environmental conditions (i.e., year effect), which allow us the use of bacterial loads and growths in comparative analyses. In addition, the study year explained variation in bacterial variables suggesting an environmental influence. Nest type, the use of feathers or plants as lining material and incubation behaviour (i.e. start incubation before or after clutch completion) explained a significant proportion of interspecific variation in density and growth of different bacteria types on the eggshells. The importance of these life history traits, however, depended of the sampling year and the considered bacterial type.

After statistically controlling for the interspecific variation, total mesophilic bacteria and relative growth of *Enterococcus* and *Enterobacteriaceae* explained significantly proportion of variance of hatching success. Nests that harboured higher load and growth of bacteria experienced lower probability of hatching success. However, *Enterococcus* load was positively related with hatching success. At the interspecific level, bacterial load or its growth did not explained differences in hatching success between species. These results suggest that although individuals with higher bacterial load experience higher probability of hatching failures at intraspecific level, each species could be adapted to the typical bacterial burden of their nests.

Given the apparently role that nest material play determining nest bacterial environment, we explore the relationship between eggshell bacterial loads, number of feathers used as lining material and hatching success in the barn swallow (*Hirundo rustica*). Since feather pigmentation are known to differ in their bacterial community, we explored the importance of selecting pigmented or unpigmented feather determining nest bacterial environment and hatching success of barn swallows. We found that swallows preferentially selected white feathers for lining their nests and experimental nests with more white feathers added at the beginning of the incubation had a lower probability of hatching failure. Furthermore, the number of nest lining feathers predicted eggshell bacterial load, mainly for nests with the highest number of white feathers. These results suggest that the preferential choice of white feathers by barn swallows for lining their nests may be adaptive because of its negative association with eggshell bacterial load and probability of hatching failures.

On the other hand, nest visitors such as parasites could act as vectors of bacteria and/or affect hygienic conditions of nests and, therefore, influence nest bacterial environment. We tested this hypothetical scenario in the great spotted cuckoo (*Clamator glandarius*) – magpie (*Pica pica*) system of brood parasitism. Great spotted cuckoos visit the nests of their magpie hosts and, frequently, damage some of the host eggs during the eggs laying or in subsequent visits, which may favour bacterial colonization and growth. In accordance with this hypothesis, we found that bacterial load of magpie eggshells was greater in

parasitized nests. Because we found evidence supporting the assumption that bacterial load of magpie eggshells is related to probability of embryo infection, these results suggest that brood parasitism increases the probability of bacterial infection of magpie eggs. Moreover, comparisons of bacterial loads of cuckoo and magpie eggs revealed that (i) cuckoo eggshells harboured lower bacterial density than those of their magpie hosts in the same nests and (ii) prevalence of bacteria inside unhatched eggs was higher for magpies than for great spotted cuckoos. These interspecific differences were predicted because brood parasitic eggs, but not host eggs always experience the bacterial environments of parasitized nests and, therefore, our results suggest that parasitic eggs are better adapted to environments with high risk of bacterial contamination than those of their magpie host.

Our study of the bacterial community of avian eggshells is limited to culture-dependent techniques, which imply some limitations to characterize communities. The use of the molecular identification of bacteria has great advantages but need of specific protocols that depend of characteristic of material sampled. The molecular analyses of our samples from eggshells imply the use of protocols appropriated for samples with scarce genetic material or difficult to extract. We implemented protocols for sampling, extraction and amplification of DNA from swabs used for cleaning the complete egg surface. Our modification the method of extraction of DNA by *Chelex100* resulted in a considerable increase of success in DNA extraction (from 20% to 100%). The new protocol would surely allow new studies of the bacterial community and their effects on bird reproduction.

In this thesis, we present results pointing out the influence of bacterial environments on breeding birds and the evolution of life history traits that control and reduce bacterial contamination of the embryos. Further researches on the detected relationships are necessary to distinguish between causes and effects, which will certainly open new and fascinating issues in evolutionary biology.

INTRODUCCIÓN GENERAL

Las aves son un grupo de organismos bastante bien estudiados que ocupan la mayoría de los ambientes existentes en el planeta. Tanto científicos como aficionados a la ornitología han recolectado información sobre la biología de este grupo, generando una inmensa cantidad de datos y de bibliografía (Bennett & Owens 2002). Todo esto ha hecho de las aves uno de los grupos más apropiados de organismos para testar y comprobar diferentes hipótesis y teorías en ecología, comportamiento y evolución. Así como para determinar el origen evolutivo de numerosos caracteres, las bases ecológicas de la diversidad en sus historias evolutivas y los procesos de extinción y los patrones de riqueza de especies (Bennett & Owens 2002). Además, su filogenia está especialmente bien estudiada comparada con otros grupos, lo que facilita la utilización de metodologías apropiadas en estudios comparativos. Por ello, se han utilizado como modelo para explorar la evolución de rasgos de estrategias vitales (Stark & Ricklefs 1998; Deeming 2002b) dentro de los cuatro marcos conceptuales que modelan la variación de estos rasgos (Stearns 1992): demografía, genética cuantitativa, trade-offs, y efectos filogenéticos.

La influencia del ambiente en la eficacia de los rasgos de estrategias vitales es un aspecto fundamental para comprender su evolución (Stearns 1992). En este sentido, las relaciones con parásitos y patógenos son claves para entender la evolución de estos rasgos con sus hospedadores (Clayton & Moore 1997; Møller 1997). Las bacterias en particular son simbiontes de animales, estableciendo relaciones ecológicas con ellos muy diversas (Nuttall 1997; Madigan et al. 2004). Sin embargo, lo que se conoce de las relaciones entre bacterias y aves proceden de trabajos en avicultura, principalmente relacionado con el riesgo de contagio e infección (Board & Fuller 1994). La porosimetría del huevo (Tyler 1953; Board & Scott 1980), la mineralización del mismo (Nys 2005), sus defensas (Tranter & Board 1982; Board et al. 1982), la infección por *Salmonella* y otras bacterias patógenas (Humphrey 1994; De Reu et al. 2006), o la mejora de los sistemas de producción (Baker & Bruce 1994; Nys 2005), han sido ampliamente estudiados, principalmente por su repercusión económica y en la

salud pública. Sin embargo, trabajos que estudien la relación entre bacterias y aves en la naturaleza son muy escasos en la literatura científica.

La diversidad bacteriana es probablemente la más rica en la naturaleza (Rossello-Mora & Amann 2001). Lo que conocemos de la influencia de bacterias sobre aves silvestres (Moreno et al. 2003; Moller et al. 2010) y de corral (Bruce & Drysdale 1991) sugieren un papel primordial como agente selectivo actuando sobre el fitness de las aves silvestres, especialmente durante su reproducción. Las bacterias que pueden provocar enfermedades en aves son filogenéticamente muy diversas y pertenecen a diferentes grupos bacterianos (Nuttall 1997) transmitiéndose por distintas rutas. Las aves están expuestas en todo momento a la adquisición de bacterias, tanto del ambiente como de contactos con individuos de la misma o de distinta especie (Lombardo et al. 1999; 2008). En este sentido, el nido de las aves es un ambiente propicio para el crecimiento bacteriano debido, sobre todo, a la actividad fisiológica de adultos y pollos que afectarían a las condiciones higiénicas del nido y, por tanto, a la colonización y crecimiento bacteriano en huevos, adultos y pollos. Es por ello que algunas estrategias vitales en aves se han asociado con una mejora del saneamiento del nido, y consecuentemente, con una disminución del riesgo de infección. Probablemente esta sea una de las principales causas que hace que la construcción de nuevos nidos para cada evento reproductor sea tan extendida en aves (Singleton & Harper 1998). La construcción de nidos abiertos o el uso de agujeros para la reproducción, puede también implicar distintos costes asociados con el riesgo de infección de los huevos (Møller & Erritzøe 1996). Por ejemplo, se sabe que los huevos de gallina artificialmente expuestos en nidos abiertos adquieren una mayor carga bacteriana, sufren una mayor probabilidad de infección trans-cáscara y un menor éxito de eclosión que aquellos dejados en cajas nidos (Godard et al. 2007).

El uso de distintos materiales a la hora de forrar el nido es un comportamiento que también ha sido relacionado con el saneamiento y control bacteriano del nido. El aporte continuo de plantas frescas al nido durante la incubación e incluso durante la estancia de los pollos en el nido, parece especialmente importante, ya que muchas de estas plantas tienen propiedades antibacterianas (Clark & Mason 1985) y, en algunos casos, su aporte está

relacionado con el éxito reproductor (Mennerat et al. 2009a, 2009b). Se sabe, por ejemplo que las plantas verdes utilizadas por los estorninos pintos (*Sturnus vulgaris*) en la construcción y mantenimiento de sus nidos poseen propiedades antibacterianas contra conocidos patógenos de aves: *Escherichia coli*, *Streptococcus aurealis*, *Staphylococcus epidermis* y *Pseudomonas aeruginosa* (Nuttall 1997). Las plumas, otro tipo de material frecuentemente utilizado por las aves para forrar sus nidos (Cramp 1998; Hansell 2000), gracias a sus propiedades aislantes aumentarían la eficiencia de la incubación (Møller 1984; Hilton et al. 2004) y supondrían efectos beneficiosos en el crecimiento de los pollos y en la reducción de la carga de parásitos como se ha puesto de manifiesto en algunas especies (Winkler 1993). Además, las plumas que las aves utilizan en sus nidos portan microorganismos, en la mayoría de los casos bacterias y hongos degradadores de queratina (Pugh & Evans 1970; Onifade et al. 1998; Gunderson 2008), que pueden infectar a adultos que están incubando, a los huevos, o incluso a los pollos. Por ello, el aporte de plumas podría implicar efectos negativos mediados por bacterias. Sin embargo, algunas de estas bacterias degradadoras de plumas son productoras de sustancias que impiden el crecimiento de otras bacterias e incluso hongos que también podrían implicar efectos positivos para las aves (ver revisión en Soler et al. 2010). De entre estas bacterias, especial atención se ha prestado a *Bacillus licheniformis*, un bacilo Gram positivo con una gran prevalencia en las plumas de las aves (Burtt & Ichida 1999; Whitaker et al. 2005) y productor de sustancias antagónicas contra un gran número de microorganismos (Katz & Demain 1977; Galvez et al. 1994; Lebbadi et al. 1994). Esta bacteria crece de forma diferencial en plumas pigmentadas y no pigmentadas (Goldstein et al. 2004; Grande et al. 2004; Gunderson et al. 2008), por lo que el uso de plumas de un color u otro para forrar el nido podría tener distintos efectos sobre el ambiente bacteriano del nido.

Otro aspecto que podría afectar al ambiente bacteriano del nido son las secreciones glandulares de los adultos, ya que distintos componentes químicos de la secreción uropigial tienen actividad antimicrobiana (Shawkey et al. 2003; Martin-Vivaldi et al. 2010). Un caso extremo es el de la secreción uropigial de la abubilla (*Upupa eppops*), la cual cambia de aspecto y de composición química

durante la época reproductiva (Martin-Vivaldi et al. 2009, 2010). Estos cambios están asociados a bacterias simbiontes que viven en su glándula uropigial, jugando un papel fundamental en las propiedades antibacterianas de la secreción (Soler et al. 2008; Martin-Vivaldi et al. 2010).

Además de estos rasgos de estrategia vital asociados al saneamiento y limpieza del nido y a una posible reducción de los riesgos de infección bacteriana de los huevos, también se ha comprobado una influencia de las condiciones ambientales en la comunidad bacteriana de los nidos. Se sabe, por ejemplo, que los huevos más expuestos a las condiciones ambientales incrementan rápidamente la densidad de de microorganismos en su superficie (Godard et al. 2007). La alta carga bacteriana implica una mayor probabilidad de paso trans-cáscara y posterior infección microbiana del embrión (Cook et al. 2003), por lo que cualquier comportamiento (o rasgo de estrategia vital) que disminuya los efectos de las condiciones ambientales externas al nido en la carga bacteriana de los huevos, supondrá una ventaja y será fijado rápidamente en la población. Uno de estos comportamientos es el comienzo de la incubación de los huevos antes de que la puesta de huevos termine. Comenzar la incubación antes de terminar la puesta produce una asincronía en la eclosión de los huevos y, aunque son muchas las hipótesis funcionales que se han propuesto para explicar este comportamiento (Beissinger & Stoleson 1997), recientemente se ha demostrado que afecta al ambiente bacteriano del nido. En concreto se ha detectado experimentalmente un descenso en la carga microbiana en la superficie de la cáscara asociado al comienzo de la incubación (Cook et al. 2003; 2005a; 2005b). La cáscara de los huevos constituye una serie de barreras físicas y químicas frente al paso de bacterias hasta el embrión (Bruce & Drysdale 1994). La probabilidad de que una bacteria penetre a través de esta cáscara al interior del huevo aumenta con la presencia de agua en el medio, que proporcionaría las condiciones idóneas para el crecimiento bacteriano. El agua actuaría como vehículo de bacterias a través de los poros de la cáscara del huevo (Board et al. 1979; Bruce & Drysdale 1994). Estos poros se encuentran por lo general protegidos una cutícula de naturaleza proteica y lipídica, con propiedad hidrófoba (Jacob et al. 1979) y que en algunos casos se llega a introducir en su interior (Board & Board 1967; Becking 1975). En

condiciones de alta humedad, los hongos pueden proliferar en la superficie de los huevos y su crecimiento puede suponer la degradación y rotura de la cutícula, favoreciendo la penetración de bacterias a través de estos poros. También bacterias fermentadoras Gram negativas, presentes en las cáscaras, pueden digerir la cutícula y facilitar la infección eliminando las propiedades impermeables de la cáscara y aumentando enormemente el número de poros accesibles (Board & Fuller 1974). La incubación podría proteger a los huevos de esta humedad, al prevenir el crecimiento bacteriano incontrolado en la cáscara de los huevos y reducir el riesgo de infección (Cook et al. 2003; 2005a), ya que se ha comprobado que la carga y la presencia de hongos en la superficie de la cáscara están íntimamente relacionadas con la probabilidad de traspaso de bacterias a través de la cáscara (Board & Tranter 1986; Cook et al. 2005a).

Las bacterias que consiguen penetrar al interior de los huevos pertenecen a diferentes grupos bacterianos (Pinowski et al. 1994; Houston et al. 1997; revisadas en Stewart & Rambo 2000), por lo general saprofíticos y oportunistas (Cook et al. 2005a) presentes de forma natural en el nido (Singleton & Harper 1998; Goodenough & Stallwood 2010). Una vez que las bacterias han penetrado en el interior de los huevos a través de la cáscara, se encuentra con una batería de defensas físicas (membranas) y químicas frente a bacterias (Wellman-Labadie et al. 2008). El albumen es la principal fuente de defensas químicas antimicrobianas del huevo, jugando un papel fundamental antes de la incubación y durante sus etapas iniciales (Deeming 2002a). Varias de sus proteínas son potentes inhibidores del crecimiento bacteriano, como defensinas, catelicidinas, tranferrinas o lisozimas (Wellman-Labadie et al. 2007). Pero además, diferentes viscosidades y valores de pH inhiben el crecimiento microbiano (Brady et al. 2003). Por ello, es probable que la mayoría de las bacterias encontradas en el interior de los huevos hayan llegado tras la muerte del embrión o hayan entrado en huevos infértiles tras la descomposición de las sustancias defensivas (Houston et al. 1997; Stewart & Rambo 2000). Por todo esto, aunque la infección bacteriana se cree que es la principal causa de muerte de embriones en aves (Pinowski et al. 1994; Stewart & Rambo 2000), no ha sido posible saber hasta ahora si la muerte

del embrión es debida a las bacterias, o estas, tras la muerte del embrión, infectan al huevo.

En este contexto teórico y teniendo en cuenta el papel que el ambiente bacteriano debe jugar en el éxito reproductor de las aves y la urgencia de ponerlo de manifiesto, se encuadran los objetivos de esta tesis que se describen a continuación.

OBJETIVOS

En el apartado anterior hemos puesto de manifiesto el interés del estudio de la microbiota bacteriana en la superficie de los huevos de aves silvestres como variable que se aproxima a la probabilidad de paso de bacterias a través de la cáscara, y por tanto a la de infección del embrión. Las bacterias son organismos ubicuos potencialmente patógenos para los embriones y a los que las aves tienen que hacer frente siguiendo distintas estrategias.

En esta tesis nos hemos planteado el estudio interespecífico de la carga bacteriana que alberga la superficie de los huevos de diferentes especies **(Capítulo 1 y 2)**. En función de esto, las aves, por ejemplo, pueden seleccionar distintos lugares para hacer el nido (abiertos o en agujeros), distintos materiales para forrarlos, como son plantas y plumas o decidir el inicio de la incubación. Estas estrategias o comportamientos, podrían afectar las condiciones ambientales del nido, junto a la probabilidad de colonización y crecimiento bacteriano en el mismo. Estas estrategias o comportamientos, además, tendrían un marcado carácter específico y, por tanto, nidos de diferentes especies podrían albergar distintas comunidades bacterianas en sus huevos. En el **capítulo 1** estudiamos las diferencias interespecíficas en la comunidad bacteriana de las cáscaras de los huevos y su asociación con las estrategias que las distintas especies siguen en sus nidos. La importancia de estas asociaciones entre ambientes bacterianos y estrategias vitales de las aves se basa en la asunción de que la comunidad bacteriana asociada a las cáscaras de los huevos estaría relacionada con la probabilidad de infección del embrión y, por tanto, con el éxito de eclosión. Esta asunción la comprobaremos en el **capítulo 2**.

Estudios de huevos no eclosionados o del efecto de las bacterias sobre la eclosión de azotador de ojos perlados (*Margarops fuscatus*), confirman que las bacterias traspasan las defensas de los huevos y llegan a su interior, infectando al embrión (Pinowski et al. 1994; Houston et al. 1997; Cook et al. 2005a; Cook et al. 2005b). Si la carga bacteriana varía entre especies **(Capítulo 1)** es de esperar que estas diferencias puedan desembocar en diferencias en el éxito de eclosión que se

aprecia tanto a nivel intraespecífico **(Capítulo 2 y 5)** como interespecífico **(Capítulo 2)**. ¿Afectan las bacterias a la eclosión? ¿Estarán adaptadas las aves a la carga bacteriana que soportan sus huevos? ¿Habrá costes para aquellos individuos que superen esta carga?

Las plumas son un material ampliamente utilizado por las aves para forrar sus nidos (Hansell 2000). Como ya explicamos en la introducción, su uso se relaciona con sus propiedades aislantes que facilitaría la incubación, aunque también podría influir en el ambiente bacteriano del nido. Por un lado, en las plumas pueden existir microorganismos potencialmente patógenos para los embriones. Por otro lado, los microorganismos presentes en las plumas podrían invadir las cáscaras de los huevos e impedir la colonización de las mismas por otras bacterias patógenas. Algunos de los microorganismos degradadores de plumas son productores de sustancias antagónicas que podrían ayudar a controlar la flora bacteriana del nido, y por tanto, de la superficie de los huevos. Las plumas de distinto color también difieren en la carga bacteriana que presentan (Goldstein et al. 2004; Grande et al. 2004; Gunderson et al. 2008), y por tanto, el efecto sobre el ambiente bacteriano del nido podría depender del color de las plumas utilizadas. Este escenario lo exploramos experimentalmente en la golondrina común que es un ave que usa plumas para forrar sus nidos (Cramp 1998) **(Capítulos 3 y 4)**. El experimento consistió en alterar la composición de plumas de cada color presentes en los nidos. Esperamos encontrar un efecto en la carga bacteriana de los huevos según el color de las plumas experimentales **(Capítulo 3)** que pudiera provocar diferencias en el éxito de eclosión **(Capítulo 4)**.

El ambiente bacteriano del nido también podría depender de la tasa de visitas al nido de agentes (i.e. vectores) que favorezcan la colonización y crecimiento bacteriano de la cáscara de los huevos. El sistema que forman el parásito de cría, el críalo *Clamator glandarius* y su hospedador, la urraca *Pica pica* es un modelo idóneo para comprobar esta posibilidad por varias razones **(Capítulo 5)**. Los críalos visitan frecuentemente los nidos de urraca y, durante estas visitas, además de poner sus huevos, rompen algunos huevos de urraca. En el **Capítulo 5** comprobamos la hipotética asociación entre parasitismo y el ambiente bacteriano de la cáscara de los huevos en nidos de urraca. Estas visitas

pueden implicar un aumento de la carga bacteriana de la cáscara de los huevos al quedar los huevos de ambas especies manchados con clara y yema de huevos rotos. Además, si el parasitismo afecta al ambiente bacteriano del nido, los huevos del parásito siempre van a experimentar este ambiente, mientras que los huevos de urraca solo lo van a encontrar en algunos casos. Por tanto, los críalos se ven sometidos a una mayor presión selectiva mediada por bacterias, y es de esperar que sus huevos estén mejor preparados que los de urraca para evitar la contaminación bacteriana de sus embriones. En este escenario, el parasitismo de cría supondría un coste extra para la urraca. En el **Capítulo 5** exploramos esta posibilidad.

Para la comprobación de las hipótesis anteriores hemos utilizado estimas de bacterias cultivables. Se cree que el número de especies bacterianas cultivables está por debajo del 1% (Amann et al. 1995) y, por tanto, estimas de comunidades independientes de cultivo son esenciales para profundizar en las relaciones ecológicas y evolutivas. Existen técnicas moleculares apropiadas para el estudio de la comunidad bacteriana. Sin embargo, la escasa cantidad de material genético obtenido de la superficie de las cáscaras de los huevos y la presencia de sustancias que inhiben la Taq polimerasa, platean dificultades metodológicas en la extracción y amplificación por PCR del ADN que en el **capítulo 6** nos propusimos solventar, intentado diseñar protocolos eficientes en la extracción de DNA de microorganismos asociados a la superficie de huevos, y que permitan la realización de estudios independientes de cultivo, para establecer los efectos sobre la eclosión de los huevos en las aves.

MATERIAL Y MÉTODOS GENERALES

En este apartado se describen los aspectos más generales de la metodología utilizada, centrándose principalmente en el área de estudio y los protocolos de muestreo de bacterias en condiciones naturales y de análisis en el laboratorio. En cuanto a los métodos estadísticos, describo someramente la aproximación comparativa que utilizamos. Una descripción más detallada de los métodos se puede encontrar en los capítulos-artículos que componen esta tesis.

A. Área de estudio y especies muestreadas

El trabajo de campo se realizó durante las primaveras de 2006, 2007 y 2008 en dos zonas geográficamente distantes: la Hoya de Guadix (España) y la región de Kraghede (Dinamarca). La Hoya de Guadix (37º18’N, 3º11’W) es una altiplanicie alrededor de los 1000m sobre el nivel del mar y dominada por un clima semiárido. La vegetación típica de la zona consiste en campos de cultivo de cereal (principalmente cebada *Hordeum vulgare*), plantaciones de almendros (*Prunus amygdalus*), algunas dehesas de encina (*Quercus ilex subsp. ballota*) y restos de la vegetación mediterránea autóctona. La zona presenta en menor escala bosques de ribera mediterráneos, plantaciones de álamos (*Populus canaescens*) en las riberas y poblaciones rurales. En la zona hay colocadas unas 600 cajas nido de corcho (40x20x20 cm) diseñadas originalmente para el uso de la abubilla, pero numerosas especies usan las cajas habitualmente para criar (para más detalles ver Martín-Vivaldi et al., 2006). Para localizar nidos de especies que no usan cajas en la Hoya de Guadix, se prospectó intensamente el área de estudio. Durante la primavera de 2006 y 2007 se visitaron los nidos un par de veces por semana para determinar la fecha y tamaño de puesta. Tras la finalización de la puesta, los nidos se volvieron a visitar poco antes de la eclosión diariamente para determinar fecha de eclosión y su éxito (**Capítulos 1 y 2**). En el caso de los nidos de urraca (**Capítulo 5**), los nidos se visitaron con mayor frecuencia durante los primeros días de incubación para localizar y determinar el parasitismo de cría por parte del críalo. En total, se muestrearon 754 nidos pertenecientes a 38 especies (Tabla I.1 para diferencias de muestreados entre años).

Tabla I.1- Número de nidos muestreados por especie en las temporadas de cría de 2006 y 2007.

Especie	Tipo de nido	N (2006)	N (2007)	Total de nidos
Asio otus	abierto	1	4	5
Athene noctua	agujero	9	13	22
Burhinus oecdinemus	abierto	1		1
Carduelis carduelis	abierto	3	1	4
Carduelis chloris	abierto	4	1	5
Columba livia	abierto	26	16	42
Columba palumbus	abierto	5	6	11
Coracias garrulus	agujero	13	12	25
Corvus corone	abierto	27	1	37
Corvus monedula	agujero	8	8	16
Cyanistes caeruleus	agujero	4		4
Emberiza cirlus	abierto		1	1
Falco tinnunculus	abierto	16	11	27
Fringilla coelebs	abierto	1	1	2
Galerida cristata	abierto	2	3	5
Garrulus glandarius	abierto		1	1
Hirundo rustica	abierto	22	15	37
Lanius senator	abierto	3	6	9
Melanocorypha calandra	abierto		1	1
Oenanthe leucura	agujero	11	8	19
Oenanthe oenanthe	agujero	1		1
Otus scops	agujero	15	16	31
Parus ater	agujero	1		1
Parus major	agujero	16	17	33
Passer domesticus	agujero	3	7	10
Passer montanus	agujero	26	13	39
Petronia petronia	agujero	4	6	10
Pica pica	abierto	54	67	121
Picus viridis	agujero	1	1	2

Una vez localizado un nido, se visitó cada 2-3 días para determinar fecha y tamaño de puesta. Teniendo en cuenta el periodo de incubación medio de cada especie (tomado de Harrison 1975), se calculó la fecha aproximada de puesta, y por tanto, la siguiente visita al nido se realizó 2-3 días antes de la eclosión para la toma de la segunda muestra de bacterias (ver más adelante). Tras la visita antes de la eclosión, el nido se visitaba diariamente para determinar con precisión la fecha de eclosión y número de huevos eclosionados. El éxito de eclosión se determinó como porcentaje de huevos eclosionados. La información sobre rasgos de estrategia vital de cada especie, tales como tipo de nido (abierto o en agujero), el uso de plantas o plumas y el inicio de la incubación (determinando que la eclosión sea sincrónica o asincrónica) fue recopilada de Snow et al. (1998).

La segunda área de estudio se localizó en la región de Kraghede (57º12'N; 10º00'E), en las proximidades de la localidad de Brönsderslev. El hábitat de la zona es el típico del Centro-Norte europeo: amplias llanuras altamente manejadas por el hombre, dominadas por campos de cultivo, principalmente hortalizas, colza (*Brassica napus*) y trigo (*Triticum spp.*), y explotaciones ganaderas, y salpicadas de rodales de árboles caducifolios o plantaciones de coníferas, dando un aspecto al paisaje de mosaico. Las granjas de vacas son muy comunes en el área y muy utilizadas por la golondrina común para criar dentro de sus instalaciones. En un primer experimento realizado en 1982 para estudiar la preferencia de las golondrinas por las plumas de un determinado color para forrar sus nidos, el Doctor Anders Pape Møller, ofreció a las golondrinas 250 plumas blancas y 250 plumas coloreadas, todas ellas marcadas. Diez días más tarde se contaron el número de plumas marcadas de cada color presentes en 26 nidos de golondrinas de la misma granja. Para tener en cuenta la variación natural de la composición en el color de las plumas usadas por esta especie para forrar sus nidos se contaron las plumas blancas y coloreadas de otros 56 nidos en granjas donde no se ofrecieron plumas a las golondrinas comunes. En un segundo experimento, prospectamos cuatro de estas granjas para localizar y manipular un total de 40 nidos **(Capítulos 3 y 4)**. Brevemente, el experimento consistió en cambiar la composición de plumas blancas y pigmentadas que forraban los nidos. Para ello, en un primer nido se retiraron todas las plumas blancas. En el siguiente nido, se añadieron estas plumas y se retiraron las pigmentadas, añadiéndolas al siguiente nido, y así sucesivamente. Así creamos dos grupos experimentales: uno en el que todos los nidos tuvieron plumas blancas; y un segundo grupo de nidos con todas las plumas pigmentadas.

B. Protocolo de muestro bacteriano de la superficie de los huevos

Los nidos se muestrearon dos veces, una al principio y otra al final de la incubación, intentando evitar en todo momento la contaminación por parte del observador. Se usaron guantes de látex previamente lavados con etanol 96º. Seguidamente con un único hisopo estéril y ligeramente humedecido en tampón fosfato estéril (pH 7.2) se limpiaron completamente la superficie de todos los

huevos de la puesta. El hisopo se guardó en un tubo de microcentrífuga de 1,5ml con 1,2 ml de tampón fosfato estéril y lo conservamos en una nevera portátil por debajo de 4º hasta su llegada al laboratorio, menos de 12 horas después. Una vez en el laboratorio las muestras se mantuvieron a 4º hasta su posterior siembra. Para corregir por el tamaño de puesta, la carga bacteriana fue referida al ancho y largo de los huevos, que los medimos con un calibre (precisión 0.01 mm) para posteriormente estimar la superficie muestreada utilizando las fórmulas:

$$S = (3.155-0.0136*L+0.0115*W)*L*W$$

(para los datos de 2006 y 2007 de Narushin (2005))

y

$$S = 3*L^{0.771}*W^{1.229}$$

(para los datos de golondrinas de 2008 de Narushin (1997))

donde S es la superficie del huevo, L el largo y W el ancho. Con este método se obtuvo una estima de la carga bacteriana de la puesta.

C. El trabajo en el laboratorio

Las muestras se sembraron en placas Petri con medio sólido para el crecimiento bacteriano. Para ello, se pipetearon y sembraron 0.1ml de muestra y de sus diluciones seriadas (hasta 10^{-6}) con espátula de Drigalsky en cada uno de los medios empleados. La determinación de bacterias mesófilas se realizó mediante la siembra en un medio general como es el agar de tripticasa de soja (TSA), que ofrece una estima de la carga microbiana total. Además se utilizaron tres medios selectivos y diferenciales: agar Kenner Fecal (KF), para el crecimiento de enterococos, agar Vogel-Johnson (VJ) para el crecimiento de estafilococos, y agar Hektoen (HK) para el crecimiento de enterobacterias. Todos los medios fueron suministrados por Scharlay Chemie, S.A., Barcelona. Los tipos bacterianos crecidos en medios selectivos tienen la capacidad de traspasar la cáscara (Board et al. 1994; Cook et al. 2003). Por tanto, pueden ser buenos indicadores del riesgo de infección del embrión. De hecho, existen evidencias de que la cantidad de bacterias mesófilas totales (TSA) está directamente relacionada con el riesgo de

paso de bacterias a través de la cáscara y la infección del embrión (Bruce & Drysdale 1994; Cook et al. 2003; Cook et al. 2005b). *Staphylococcus spp*. (VJ) y *Enterobacteriaceae* (HK) son microorganismos oportunistas y saprofíticos (Houston et al. 1997; Singleton & Harper 1998; Cook et al. 2005a) que viven en la piel, el pelo y las plumas de aves y mamíferos (Krieg & Holt 1984) y también incluye especies patógenas presentes en los huevos de las aves (Bruce & Drysdale 1994). Por último, los enterococos son bacterias Gram positivas que se han encontrado en el interior de huevos no eclosionados (Bruce & Drysdale 1994) y que podrían tener tanto efectos negativos (Franz et al. 1999) como beneficiosos (Soler et al. 2008) sobre el embrión.

Una vez sembradas, las placas fueron incubadas a 32ºC durante 72 horas. Tras la incubación estimamos el número de unidades formadoras de colonias (CFU's, de Colonies Forming Units) en aquella dilución que permitía el adecuado recuento de colonias en las placas Petri.

El tiempo transcurrido desde la toma de la muestra hasta los análisis microbiológicos no fue el mismo para todas las muestras, pero ni la fecha de muestreo, ni la permanencia en el frigorífico, explicó significativamente los recuentos bacterianos (GLM: TSA, KF o HK como variables dependientes; días almacenados y fecha de muestreo como variables independientes; todas $p > 0.1$). Consecuentemente, la variación en el tiempo de almacenaje de las muestras no se tuvo en cuenta en posteriores análisis.

D. Estadística

Además de obtener dos medidas de la carga bacteriana de los huevos, una al principio de la incubación y otra al final de ésta, se calculó el crecimiento bacteriano relativo, como la diferencia de cargas bacterianas (final – principio) dividido por la carga bacteriana al principio de la incubación.

Las variables relacionadas con la carga bacteriana a nivel de nido raramente siguieron una distribución normal, incluso después de transformar las variables. En nuestro caso, la carga bacteriana estimada para distintas especies seguía una distribución multimodal (aproximándose a normal dentro de cada especie). Por ello, en estas aproximaciones se repitieron todos los análisis con los

datos transformados a rangos para comprobar que los resultados no dependían del tipo de distribución. Para los análisis comparativos entre especies, se obtuvo la media geométrica de las cargas bacteriana por especie, y estas medidas sí se aproximaron a una distribución normal. Las frecuencias de distribución de variables reproductoras, por lo general, no difirieron de las de una distribución normal y cuando fue necesario, se transformaron hasta alcanzar la normalidad.

Los valores para cada especie de rasgos de estrategia vital en general, y de recuentos de bacterias en particular, no se pueden considerar independientes ya que las especies comparten una historia evolutiva común. El método comparativo permite establecer si los patrones de variación que se encuentran en estos rasgos son debidos a respuestas independientes de cada especie a procesos evolutivos o ecológicos, o en cambio, son producto de compartir una historia evolutiva común entre especies emparentadas. Por ello, hemos tenido en cuenta en nuestros análisis las relaciones filogenéticas entre especies de las distintas especies estudiadas considerando las relaciones filogenéticas existentes entre ellas. Información que extrajimos de los trabajos de Sibley y Ahlquist (1990) y Jonsson y Fjeldsa (2006) (Figura I.1).

El efecto de la filogenia se tuvo en cuenta en nuestros análisis utilizando modelos lineales generalizados. Utilizamos modelos PGLS (Phylogenetic Generalized Least Square) (Pagel 1997; Pagel 1999), puestos a punto en el ambiente de programación R (R Development Core Team 2010), con librerías específicas MASS (Venables & Ripley 2010), ape (Paradis et al. 2004) y mvtnorm (Genz et al.) y la función pglm3.3r (función sin publicar, R. Freckleton). La ventaja de utilizar este método es que en las regresiones permite incluir la relación filogenética en el término error. Este método permite comprobar hipótesis evolutivas mediante el cálculo de estadísticos de máxima verosimilitud (Pagel 1997). Nosotros evaluamos la importancia de la filogenia mediante el coeficiente de señal filogenética λ (lambda). Este coeficiente nos revela si la filogenia se asocia con el patrón de covarianza entre especies de un rasgo dado. Si λ toma un valor de 0, nos indicaría que la filogenia no influye en el carácter y que, por tanto, los valores de distintas especies se podrían considerar como independientes. Un valor de lambda de 1 nos indicaría que el valor del carácter tiene un fuerte

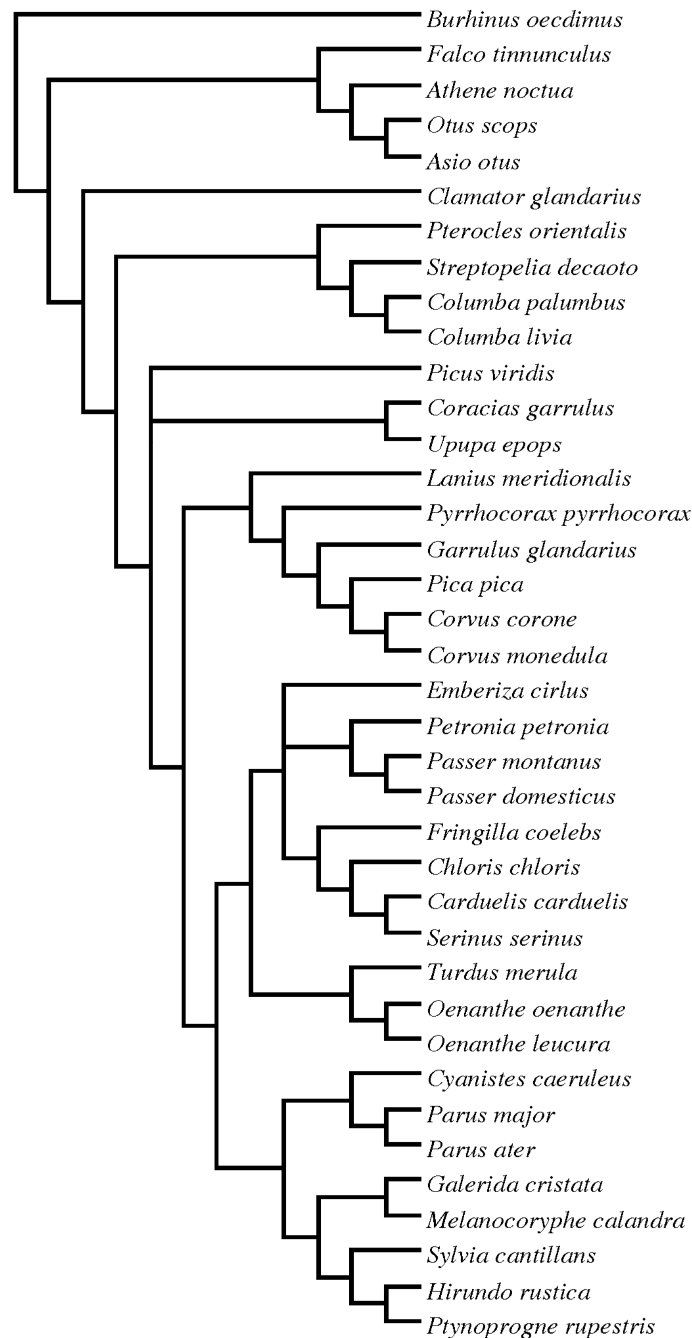

Figura I.1.- Relación filogenética de todas las especies muestreadas durante las temporada de cría de 2006 y 2007.

componente filogenético (i.e., el rasgo evoluciona en relación a la topología del árbol filogenético) y que es constante a lo largo de la historia evolutiva del grupo de especies considerado (modelo de varianza constante o Browniano). Cuando lambda varía entre 0 y 1, indica que las relaciones filogenéticas (i.e., la topología del árbol) sobrestima la covarianza del rasgo entre especies. Los análisis PGLM

con la función pglm3.3r permiten el cálculo por máxima verosimilitud del coeficiente lambda del modelo completo.

RESULTADOS GENERALES Y DISCUSIÓN INTEGRADORA

A. Variación interespecífica en la comunidad bacteriana de las cáscaras de los huevos de las aves (Capítulo 1 y 2)

Para la carga bacteriana al principio de la incubación y su variación a lo largo de este periodo (i.e. crecimiento), encontramos que las variaciones dentro de cada especie era significativamente menor que la existente entre especies; tanto para el medio general (TSA) como en los tres medios específicos (KF, VJ y HK) **(Capítulo 1)**. Este resultado avala el uso de las estimas de carga bacteriana y crecimiento a lo largo de la incubación en análisis comparativos **(Capítulo 1 y 2)**.

La carga bacteriana explicó significativamente la variación en el éxito de eclosión de los nidos una vez corregidos los análisis por la especie de ave a la que pertenecían **(Capítulo 2**, Figura I.2). Este resultado es muy interesante, y nos indica que dentro de cada especie los huevos con más bacterias experimentan una mayor probabilidad de no eclosionar. Aunque ya había evidencias anteriores de esta asociación para huevos de azotador de ojos perlados (*Margarops fuscatus*) en distintos regímenes de incubación (Cook et al. 2005b), y sobre todo, en aves de

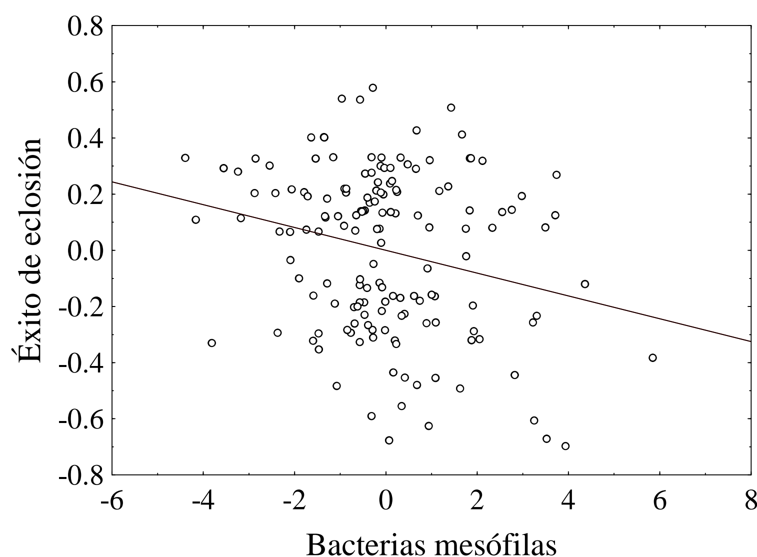

Figura I.2.- Relación entre el éxito de eclosión de los nidos muestreados y la carga bacteriana total (mesófilas totales-TSA) estimada en la cáscara de los huevos. Los valores son residuos después de controlar por los efectos de las estimas de cargas de los demás tipos bacterianos (*Enterococccus*, *Staphylococcus* y *Enterobacteriaceae*).

corral (Bruce & Drysdale 1994), nuestros resultados sugieren que el efecto es general en aves en condiciones naturales.

Cuando analizamos la variación interespecífica en carga bacteriana y éxito de eclosión (valores medios) y, además, tenemos en cuenta la relación filogenética entre las especies, la carga bacteriana no explica el éxito de eclosión **(Capítulo 2)**. Estos resultados sugieren que cargas bacterianas similares no tienen los mismos efectos en la probabilidad de eclosión de los huevos de distintas especies, lo que sugiere que distintas especies de aves varían en su eficacia para contrarrestar los efectos de una determinada carga bacteriana **(Capítulo 2 y 3)**. Estas diferencias en eficacia podrían ser consecuencia de que las aves han sufrido presiones selectivas específicas (carga bacteriana) relacionadas con el ambiente que soportan sus huevos.

En este escenario de posible variación interespecífica en la carga bacteriana en relación a comportamientos y estrategias seguidas por distintas especies en sus nidos **(Capítulo 1)**, encontramos evidencias que sugieren que el tipo de nido (Singleton & Harper 1998; Godard et al. 2007), el uso de un tipo u otro de material con posibles propiedades antibacterianas (Clark & Mason 1985), o el comportamiento de incubación (Cook et al. 2005a) modulan la carga bacteriana de los huevos (Tabla I.2). Por tanto, las presiones selectivas ejercidas por las bacterias pueden haber jugado un papel decisivo en la evolución de los rasgos de estrategia vital.

El efecto del año de muestreo explicó una parte significativa de la variación en carga bacteriana a nivel de nido al principio de la incubación, y la de algunos crecimientos a lo largo de la incubación (mesófilas totales y *Enterobacteriaceae*) **(Capítulo 1)**. Las estimas de carga bacteriana fueron significativamente mayores en 2007 que en 2006, así como los crecimientos de bacterias mesófilas totales y enterobacterias fueron mayores en 2007 que en 2006. Estos resultados sugieren una asociación entre variables climáticas que varían anualmente y la carga bacteriana de las cáscaras de los huevos de las aves y su crecimiento. De hecho, el año 2006 fue más caluroso y seco que el 2007 (AEMET 2006, 2007) y se conoce que la temperatura y la humedad juega un papel determinante en el riesgo de infección de los huevos (Bruce & Drysdale 1994;

Cook et al. 2005b). Nuestros resultados se ajustarían a los esperados ya que en el año más húmedo y frío es cuando encontramos las mayores cargas bacterianas. Sin embargo, solo tenemos datos de dos años y las inferencias que podemos hacer con esos resultados son muy limitadas y deben considerarse con cautela.

Tabla I.2. Resultados de las regresiones PGLS (Phylogenetic Generalized Least Square) testando el efecto de rasgos de estrategia vital tales como tipo de nido (abierto o en agujero), el uso o no de plantas o plumas como material para forrar los nidos y el comienzo de la incubación (con la puesta completa o incompleta) sobre el éxito de eclosión. Se muestran los valores de P y Beta y su desviación estándar, así como el coeficiente de señal filogenética (λ), y el porcentaje de varianza explicada del modelo completo (r^2) junto a su valor de probabilidad asociado (p).

			Tipo de nido	Plantas	Plumas	Incubación
TSA	2006					
	λ<0.001;	P	0.035	0.115	0.887	0.332
	r^2 = 0.150; p=0.163	Beta(SE)	-1.37(2.70)	0.98(2.70)	-0.07(2.34)	-0.50(2.29)
	2007					
	λ=0.241;	P	0.060	0.821	0.954	0.427
	r^2 = 0.104; p=0.241	Beta(SE)	-0.96(2.25)	0.09(1.88)	0.02(1.88)	-0.23(1.69)
KF	2006					
	λ<0.001;	P	0.115	0.762	0.035	0.300
	r^2 = 0.413; p=0.012	Beta(SE)	-0.81(2.25)	-0.15(2.25)	-0.96(1.92)	-0.44(1.88)
	2007					
	λ<0.001;	P	<.001	0.457	0.568	0.029
	r^2 = 0.585; p<0.001	Beta(SE)	-1.85(1.83)	0.25(1.55)	-0.19(1.50)	-0.71(1.41)
VJ	2006					
	λ<0.001;	P	0.341	0.734	0.514	0.514
	r^2 =-0.029; p=0.508	Beta(SE)	-0.88(4.08)	-0.21(2.80)	-0.40(2.70)	-0.11(2.29)
	2007					
	λ=0.214;	P	0.213	0.922	0.738	0.836
	r^2 =-0.050; p=0.582	Beta(SE)	-0.64(2.30)	-0.04(1.92)	-0.14(1.92)	-0.08(1.78)
HK	2006					
	λ<0.001;	P	0.004	0.057	0.027	0.873
	r^2 = 0.319; p=0.036	Beta(SE)	-1.76(2.43)	1.08(2.43)	-1.09(2.06)	-0.07(2.06)
	2007					
	λ<0.001;	P	0.133	0.590	0.799	0.827
	r^2 = 0.123; p=0.189	Beta(SE)	-0.76(2.25)	-0.22(1.88)	0.10(1.88)	-0.08(1.69)

B. Efectos del color de las plumas que tapizan los nidos de golondrinas comunes en el ambiente bacteriano y en el éxito de eclosión de sus huevos (Capítulo 3 y 4)

Los nidos de golondrinas comunes experimentales que después del experimento sólo quedaron con plumas blancas en sus nidos tuvieron una menor probabilidad de que algún huevo no eclosionara, sugiriendo un efecto positivo del uso de este tipo de plumas (**Capítulo 4**, Tabla I.3). Este efecto podría ir mediado por la influencia de las plumas en el ambiente bacteriano del nido. Las plumas tienen una microbiota bacteriana característica, degradadora de queratina (Pugh & Evans

1970; Onifade et al. 1998; Gunderson 2008) y productora de sustancias que impiden el crecimiento de otras bacterias e incluso hongos (Soler et al. 2010). Entre estas bacterias, *Bacillus licheniformis*, un bacilo Gram positiva, es común en las plumas (Burtt & Ichida 1999; Whitaker et al. 2005) y degrada con mayor facilidad las plumas blancas que las de color (Goldstein et al. 2004; Gunderson et al. 2008), por lo que las plumas blancas albergarían un mayor número de esta especie de bacteria que las plumas pigmentadas. Debido a los efectos beneficiosos de este bacilo, un nido con mayor número de plumas blancas podría implicar un ambiente más "limpio" frente a otros microorganismos potencialmente patógenos que pudieran colonizar y por lo tanto, crecer en la superficie de los huevos.

Tabla I.3. Efectos del número de plumas blancas y pigmentadas en nidos de golondrinas comunes que experimentaron o no fallos de eclosión. Todos los modelos incluyeron tratamiento como factor. El término "Nº de plumas exp. añadidas" se refiere al número de plumas tras el tratamiento menos las que tenía antes del mismo. El término "Nº de plumas añadidas" se refiere al número de plumas encontradas en el nido de las golondrinas en la eclosión menos el número experimental de plumas. Los resultados provienen de modelos lineales generalizados con error binomial y función de enlace logística.

	Con fallos (N = 13) *Media (SE)*	Sin fallos (N = 23) *Media (SE)*	*Estadístico Wald*	P
Modelo I				
Nº plumas en la puesta	36.46 (5.08)	28.39 (3.02)	0.21	0.646
Nº plumas exp. añadidas	-1.00 (5.68)	1.30 (2.51)	1.65	0.199
Nº plumas añadidas	13.77 (1.66)	9.78 (1.38)	3.46	0.063
Nº plumas en la eclosión	25.85 (3.20)	22.04 (2.75)	0.01	0.921
Tratamiento			1.65	0.198
Modelo II				
Nº plumas pigmentadas en la puesta	21.92 (3.65)	17.52 (2.53)	1.56	0.211
Nº plumas pigmentadas exp. añadidas	1.00 (7.83)	-2.39 (4.44)	0.18	0.671
Nº plumas añadidas	13.77 (1.66)	9.78 (1.38)	2.19	0.139
Nº plumas pigmentadas en la eclosión	10.38 (3.20)	13.96 (2.95)	1.49	0.222
Tratamiento			0.07	0.790
Modelo III				
Nº plumas blancas en la puesta	14.54 (2.23)	10.87 (1.16)	0.04	0.840
Nº plumas blancas exp. añadidas	-2.00 (2.75)	3.70 (2.44)	4.46	0.035
Nº plumas añadidas	13.77 (1.66)	9.78 (1.38)	2.13	0.145
Nº plumas blancas en la eclosión	15.46 (2.00)	8.09 (1.22)	5.60	0.018
Tratamiento			3.96	0.047
Modelo IV (mejor modelo)				
Nº plumas blancas exp. añadidas	-2.00 (2.75)	3.70 (2.44)	6.53	0.011
Nº plumas blancas en la eclosión	15.46 (2.00)	8.09 (1.22)	5.05	0.025
Tratamiento			4.11	0.043

De acuerdo con el posible efecto positivo de las plumas en el ambiente bacteriano de los nidos de golondrina encontramos que el número experimental de plumas en el nido se relacionó negativamente con la carga bacteriana de los huevos **(Capítulo 3**, Figura I.3). El efecto positivo de las plumas fue más evidente para plumas de color blanco ya que el número de plumas blancas al principio de la incubación (y por tanto antes del tratamiento) predijo la carga bacteriana de los huevos al final de la incubación **(Capítulo 3)**. Además los nidos blancos experimentales tuvieron una menor carga bacteriana en los huevos que los nidos experimentales con plumas negras **(Capítulo 3**, Tabla I.4).

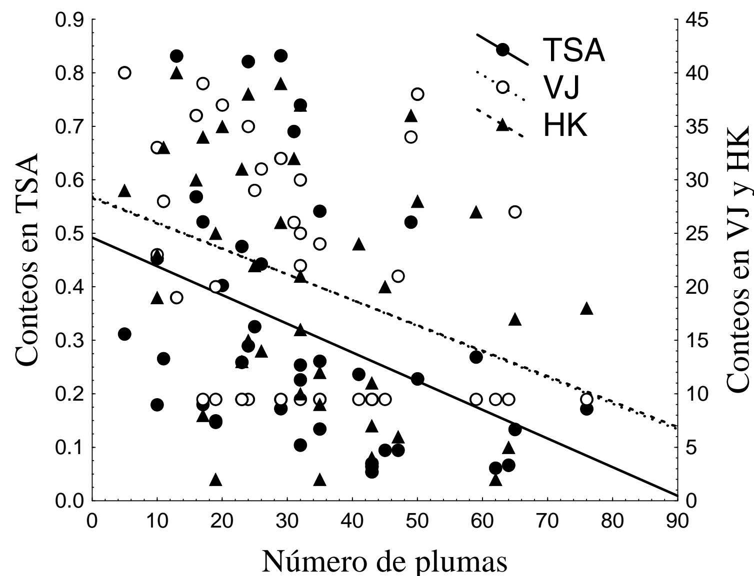

Figura I.3. Relación entre el número de plumas en los nidos de golondrina común al principio de la incubación y la densidad bacteriana de mesófilos totales transformados dos veces mediante logaritmos naturales (TSA) y la densidad en rangos de *Staphylococcus* (VJ) y *Enterobacteriaceae* (HK). Las líneas son rectas de regresión.

Se sabe que algunas especies de aves prefieren plumas de distintas características cromáticas para sus nidos, lo que se ha tratado de explicar por una posible función de señalización (e.g. en el estornino negro (*Sturnus unicolor*), Veiga & Polo (2005)). Estos resultados, sugieren además un probable efecto antibacteriano debido al tipo de plumas seleccionado en el recubrimiento de los nidos. En el experimento de selección de plumas que realizamos con golondrinas **(Capítulo 4)**, pusimos de manifiesto una predilección por las de color blanco, aquellas con mayores efectos beneficiosos sobre el ambiente bacteriano del nido.

Tabla I.4 - Efecto del tratamiento de cambio de la composición de color y del total de las plumas del nido (modelo 1) y número de plumas blancas y negras (modelo 2) en la carga de mesófilas totales, (TSA), *Staphylococcus* (VJ) y *Enterobacteriaceae* (HK) en la cáscara de los huevos, antes y después del tratamiento (i.e., al final de la incubación).

	TSA			VJ			HK		
	Beta (SE)	$F_{(1,37)}$	P	Beta (SE)	$F_{(1,37)}$	P	Beta (SE)	$F_{(1,37)}$	P
Antes del tratamiento									
Número de plumas blancas	-0.35 (0.16)	4.85	0.034	-0.39 (0.16)	6.03	0.019	-0.46 (0.16)	8.58	0.006
Número de plumas negras	-0.15 (0.16)	0.92	0.343	-0.09 (0.16)	0.35	0.558	-0.02 (0.16)	0.01	0.910
Después del treatment									
Modelo 1	Beta (SE)	$F_{(1,29)}$	P	Beta (SE)	$F_{(1,29)}$	P	Beta (SE)	$F_{(1,29)}$	P
Número de plumas	-0.34 (0.17)	4.22	0.049	-0.40 (0.18)	4.86	0.036	-0.28 (0.15)	3.46	0.07
Tratamiento	0.53 (0.17)	9.88	0.004	0.23 (0.18)	1.57	0.22	0.67 (0.15)	19.37	0.0001
Modelo 2	Beta	$F_{(1,28)}$	P	Beta	$F_{(1,28)}$	P	Beta	$F_{(1,28)}$	P
Número de plumas blancas	-0.14 (0.18)	0.58	0.453	-0.17 (0.19)	0.71	0.41	-0.10 (0.17)	0.38	0.544
Número de plumas negras	-0.38 (0.18)	4.28	0.048	-0.44 (0.20)	4.92	0.035	-0.32 (0.17)	3.56	0.069
Tratamiento	0.51 (0.17)	9.02	0.006	0.21 (0.19)	1.34	0.26	0.66 (0.16)	17.88	0.0002

Sin embargo, más experimentos en otras especies que utilicen plumas para forrar sus nidos serían necesarios para alcanzar conclusiones más robustas sobre la función antibacteriana del tipo de plumas.

C. Efectos del parasitismo en la carga bacteriana de los huevos de urraca y de crílo (Capítulo 5)

En primer lugar encontramos que los huevos de urraca en nidos parasitados al principio de la incubación adquirieron mayor carga bacteriana que aquellos que estaban en nidos que no fueron parasitados (GLM: efecto general en carga bacteriana como variable dependiente y si fue parasitado o no como factor; Wilks = 0.90; $F_{4,79}$ = 1.73; P = 0.002) (Figura I4). Segundo, detectamos que los huevos de urraca albergaron mayor carga bacteriana en sus cáscaras que los de críalo dentro del mismo nido (GLM: efecto general en carga bacteriana como variable dependiente y especie como factor; Wilks = 0.69; $F_{4,28}$ = 3.18; P = 0.028) (Figura I4). Por último, al comparar las bacterias encontradas en el interior de huevos no eclosionados, se detectó una mayor paso de bacterias en huevos de urraca. En concreto detectamos bacterias en el interior de los huevos no eclosionados, tanto de urraca como de críalo, sin embargo, ambas especies variaron en la probabilidad de infección (Fisher exact test, P = 0.007). En la mayoría de los huevos de urraca no eclosionados (12 de un total de 13; 92,3%) encontramos bacterias, mientras que la probabilidad de infección en los huevos de críalo fue mucho menor (3 de un total de 9; 33%).

En el sistema críalo-urraca, el parasitismo de nidos por parte del críalo conlleva en numerosas ocasiones roturas de huevos de la urraca. Los huevos quedan parcialmente cubiertos (tanto lo de críalo como los de urraca) con clara y albumen (Soler et al. 1997), caldo de cultivo idóneo para bacterias patógenas (Stadelman 1994). Además, el críalo visita varias veces el nido de urraca tras la puesta del huevo parásito (Soler et al. 1995) por lo que ambos comportamientos pueden ser los responsables de la alteración del ambiente bacteriano de los nidos de urraca parasitados. Aunque nuestros resultados son correlativos, la relación encontrada entre el parasitismo de cría y la carga bacteriana de los huevos de la urraca sugiere la existencia de costes para el hospedador, mediados por el efecto del parásito en el ambiente bacteriano de los nidos del hospedador.

Uno de los resultados más llamativos es que los huevos de críalo mantengan cargas bacterianas menores que los de urraca incluso al comparar aquellos que comparten el mismo ambiente bacteriano del nido (Figura I.3.) Estos

resultados deben estar relacionados con características físicas y químicas de los huevos de esta especie. Es conocido que existen diferencias en los caracteres de

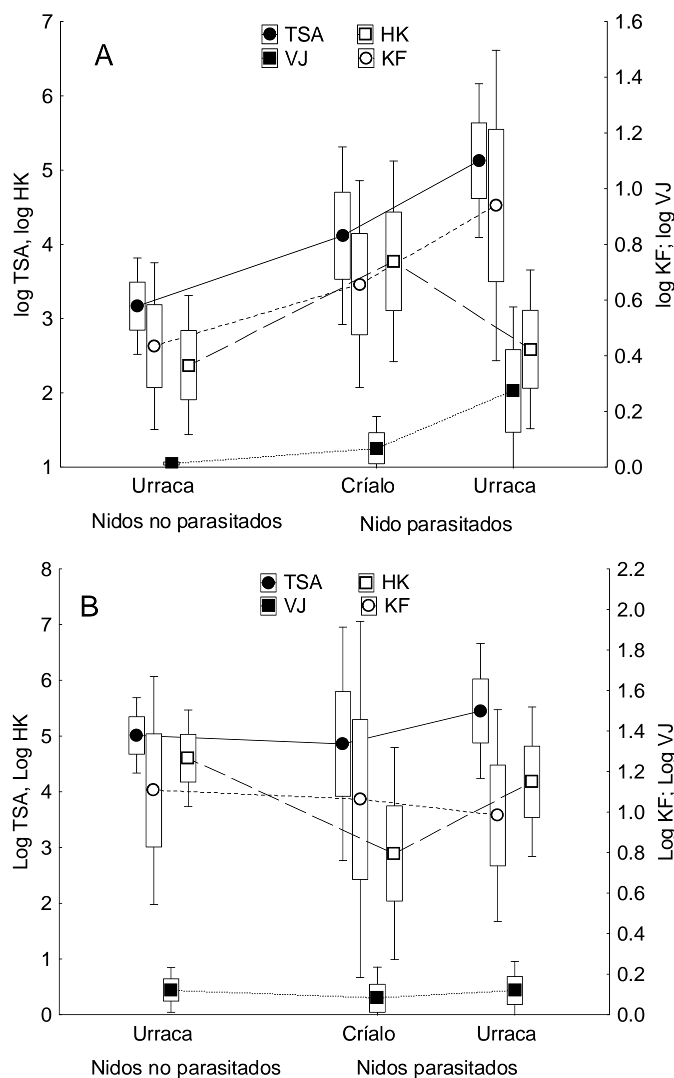

Figura I.4. Medias (± SE (barras) e intervalos de confianza (corchetes)) de las estimas de la carga bacteriana (transformadas mediante logaritmo natural) de la superficie de los huevos de críalo y urraca en nidos parasitados y no-parasitados al principio (A) y al final de la incubación (B). Las estimas fueron realizadas para un medio no selectivo (TSA) así como para medios específicos (*Enterococcus spp.* (KF), *Staphylococcus spp.* (VJ) y *Enterobacteriaceae* (HK)).

las cáscaras de huevos de diferentes especies (Becking 1975) y que algunas de estas características podrían afectar a la probabilidad de colonización y crecimiento de bacterias, y al traspaso e infección posterior del embrión. Ya que los huevos de críalo se desarrollan en nidos parasitados, estarían bajo presiones

selectivas más fuertes que los de urraca y, por tanto, también deberíamos encontrar características en sus huevos que disminuyan la probabilidad de que los huevos sufran infecciones bacterianas. Futuros estudios comparativos de las características de las cáscaras de huevos de estas especies deberían de poner de manifiesto estas adaptaciones.

D. Puesta a punto del protocolo de aislamiento y amplificación de ADN bacteriano procedentes de muestreos de cáscaras de huevos en condiciones naturales (Capítulo 6)

Las técnicas moleculares han abierto nuevas vías para el estudio de la diversidad y del efecto de los microorganismos sobre otros seres vivos. Para poder aplicar estas técnicas deben de ponerse a punto protocolos de muestreo, de aislamiento y de purificación de ADN que van a depender de las características del sustrato de muestreo. En el caso de las cáscaras de los huevos de las aves, el disponer de métodos rápidos y eficientes de extracción de ADN, permitiría la detección de un mayor tipo de bacterias presentes en la comunidad (cultivables y no cultivables), y por lo tanto permitiría, entre otros muchos, un estudio mucho mas completo y más pormenorizado entre procesos coevolutivos entre estos tipos bacterianos y especies de aves. Para los análisis moleculares de las muestras recogidas en el campo nos planteamos el uso de métodos tradicionales y kits comerciales ampliamente utilizados en la extracción del ADN. Sin embargo, en la mayoría de los casos, los rendimientos y calidad del ADN extraído fueron bajos, de forma que sólo se obtuvieron amplificaciones en un 20% del total de muestras analizadas (resultados no mostrados en esta tesis). Por ello, hemos puesto a punto un protocolo, modificando parcialmente un método de extracción de ADN existente (*Chelex100*) con el que maximizamos la probabilidad de extraer ADN bacteriano (próxima al 100%) **(Capítulo 6**, Figura I.5).

Básicamente, las modificaciones del método convencional consistieron en la adición de dos pasos para mejorar la extracción del ADN. El primero fue la adición de lisozima durante 45 minutos. En este paso, la lisozima actúa hidrolizando parcialmente la pared celular bacteriana, y en especial de las

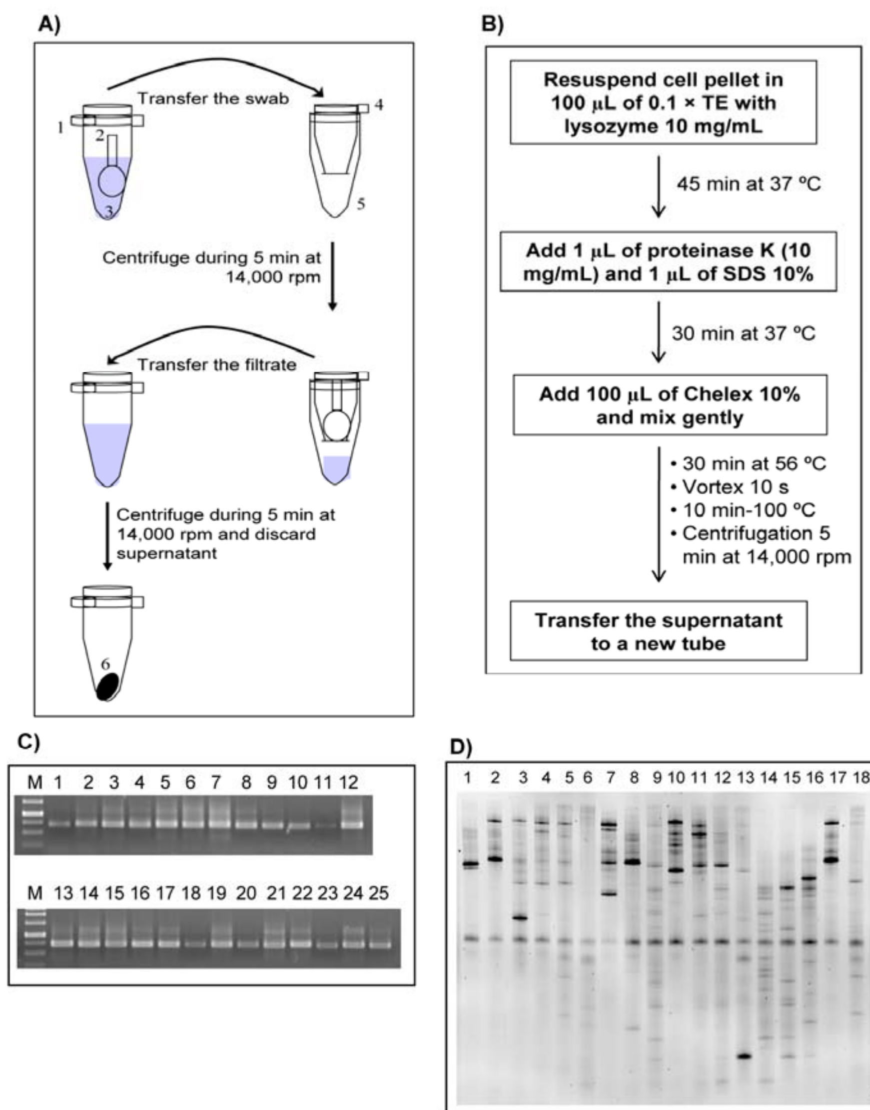

Figura I.5. Diagrama de flujo del aislamiento de ADN A) Recuperación de células de las muestras. 1, tubo de microcentrífuga de 1.5 ml; 2, hisopo (con la mayoría del palo roto); 3, tampon fosfato; 4, tubo de microcentrífuga de 0.5 ml con la tapa y el fondo cortados; 5, tubo de microcentrífuga de 1.5 ml con la tapa cortada; 6, pellet de células. B) Procedimiento de aislamiento de ADN. C) Amplificación del gen eubacteriano ARN ribosómico 16S (rRNA). M, 1 Marcador Kb (Biotools); D) Perfiles de TTGE V3 de fragmentos de ADN ribosómico 16S.

bacterias Gram positivas. La adición de proteinasa K en el segundo paso, favorece la inactivación de la lisozima así como la degradación de posibles proteasas contaminantes, que luego se eliminarán mediante una lisis térmica posterior a 100ºC. Estos dos pasos facilitan la acción del *Chelex100*, permitiendo la obtención de ADN de calidad adecuada para su amplificación por PCR.

CONCLUSIONES

1. La variación en carga bacteriana en la superficie de los huevos es mayor a nivel interespecífico que intraespecífico, lo que sugiere que características de la especie determinan al menos parcialmente el ambiente bacteriano de los nidos.
2. Rasgos de estrategia vital tales como el tipo de nido (abierto o en agujero), el uso de plumas o plantas para forrar el nido, o el comportamiento incubador, juegan un papel importante a la hora de explicar la variación interespecífica en la carga bacteriana de los huevos de distintas especies de aves.
3. La carga bacteriana de los huevos explica el éxito de eclosión a nivel intraespecífico. A nivel interespecífico esta relación no es estadísticamente significativa, lo que sugiere que las distintas especies están adaptadas al ambiente bacteriano en el que se desarrollan sus embriones, al menos parcialmente.
4. El uso de plumas para forrar los nidos por parte de las golondrinas está relacionado con el ambiente bacteriano del nido, como indica la asociación entre número de plumas en los nidos y carga bacteriana de los huevos de esta especie.
5. Las preferencias de las golondrinas comunes por plumas blancas para forrar sus nidos es posiblemente una adaptación que permite reducir la carga bacteriana de sus huevos y la probabilidad de fallos en la eclosión.
6. El parasitismo de cría por parte del críalo influye en el ambiente bacteriano de los nidos de su hospedador la urraca, implicando unos costes del parasitismo de cría hasta ahora no explorados.
7. En comparación con los huevos de urraca, los de críalo están mejor adaptados al ambiente bacteriano de los nidos parasitados como indican las comparaciones de la carga bacteriana de los huevos de ambas especies, incluso cuando comparten el mismo nido.

8. Presentamos un protocolo de muestreo, extracción y purificación de ADN bacteriano presente en la cáscara de los huevos de aves silvestres que permitirá el estudio más pormenorizado de la comunidad bacteriana y sus efectos en la reproducción de las aves.

CONCLUSIONS

1. Interspecific variation of eggshell bacterial load is greater than the intraspecific variation, suggesting that the nest bacterial environment is partially determined by species traits.
2. Life history traits such as type of nest (open or hole), use of feathers or plants as lining material, or incubation behaviour, play a important role in explaining the interspecific variation in bacterial loads of the eggshells of wild birds.
3. Eggshell bacterial loads resulted in association with hatching success at the intraspecific level. At the interspecific level, this relationship did not result statistically significant, suggesting that different species are partially adapted to the bacterial environment where their embryos develop.
4. The use of feathers by barn swallows for lining their nests is related to bacterial nest environment as showed by the detected association between number of nest lining feathers and bacterial load on their eggshells.
5. The preference of barn swallow for lining their nests with white feathers is surely an adaptation that allows them to reduce bacterial load of their eggshells and the probability of hatching failure.
6. Brood parasitism by great spotted cuckoos affects nest bacterial environment of their magpie hosts, which suggest hitherto unexplored costs of brood parasitism.
7. In comparison with magpie eggs, those of cuckoo are better adapted to the bacterial environment of a parasitized nest as indicated by the interspecific comparisons of eggshell bacterial loads, even when they shared the same nest.
8. We optimized a protocol for sampling bacteria on the eggshell of wild birds, and for extraction, and purification of bacterial DNA. This protocol will surely allow studies about the bacterial community of eggshells and their effects on reproduction of birds more detailed.

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CAPÍTULO I:

Behaviour at the Nest and Bacterial Load of Bird Eggshells: A Comparative Study

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Abstract: The relationships between bacterial community and the evolution of organisms' life history traits remains poorly investigated. We here explore interspecific variation of eggshell bacterial community (density of total mesophylic bacteria, *Enterococcus*, *Staphylococcus* and *Enterobacteriaceae*) and its association with breeding traits of 24 species of birds. First, we found that inter-specific variation in density and relative growth of bacteria on the eggshells is much higher than the intra-specific variation even after controlling for a significant effect of environmental conditions (i.e., year effect), which allow to explore interspecific associations between average values describing eggshell bacterial community and life history traits of different species in a comparative approach. Second, using Phylogenetic Generalized Least Square models (PGLS), we found that nest type, the use of feathers or plants as lining material and incubation behaviour (i.e. start incubation before or after clutch completion) explain interspecific variation in density and growth of different bacteria types on the eggshells. The effects of these traits depends of the study year, suggesting that environmental conditions affecting bacterial colonization and growth would influence the strength of the expected relationships between life history traits and nest bacterial environment. Eggshell bacterial load has been shown to predict hatching failures (i.e., probability of embryo infection) and, consequently, all these results suggest a possible role of bacterial environment at the nest in the evolution of some life history traits of bird species.

Keywords: bacterial community, breeding biology, comparative approach, hole-nesting, nest building material.

The Auk, submitted

Introduction

Selection pressures due to parasitism are important processes driving the evolution of life history traits of birds in general (Stearns 1992), and of behaviours at the nest in particular (Deeming 2002). Accordingly, some reproductive and behavioural features of bird species have been interpreted as minimizing the probability of offspring infection by opportunistic pathogens (Clark and Mason 1985; Christe et al. 1994; Merilä and Allander 1995; Singleton and Harper 1998). Empirical tests exploring the effects of microorganisms such as bacteria and viruses on the evolution of birds life history traits are however scarce (Hansell and Deeming 2002). Bacteria are ubiquitous organisms that colonize avian nests and eggshells, where conditions and availability of food and water is enough for growth (Singleton and Harper 1998). Opportunistic pathogenic bacteria might cross eggshells and membranes and provoke embryo mortality (Pinowski et al. 1994; Houston et al. 1997; Stewart and Rambo 2000). Probability of trans-shell infection increases with the abundance of microorganisms on the eggshell (Cook et al. 2003; Cook et al. 2005b; Shawkey et al. 2009), and, thus, any trait that diminishes density of opportunistic bacteria on the eggshell would rapidly become fixed in the population.

Several environmental factors have been proposed to influence eggshell bacterial load and, therefore, the probability of trans-shell embryo infection. For instance, it is known that the exposition to outside nest environment affected bacterial colonization and load on eggshells, presenting experimental eggs in open nests higher trans-shell infection rates than those in hole nests (Godard et al. 2007). Nest material may also affect bacterial environment of nests, given that different nest materials may harbour different bacterial populations, as Goodenough et al. (2010) have shown studying bacterial assemblages of nest material used by two common and related hole nesting species, the Blue Tit (*Cyanistes caeruleus*) and Great Tit (*Parus major*). Material used for lining the nest cup may indirectly influence the bacterial environment of nests throughout its effects on thermal conditions (Mertens 1977). In addition, nest material may directly affect bacterial environment of nests through the antibacterial properties.

For instance, some plants that starlings use selectively for nest building have antimicrobial properties and therefore may suppress bacterial proliferation (Clark and Mason 1985). Feathers are commonly used by birds for lining their nest (Cramp 1998), and have been recently found to be related to bacterial density on the eggshells of Barn Swallows *Hirundo rustica* (Peralta-Sanchez et al. 2010). The use of feathers as nest material might reduce bacterial infection because, for instance, they are coated with uropygial oil via preening, which has been shown to inhibit feather-degrading bacteria (Shawkey et al. 2003). Otherwise, bacterial community of nest lining feathers, which are mainly non-pathogenic bacteria for embryo but produce antimicrobial substances against pathogenic microorganisms (Soler et al. 2010), might also prevent the establishment of pathogenic bacteria on the eggshells of birds (Soler et al. 2010; Peralta-Sanchez et al. 2010). Thus, there are good reasons to hypothesize that nest building behaviour of birds might modulate and control bacterial load on eggshells, and hence, reduce probability of embryo infection.

Incubation behaviour is also known to reduce bacterial density and modulate the growth of bacteria on the eggshells of birds (Cook et al. 2003; Shawkey et al. 2009). Accordingly, it has been suggested that one of the adaptive reasons of early incubation is to reduce the time that early eggs are unprotected in the nest from bacterial colonization (Cook et al. 2003). Bird species show interspecific variation in the onset of the incubation (Cramp 1998), and, then, it is likely that this variation is related to the interspecific differences in bacteria load and growth on eggshells.

Most of these birds behaviour at the nest that may influence bacterial community on the eggshells are species-specific characteristics. At the interspecific level, if the evolution of those life history traits was related to the bacterial environment of nest locations (i.e. probability of trans-shell infection), we should expect an interspecific covariation between life history traits and eggshell bacterial load. We here explore such relationships between the life history traits (associated to behaviours at the nest) and eggshell bacterial load of 24 species of birds in two different study years. Briefly, we first test whether among species variation on bacterial density of the eggshell is larger than within

species variation (species effect), which is a requisite for performing comparative analyses. Second, we tested for year effects on estimates of bacteria load on eggshells as evidence of local environmental conditions affecting bacterial community of eggshells. Finally, we studied the predicted interspecific associations between eggshell bacterial loads and growths, and nesting habits (hole *vs.* open nesting species), the use of lining material and its type (plants or feathers) and the onset of incubation (before or after clutch completion).

Material and methods

The study was performed during the breeding seasons 2006-2007 in Hoya de Guadix (Spain: 37º18'N, 3º11'W), a high altitude plateau, 1000 m a. s. l., dominated by a semi-arid climate. Typical vegetation in the area includes cultivated crops, olive and almond plantations, sparse holm oaks remaining from the original Mediterranean forest, small shrubs in abandoned fields, and deciduous trees in temporary streams and villages. About 600 nest-boxes were present in the study area from previous years that were checked twice per week during March-June (for more details see Martin-Vivaldi et al. (2006)). Open nests of birds that did not breed in boxes were detected by intensively searching suitable habitats in the study area.

Information on nest site (open vs. hole nests), use of plants or feathers as lining material and onset of incubation (before or after clutch completion) of different species were collected from Cramp (1998) and information is included in the Appendix 1.

Bacterial sampling protocol

Bacteria on the eggshells were sampled twice; one at the beginning of the incubation period (2-3 days after clutch completion, which assured that all the eggs were incubated), and another one few days before egg hatching. Once a new nest was found, we visited it every 2-3 days, which allowed us to estimate laying date and clutch size. We obtained data from 289 nests belonged to 21 species in 2006 and 292 nests belonged to 22 species in 2007, with at least three nests

sampled. Some nests however were found later during the incubation or were predated before second sampling and thus sample size of eggshell bacterial loads and growth. From these pools of nests and species, 19 species were sampled in both years at the beginning of the incubation and 15 species were sampled in both years for bacterial growth between first and second sample. Appendix 1 summarizes collected data for the two study years. Bird and egg manipulations were performed according to Junta de Andalucía (Spanish Regional Government) permissions.

Samples were taken in the field trying to maintain sterile conditions and preventing inter-nest contamination by using new latex gloves sterilized with 96% ethanol for each nest. When gloves were dry, we gently handled the eggs, and cleaned the complete eggshell with a sterile swab slightly wet with sterile sodium phosphate buffer (0.2 M; pH = 7.2). All the eggs in the clutch were sampled with a single swab that afterwards was introduced in a microfuge tube containing 1.2 ml of sterile phosphate solution and stored in a portable refrigerator at 4-6 ºC. Once in the laboratory, samples were stored at 4 ºC until processing. Estimates of bacterial load were standardized to number of colonies grown from inoculations in growing media (see below) (CFU's, Colonies Forming Units) per cm^2 by taking into account number and surface of the sampled eggs in the nests. Eggshell surface was estimated according to the formula:

$$S = (3.155-0.0136*L+0.0115*W)*L*W$$

from Narushin (2005), where S is the surface in cm^2, L the length of the egg, and W the width of the egg. Length and width of all eggs were measured with a calliper (accuracy 0.02 mm).

Eggshell bacterial growth was calculated as the difference of bacterial density estimated at the end minus that estimated at beginning of the incubation period divided by bacterial load at the beginning of the incubation.

Laboratory work

Samples were collected from microcentrifuge tubes after vigorously

shaking them in vortex for at least three periods of 5 seconds. We performed serial dilutions until 10^{-6} times, and microorganism cultivation was performed by spreading homogenously 100 μl of each dilution of the serie, measured with an automatic pipette, in each of four Petri dishes containing four different sterile solid growth media (Scharlau Chemie S.A. Barcelona). We used Tryptic Soy Agar (TSA), a broadly used general medium to grow total mesophylic bacteria, and three selective media: Kenner Fecal Agar (KF) for growing bacteria belonging to the genus *Enterococcus*; Vogel-Johnsson Agar (VJ) for bacteria of the genus *Staphylococcus*; and Hecktoen Enteric Agar (HK) for Gram negative bacteria of the family *Enterobacteriaceae*. Dishes were incubated at 32ºC for 72 hours, and afterwards, the number of CFU's on the dish was counted. Eggshell bacterial load in aerobic heterotrophic medium (TSA) is positively related to probability of embryo infection (Bruce and Drysdale 1994; Cook et al. 2003; Cook et al. 2005b). We have also selected selective media for the most common groups of bacteria known to live on avian eggshells and known to reduce embryo viability based on extensive studies of bacteria on domestic and wild bird eggs (Board and Tranter 1986; Kozlowski et al. 1989; Bruce and Drysdale 1991; Bruce and Drysdale 1994; Houston et al. 1997; Cook et al. 2003; Cook et al. 2005a; Cook et al. 2005b; Soler et al. 2008; Shawkey et al. 2009; Peralta-Sanchez et al. 2010). *Staphylococcus* sp. and *Enterobacteriaceae* are saprophytic and opportunistic bacteria (Houston et al. 1997; Singleton and Harper 1998; Cook et al. 2005a) that live in skin, hair and feathers of mammals and birds (Krieg and Holt 1984), and they are known to be pathogenic for avian embryos (Bruce and Drysdale 1994). Enterococci, the third analysed group of bacteria, are also frequently found inside unhatched eggs (Bruce and Drysdale 1994) and, although they are opportunistic pathogens (Franz et al. 1999), they might also have beneficial effects for embryos (Soler et al. 2008). Most of these bacteria are able to penetrate eggshells (Board et al. 1994; Cook et al. 2003). Therefore, used together, these media should adequately characterize the relative load of bacterial groups living on the avian eggshell that are known to produce pathogenic infection of embryos.

Culture-based techniques do not characterize microbial community as

molecular techniques do since only around 1% of micro-organisms are cultivable (Amann et al. 1995), but culture-independent methods may also imply limitations, bias and errors (Qiu et al. 2001; Speksnijder et al. 2001; Shawkey et al. 2005). Interestingly, both methodologies have reached identical conclusions when exploring the effects of incubation on eggshell bacterial communities (Cook et al. 2005a; Shawkey et al. 2009), which validate the use of culture-based methods in our study.

Statistics

To test for the effects of species identity and the random effect of the study year, frequency distributions of bacterial colonies estimated in the different media, as well as the estimates of bacterial growth (hereafter "relative growth"), was rank-transformed.

For comparative analyses, we calculated the geometric mean of eggshell bacterial loads estimated per species at the beginning of the incubation and their relative growth. The use of geometric mean values per species in comparative analyses is justified because variation among species is much greater than variation within species in bacterial counts even after controlling for year effects (see Results and Table 1). Distribution frequencies of these geometric means did not differ from normality after $\log_{10}$-transformation and standardization of the variables. Because of the detected effect of year on the response variables, we repeated the analyses for the estimates and pool of species sampled in 2006 and in 2007 separately. Species estimates of life history traits, or of bacterial density and growth cannot be considered statistically independent data points in the analyses because, due to common ancestry, species that are phylogenetically close related are probably those with more similar life-histories (Harvey and Pagel 1991). We therefore took into account the phylogenetic relationships between species (see the phylogeny used in Appendix 2, based on Sibley & Ahlquist (1990) and Jonsson & Fjeldsa (2006)) for our comparative analyses.

To control for possible effects of common ancestry we performed phylogenetic generalized least square regression (PGLS) analyses (Pagel 1997; Pagel 1999) as implemented in the R statistical computing environment (using

"MASS", "ape" and "mvtnorm" libraries) with an additional unpublished function by R. Freckleton (University of Sheffield, pglm3.3.r available upon request). The PGLS model is a linear regression model in which phylogenetic information is incorporated to the error term and thus controlling for the shared evolutionary history among species (Harvey and Pagel 1991; Martins and Hansen 1997). This method tests evolutionary hypotheses with likelihood ratio statistics (Pagel 1997) and enabled us to estimate the importance of phylogenetic correlation signal (λ) that can vary between 0 (phylogenetic independence) and 1 (species' traits covary in direct proportion to their shared evolutionary history). Then, we conducted all the analyses by setting the degree of phylogenetic correlation to the most appropriate degree evaluated for each model. In addition, we corrected our comparative analyses for heterogeneity in data quality due to the large variation in sample sizes among species by using weights in the comparative analyses (Garamszegi and Møller 2010). Briefly, following Garamszegi & Møller (2007), we combined variance factors due to phylogenetic and weight effects as error terms in a form of a matrix using the $\boldsymbol{Q=V+cW}$ equation, where $\boldsymbol{V}$ is the phylogeny matrix; $\boldsymbol{W}$ is the diagonal matrix of 1/weights; and $\boldsymbol{c}$ is a constant (Martins and Hansen 1997). By varying $\boldsymbol{c}$ constant, we calculated the maximum likelihood of different combinations of the phylogeny and weight matrices. At the combination which resulted in the highest maximum likelihood, we determined the statistics (slope, standard error of the slope, t-statistic and p-value) of the effect in focus. This additional PGLS exercise was also performed in the R statistical computing environment, and setting the degree of phylogenetic dependence (λ) to the most appropriate degree evaluated for each unweighted model.

We estimated statistical significance (i.e., P-values) in PGLS models by using the function "summary" as implemented in R (pglm3.3.r), which performed a type III decomposition of errors and, consequently, the order of the categorical factors do not affect results. First, we explored the effects of nest building and incubation behaviour on each bacterial type at the beginning of the incubation and growth. Second, we explored general effects of life history traits over bacteria load and growth by combination of p-values (Chi-square $_{\alpha,k}$ = -2 * SUM (ln P),

where k is number of different analyses * 2) from models from each bacterial type (Sokal and Rohlf 1995).

All the analyses were two-tailed and conducted using R statistical computing environment and STATISTICA 7.1 software.

Results

Species identity explained a significant proportion of variance of eggshell bacterial density at the beginning of the incubation when using a general medium (i.e., for total mesophylic bacteria: TSA), and when bacterial counts where performed in selective media for *Enterococcus* (KF)*, Staphylococcus* (VJ) and *Enterobacteriaceae* (HK) (Table 1). We also found statistically significant evidence of interspecific differences for relative growth of total mesophylic bacteria, *Enterococcus* and *Enterobactericeae*. Consequently, eggshell bacterial density and growth were significantly more variable among than within species, which validate the use of average values for interspecific comparative analyses.

Table 1. Results of GLM's testing the effects of year (random factor) and species identity (fixed factor) on rank-transformed eggshell bacterial density estimated at the beginning of the incubation (1) and relative growth (RG) along incubation. We show univariate results for total mesophylic bacteria (TSA 1 and RG TSA), *Enterococcus*, (KF 1 and RG KF), *Staphylococcus* (VJ 1 and RG VJ) and *Enterobacteriacea* (HK 1 and RG HK). Number of sampled nests is enclosed in parentheses as subscript of the dependent variable names.

	Factor	df	F	P		Factor	df	F	P
TSA 1	Species	18	8.63	0.001	RG TSA	Species	14	2.15	0.010
(556)	Year	1	132.51	0.001	(330)	Year	1	5.33	0.022
	Species*Year	18	3.42	0.001		Species*Year	14	1.56	0.091
KF 1	Species	18	14.68	0.001	RG KF	Species	14	2.48	0.002
(556)	Year	1	61.97	0.001	(330)	Year	1	1.34	0.248
	Species*Year	18	2.21	0.003		Species*Year	14	2.48	0.002
VJ 1	Species	18	6.54	0.001	RG VJ	Species	14	1.26	0.232
(556)	Year	1	2.44	0.119	(330)	Year	1	1.96	0.162
	Species*Year	18	1.04	0.415		Species*Year	14	1.32	0.191
HK 1	Species	18	9.47	0.001	RG HK	Species	14	2.82	0.001
(556)	Year	1	9.77	0.002	(330)	Year	1	4.91	0.027
	Species*Year	18	3.74	0.001		Species*Year	14	1.22	0.263

Species identity, study year and their interactions explained significantly variation of estimated eggshell bacteria loads at the beginning of the incubation (MANOVA; TSA, KF, VJ and HK as dependant variables, Wilks > 0.43; all P < 0.001), and relative growth (MANOVA; TSA, KF, VJ and HK as dependant

variables, Wilks > 0.69; all $P < 0.001$). When looking at the univariate results, the study year explained a significant proportion of variance in eggshell bacterial density of heterotrophic, *Enterococcus,* and *Enterobacteriaceae* bacteria at the beginning of the incubation, as well as in relative growth of total mesophylic bacteria and *Enterobacteriaceae* (Table 1). In general, estimates of bacterial density and growth in 2007 were higher than that in 2006 independently of the bird species identity. Finally, the interaction between species identity and year explained significant variation in eggshell bacterial density at the beginning of the incubation of most analyzed groups of bacteria (except for *Staphylococcus* density; Table 1), as well as variance of bacterial growth for *Enterococcus*, which suggests that changes in environmental conditions differentially affects bacterial load and growth on eggshells of different bird species.

Overall, life history traits (by combination of p-values from the models for each bacterial type) significantly explained eggshells bacterial load at the beginning of the incubation ($\text{Chi-square}_{8} > 16.32$, $P < 0.038$). For bacterial growth, however, we did not find evidence of an overall association with life history traits in 2006 ($\text{Chi-square}_{0.05,8} = 3.87$, $p > 0.869$), but we found it in 2007 ($\text{Chi-square}_{0.05,8} = 18.71$, $p = 0.016$). Univariate analyses pointed out a significant interspecific covariation between nest building and incubation behaviours and eggshell bacterial load estimated for *Enterococcus* independently of the study year, and for total mesophylic bacteria and *Enterobacteriaceae* in 2006 (Table 2). Nest type resulted associated with density of total mesophylic bacteria and *Enterobacteriaceae* in 2006, while in 2007 only density of *Enterococcus* resulted associated with type of nest (Table 2). The use of plants and feathers as lining material explained variation in eggshell density of *Enterobacteriaceae* at the beginning of the incubation in 2006, while the use of feathers explained variation in density of *Enterococcus* in this study year. Incubation behaviour had a significant effect over *Enterococcus* load in 2007 (Table 2). Summarising, these results indicate that open nesting species harbour lower bacteria load at the beginning of the incubation than hole nesting species. Species that use plants or feathers as lining material experience lower bacteria density on eggshells that those that do not use them as lining material. Finally, species with the onset of

incubation before clutch completion experience higher bacteria density that those species that start the incubation once the clutch is complete.

Table 2. Results of Phylogenetic Generalized Least Square models (PGLS) testing the relationship between eggshell bacterial density at the beginning of the incubation as dependent variables and nest type (i.e., hole *vs.* non-hole nests), feathers (i.e. nest cups with *vs.* without feathers as lining material), plants (i.e. nest cups with *vs.* without plants as lining material) and incubation behaviour (i.e. onset of incubation before *vs.* after clutch completion) as independent factors. P-value and Beta and its standard error (SE) are presented from each predictor. We show in parentheses phylogenetic signal value (λ), and percentage of variance explained by the whole models (r^2) together with the associated p-values. Bold values represent significant p-values.

		Nest type	Plants	Feathers	Incubation
TSA					
2006 (λ <0.001; p(0)=1; p(1)=**0.003**; r^2 = 0.150; p=0.163)	P	**0.035**	0.115	0.887	0.332
	Beta(SE)	-1.37(2.70)	0.98(2.70)	-0.07(2.34)	-0.50(2.29)
2007 (λ=0.241; p(0)=1; p(1)=**0.006**; r^2 = 0.104; p=0.241)	P	0.060	0.821	0.954	0.427
	Beta(SE)	-0.96(2.25)	0.09(1.88)	0.02(1.88)	-0.23(1.69)
KF					
2006 (λ<0.001; p(0)=1; p(1)<**.001**; r^2 = 0.413; p=**0.012**)	P	0.115	0.762	**0.035**	0.300
	Beta(SE)	-0.81(2.25)	-0.15(2.25)	-0.96(1.92)	-0.44(1.88)
2007 (λ<0.001; p(0)=1; p(1)=**0.010**; r^2 = 0.585; p<**0.001**)	P	**<.001**	0.457	0.568	**0.029**
	Beta(SE)	-1.85(1.83)	0.25(1.55)	-0.19(1.50)	-0.71(1.41)
VJ					
2006 (λ<0.001; p(0)=1; p(1)<**.001**; r^2 =-0.029; p=0.508)	P	0.341	0.734	0.514	0.514
	Beta(SE)	-0.88(4.08)	-0.21(2.80)	-0.40(2.70)	-0.11(2.29)
2007 (λ=0.214; p(0)=0.609; p(1)<**.001**; r^2 =-0.050; p=0.582)	P	0.213	0.922	0.738	0.836
	Beta(SE)	-0.64(2.30)	-0.04(1.92)	-0.14(1.92)	-0.08(1.78)
HK					
2006 (λ<0.001; p(0)=1; p(1)<**.001**; r^2 = 0.319; p=**0.036**)	P	**0.004**	**0.057**	**0.027**	0.873
	Beta(SE)	-1.76(2.43)	1.08(2.43)	-1.09(2.06)	-0.07(2.06)
2007 (λ<0.001; p(0)=1; p(1)=**0.005**; r^2 =0.123; p=0.189)	P	0.133	0.590	0.799	0.827
	Beta(SE)	-0.76(2.25)	-0.22(1.88)	0.10(1.88)	-0.08(1.69)

With respect to the estimates of bacterial growth along the incubation period, we found that feathers as lining material resulted significantly associated to *Enterococcus* growth, while nest type and the use of plants as lining material explained variation in Staphylococcus growth (Table 3). Hole nesting species and those that use plants or feathers as lining material had lower bacterial growth rates. These associations were detected only for one of the study years (2006); the one with the lowest bacterial density (Table 2 and Table 3).

Table 3. Results of Phylogenetic Generalized Least Square models (PGLS) testing the relationship between relative growth of eggshell bacterial loads as dependent variables and nest type (i.e., hole *vs.* non-hole nests), feathers (i.e. nest cups with *vs.* without feathers as lining material), plants (i.e. nest cups with *vs.* without plants as lining material) and incubation behaviour (i.e. onset of incubation before *vs.* after clutch completion). P-value and Beta and its standard error (SE) are presented from each predictor. We also show in parentheses phylogenetic signal value (λ), and percentage of variance explained by the whole models (r^2) together with the associated p-values. Bold values represent significant p-values.

		Nest type	Plants	Feathers	Incubation
TSA					
2006 (λ<0.001; p(0)=1; p(1)=1; r^2 =-0.247; p=0.867)	P Beta(SE)	0.647 -0.53(4.34)	0.490 0.71(3.83)	0.370 -0.66(2.75)	0.788 0.27(3.83)
2007 (λ<0.001; p(0)=1; p(1)=**0.036**; r^2 =-0.067; p=0.577)	P Beta(SE)	0.428 0.56(2.76)	0.660 0.25(2.31)	0.430 0.42(2.10)	0.656 -0.28(2.52)
KF					
2006 (λ<0.001; p(0)=1; p(1)=**0.003**; r^2 = 0.025; p=0.413)	P Beta(SE)	0.155 -1.36(3.45)	0.081 1.50(2.98)	0.652 0.20(1.70)	0.227 -0.99(2.98)
2007 (λ=0.301; p(0)=0.417; p(1)=**0.003**; r^2 = 0.433; p<**0.026**)	P Beta(SE)	0.511 -0.40(2.43)	0.126 0.80(2.02)	**0.017** -1.24(1.86)	0.193 -0.73(2.19)
VJ					
2006 (λ<0.001; p(0)=1; p(1)<**.001**; r^2 =-0.078; p=0.582)	P Beta(SE)	0.561 -0.65(4.22)	0.837 0.20(3.68)	0.132 -0.88(2.09)	0.676 0.41(3.68)
2007 (λ<0.001; p(0)=1; p(1)=**0.005**; r^2 = 0.443; p=**0.024**)	P Beta(SE)	**0.008** 1.88(2.43)	**0.004** -1.75(2.06)	0.150 -0.66(1.77)	0.088 1.12(2.47)
HK					
2006 (λ=0.999; p(0)=**0.015**; p(1)=1; r^2 =-0.142; p=0.694)	P Beta(SE)	0.295 0.54(1.90)	0.623 -0.30(2.32)	0.719 -0.22(2.32)	0.547 -0.44(2.75)
2007 (λ<0.001; p(0)=1; p(1)=**0.023**; r^2 = 0.128; p=0.240)	P Beta(SE)	0.672 -0.27(2.60)	0.163 0.77(2.10)	0.900 0.06(1.94)	0.062 -1.14(2.31)

The estimates of phylogenetic signal (λ) were very low for most statistical models tested. Except for models explaining interspecific variation in total aerobic mesophyles and *Enterobacteriaceae* growth estimated in 2006, λ values differ significantly from 1 (Table 2 and Table 3). The model trying to explain variation in bacterial growth in HK media was the only one with a strong phylogenetic signal (i.e. λ differing significantly from zero; Table3).

Discussion

The main objective of this study was highlighting interspecific differences in abundance of several groups of bacteria on the eggshells of wild birds and

exploring its association with birds life history traits related to nest location, nest building and incubation behaviours. The first step was therefore pointing out interspecific differences in eggshell bacterial load and growth that allow performing the interspecific analyses. We found support for such interspecific variations even after controlling for a significant inter-year variation.

Differences in bacterial load and growth between years probably are caused by differences in environmental factors. Temperature and humidity related to the nest environment are known to affect eggshell bacterial loads (Beissinger et al. 2005), with bacteria being more abundant in wetter and cooler nests (Cook et al. 2005b). In our study area, 2007 was wet compared with the drier and hotter 2006 (AEMET 2006; AEMET 2007) and, accordingly, our estimates of eggshell bacterial density were larger in 2006.

We found support for the predicted association between species-specific life history traits and among species variation in eggshell bacterial loads and growth. At the intraspecific level there are evidences suggesting that birds, by selecting appropriate nesting places (Godard et al. 2007), deciding the onset of incubation (Cook et al. 2005a), and using nest material with thermal or antimicrobial properties (Clark and Mason 1985; Peralta-Sanchez et al. 2010), are able to reduce the probability of bacterial colonization of the eggshell. We therefore hypothesized that interspecific differences in eggshell bacterial density could be partially explained by interspecific variation in these life history traits.

Interspecific differences in estimates of bacterial loads and growths did not show considerable values of phylogenetic signals (except for HK growth, see Results), suggesting that species that are more closely related are those not experiencing more similar bacterial environment in their nests (Pagel 1999). These low values of phylogenetic signal may be interpreted as ecological conditions of nests with relatively low phylogenetic influence being the responsible of the detected interspecific variation. Moreover, the fact that the detected relationships between eggshell bacterial load and growth and life history traits varied depending of the study year (see univariate results in Table 2 and Table 3) is also in accordance with such suggestion because the inter-year variation in results could be related to inter-year variation in environmental

conditions that affect bacterial colonization and growth on the eggshell of birds. Abiotic factors such as temperature and humidity play an important role on nest environment (Beissinger et al. 2005) and affect bacterial load on eggshells (Cook et al. 2005b). The expected effects could be more easily detected in years where bacteria are intermediately abundant. We have in any case detected a global effect of life history traits on eggshell bacterial load estimated at the beginning of the incubation which supports the predicted relationship. The detected inter-year different results are then useful for highlighting the importance of estimating bacterial loads in different years due to the likely inter-annual variation in climatic conditions.

We found that eggshell bacterial load estimated for *Enterococcus* and *Enterobacteriaceae* were higher in hole nesting species that in open nesting ones. Previous experiments performed by Godard et at. (2007) with chicken eggs demonstrated that shells of eggs in open nests harboured higher density of bacteria than those in hole nests, likely because open nests were more exposed to environmental conditions. However, experimental nests used by Godard et al. (2007) were artificially sterilized, and without any previous breeding activity, and therefore were only exposed to bacteria arriving to the nest after the start of the experiment. Cleaning nest from previous breeding attempts in House Wren *Troglodytes aedon* has been proposed as a strategy to reduce harmful bacteria load on the nest (Singleton and Harper 1998). Thus, it is possible that the higher bacterial density associated with hole-nesting habits that we found indicates the influence of previous breeding activity in hole nests because of the frequent reutilization of holes, since open nests are less frequently reused (Møller and Erritzøe 1996).

The use of material for nest building was an important factor explaining loads of *Enterococcus* and *Enterobacteriaceae* density, being the latter opportunistic pathogens of embryos (Houston et al. 1997; Stewart and Rambo 2000). Nest material would protect eggs from environmental factors and from opportunistic fungi and bacteria that are common in the soil (Whitaker et al. 2005), but also antimicrobial properties of specific nest material would protect eggs from microbial infection. For instance, it is known that Blue Tits can

discriminate between plants for lining nests and use olfaction to select aromatic plants that are used by humans as house cleaners or herbal medicine (Petit et al. 2002; Mennerat et al. 2009a) and protect nestlings from bacteria (Mennerat et al. 2009a) with detected effect on phenotypic condition of nestlings (Mennerat et al. 2009b). Starlings *Sturnus unicolor* also preferentially use aromatic plants with antibacterial properties in their nests (Clark and Mason 1985). In accordance with this background, we found evidence suggesting that eggshells of species that used green plants in their nests had lower density of pathogenic *Enterobacteriaceae* than eggshells of other species.

Feathers are a common lining material in birds (Møller 1987; Cramp 1998) and our results suggest a role in modulating and reducing density and growth of *Enterococcus* and density of *Enterobacteriaceae*. We predicted such relationship because of the thermoregulatory properties of nest lining feathers that enhance incubation activity, but also because preen oils active against fungus and bacteria or antibiotic-producing bacteria might be present in the nest-lining feathers (see Introduction). The effect of feathers on eggshell bacterial load and hatching success has recently been experimentally detected in nests of Barn Swallows (Peralta-Sanchez et al. 2010; 2011). Results presented here highlight the importance of feathers as antimicrobial material of nests and causes underlying these effects should be explored further.

We sampled the eggs after clutch completion, once the incubation had already started, and we found that asynchronous-hatching species harboured higher density of *Enterococcus* than synchronous-hatching ones. This implies that eggs of species that start the incubation before clutch completion were incubated for a longer period that synchronous ones when we sampled the eggs. Experimental evidence has previously indicated that incubation effectively control eggshell bacterial loads (Cook et al. 2005a; Godard et al. 2007) and, consequently, starting incubation before clutch completion has been proposed as a strategy that reduces probability of bacterial infection of eggs (Cook et al. 2005b). Then, species that start incubation before clutch completion would be those with the highest probability of pathogenic colonization and bacterial growth rate during the incubation period (Cook et al. 2003; Cook et al. 2005a; Cook et al.

2005b) which is the contrary expectation to the results we have found. We only can speculate that asynchronous species start the incubation with a high density of bacteria and, although incubation should reduce bacterial load of their eggshells, they do not do so up to the level of eggshell bacterial loads of synchronous species. This interesting interspecific results opens new possibilities to understand the evolution of incubation strategies depending of bacterial environmental conditions associated with nest environments.

Summarising, we here highlight interspecific differences in life history traits that covary with variables describing the bacterial community of eggshells of wild birds, which is a proxy of probability of trans-shell embryo infection. We only show correlations from which causality cannot be inferred, but results stress the possibility that bacteria colonizing eggshells were important selective agents driving the evolution of some life history traits of birds. Hopefully, results presented here will encourage investigation of possible functional explanation of the detected patterns.

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CAPÍTULO II:

Nest Bacterial Environment Predict Hatching Success of Wild European Birds. A Comparative Study.

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Abstract: Bacterial environment has been considered as an important selective agent of embryo viability but up today no direct evidence of such a relationship in natural conditions is available. Trying to fill this gap we estimated density of four groups of bacteria on the eggshell and hatching success as proxies of bacterial environment and embryo viability, respectively, in 152 nests belonged to 17 European bird species. In accordance with the predicted negative relationship we found that, after controlling for inter-specific variation, density of aerobic mesophilic bacteria on the eggshell at the beginning of the incubation period predicted hatching success. Hatching success was also explained by the increase in density ("during-incubation growth") of the potential pathogenic *Enterobacteriaceae* along the incubation period. Moreover, during-incubation growth of bacteria from the genera *Enterococcus* resulted positively related to hatching success suggesting a possible beneficial role of this group of bacteria. Interspecific variation in eggshell bacterial density at the beginning of incubation and during-incubation growth did not predict interspecific variation in hatching success. These results taken together indicate that nest bacterial environment is and important selection agent acting on embryo viability, and that eggs of different species are optimized to their specific bacterial environment.

Keywords: hatching success, bacterial load, bacterial community, comparative approach, aerobic mesophiles, *Enterococcus*, *Staphylococcus, Enterobacteriaceae.*

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Introduction

Avian eggs are suitable environments for the opportunistic pathogenic growth of mesophiles bacteria, not only because of their very nutritive content, but also because temperature necessary for egg incubation is close to the optimal for bacterial exponential growth (Krieg & Holt 1984; Board & Fuller 1994; Singleton & Harper 1998). Potentially pathogenic bacteria of avian embryo are ubiquitous and has been considered as an important evolutionary force selecting for different physical, chemical and behavioral barriers that impede bacterial trans-shell colonization and growth inside the eggs (Board et al. 1994; Deeming 2002; Wellman-Labadie et al. 2007). However, as far as we know evidences of a direct link between bacterial environment of avian nests and embryo viability in natural conditions are absent in the literature. This lack of evidence could be due to the intrinsic methodological difficulties of establishing the expected relationship. In natural conditions, it is for instance not possible to detect when embryo death occurs and thus, even in the case of detecting pathogenic bacteria inside unhatched eggs, the causes of death of embryos cannot be directly established given that bacterial trans-shell infection and growth within the eggs could have occurred after embryo dead (Houston et al. 1997; Stewart & Rambo 2000; Birkhead et al. 2008). Moreover, due to the important physical and chemical barriers of eggs against bacterial infections, probability of detecting bacteria inside incubating eggs are very low (Board et al. 1994; Cook et al. 2005b) and the very large sample size necessary for studying selection pressures due to bacterial infection of embryo would be ethically inacceptable at least.

Before trans-shell colonization of eggs, bacteria should colonize and growth on the eggshell and there is evidence from poultry that eggshell bacterial load is positively related to probability of trans-shell embryo infection (Board & Fuller 1994). Accordingly, some authors have used bacterial density and community on eggshells as a proxy of probability of trans-shell embryo infection (Cook et al. 2005a; Cook et al. 2005b; Shawkey et al. 2009; Peralta-Sanchez et al. 2010). Here, we use such estimations as a proxy of bacterial environment of avian nests that should predict probability of hatching failure if pathogenic

microorganisms are the cause of embryo death.

Eggs from different species may differ in the effectiveness of physical and chemical barriers preventing successful trans-shell infection of bacteria living on the eggshells by for instance decreasing number and size of shell pores or increasing antibacterial chemicals in the albumen of eggs (see Saino et al. 2002; Shawkey et al. 2008). If that was the case, the effect of similar levels of eggshell bacterial loads on probability of embryo infection would differ for different species and, consequently, the predicted positive relationship between eggshell bacterial loads and hatching success may be not present at the interspecific level.

Here, we tested the predicted negative effects of eggshell bacterial loads on developing embryos by taking into account both among and within species variations in hatching success and eggshell bacterial load by using estimates of eggshell bacterial loads from 152 nests of 17 species. For these estimates we used four different growth media including both general and specific media for common bacteria on avian eggs and nests (e.g., *Enterococcus, Staphylococcus* and *Enterobacteriaceae*). The predicted tendencies should appear for estimates of heterotrophic bacteria, but also for estimates of density of known pathogenic bacteria.

Material and methods

Study Area

The study area was located in the Hoya de Guadix (37º18'N, 3º11'W), a high altitude plateau, 1000 m a. s. l., dominated by a semi-arid climate. Cultivated crops, olive and almond plantations, sparse holm oaks remaining from the original Mediterranean forest, small shrubs in abandoned fields, and deciduous trees in temporary streams are the dominant vegetation in the area. During the breeding season of 2007 (March-July), we checked the about 600 nest-boxes that were installed in the study area from previous years (see Martin-Vivaldi et al. 2006). In addition, we looked for open nests of birds by intensively searching suitable habitats in the study area. Once a new nest was found, we visited it every 2-3 days, allowing us to estimate laying date, clutch size and hatching success (as the

proportion of laid eggs that successfully hatched in non-depredated nests). We included data from all nests where at least one egg hatched. Bird and egg manipulations were performed according to Junta de Andalucía (Spanish Regional Government) permissions.

Bacterial sampling protocol

We sampled eggshells for bacteria twice, one at the beginning of the incubation period and another one few days before egg hatching. The first sample was taken 2-3 days after clutch completion, which assured that all sampled eggs were incubated. Briefly, trying to maintain sterile conditions and preventing inter-nests contamination, we wore latex gloves sterilized with 96% ethanol and cleaned the eggshells of the complete clutch with a sterile swab slightly wet with sterile sodium phosphate buffer (0.2 M; pH = 7.2). We introduced the swab in a microcentrifuge tube with sterile phosphate solution that was stored in a portable refrigerator at 4-6ºC. Once in the laboratory, samples were stored at 4ºC until processing. For a more detailed explanation of bacterial sampling protocol see Peralta et al. (2010).

Laboratory work

After vigorously shaking microfuge tubes in vortex we performed serial dilutions until 10^{-6} times, and performed microorganism cultivations by spreading homogenously 100 µl of each serial dilution in each of four Petri dishes containing four different sterile solid growth media (Scharlau Chemie S.A. Barcelona): Tryptic Soy Agar (TSA), a broadly used general medium to grow total aerobic mesophiles; Kenner Fecal Agar (KF) for growing bacteria belonging to the genus *Enterococcus*; Vogel-Johnsson Agar (VJ) for bacteria of the genus *Staphylococcus*; and Hecktoen Enteric Agar (HK) for Gram negative bacteria of the family *Enterobacteriaceae*. Dishes were incubated at 32ºC for 72 hours, and afterwards, the number of Colonies Forming Units (CFU) on the dish of the less diluted solution letting colony-counting was estimated. For a more detailed description of protocol of bacteria cultivation see Peralta-Sanchez et al. (2010). We estimated eggshell bacterial load (i.e. density) as the number of colonies that

grew in our four media per surface (cm^2) of eggs sampled. Eggshell surface was estimated for each of the egg sampled according to the formula in Narushin (2005):

$$S = (3.155-0.0136*L+0.0115*W)*L*W$$

where S is the surface in cm^2, L the length of the egg, and W the width of the egg. Length and width of all eggs were measured with a calliper (accuracy 0.02 mm). Eggshell bacterial growth was estimated as the signed differences in bacterial density estimated at the end of the incubation minus that estimated at the beginning of the incubation divided by bacterial load at the beginning of the incubation. For a more detailed description of estimates of eggshell bacterial density and growth see Peralta-Sanchez et al. (2010).

Sample size and statistical analyses

We collected data from 152 nests belonged to 17 species (a minimum of three nests per species were sampled, Appendix 3). Some of the sampled nests at the beginning of the incubation became depredated or abandoned during incubation. Consequently, we were unable to estimate eggshell bacterial growth along incubation for these nests, which resulted in different sample size for different variables. Information of eggshell bacterial loads and growth estimated for different species are presented in Appendix 3.

At the intraspecific level, after log_{10}-transformation of bacterial density and bacterial growth estimates, and arcsin-transformation of hatching success, none of these variables were normally distributed (Kolmogorov-Smirnov tests for continuous variables, $p < 0.05$). Thus, to check whether results from statistical analyses were biased due to non-normality of these variables, we repeated all analyses by using rank values rather than log_{10}-transformed bacterial counts. Because results did not change qualitatively (i.e., identical significant variables; results not shown), results from analyses using log_{10}-transformed variables are presented. Distribution frequencies of geometric means of eggshell bacterial loads and growth estimates from different culture media for different species did not

differ from normality after $\log_{10}$-transformation. Furthermore, mean hatching success per species did not departure from normality and we therefore use parametric statistics in our phylogenetically controlled comparative analyses.

Predicted influence of eggshell bacterial load and growth on hatching success of sampled nests was tested by means of GLM models with values of hatching success as dependent variable, species identities as independent random factors, and bacterial loads and growths as covariables. To test whether the predicted effects of covariables differed for different species, we explored the effect of the interaction between species identity (random factor) and bacterial counts and growth explaining probability of hatching failure.

For interspecific comparative analyses exploring the association between interspecific differences in bacterial load and growth and hatching success, we used the geometric means values estimated for each species. These values, however cannot be considered statistically independent data points due to common ancestry and phylogenetic relationships between species should be taken into account in comparative analyses (Harvey & Pagel 1991). We incorporated phylogenetic relationships (Appendix 4) based on Jonsson & Fjeldsa (2006), while polytomies of basal nodes were solved following Sibley & Ahlquist (1990).

To control for possible effects of a common ancestor we performed phylogenetic generalized least square regression (PGLS) analyses (Pagel 1997; Pagel 1999) as implemented in the R statistical computing environment (using "MASS", "ape" and "mvtnorm" libraries) with an additional function by R. Freckleton (University of Sheffield, pglm3.3.r available upon request). The PGLS approach characterizes evolutionary changes along each branch of a phylogeny through the variance components of traits and controls for the non-independence among species by incorporating a matrix of the covariances among species based on their phylogenetic relationships (Martins & Hansen 1997; Pagel 1997; Pagel 1999). Thus, phylogenetic information is incorporated to the error term and thus controlling for the shared evolutionary history among species (Harvey & Pagel 1991; Martins & Hansen 1997). The method applies likelihood ratio statistics to test evolutionary hypotheses and also to estimate the importance of phylogenetic corrections in the models (λ) (Freckleton et al. 2002), which vary between 0

(phylogenetic independence) and 1 (species' traits covary in direct proportion to their shared evolutionary history) (Pagel 1997). Then, we conducted all the analyses setting the degree of phylogenetic dependence (λ) to the most appropriate degree evaluated for each model.

Partial contribution of each eggshell bacterial load or growth included in the models was tested by partial regression coefficients with a type III decomposition of errors in PGLS models. All analyses were two-tailed P-values throughout and conducted using R statistical computing environment and STATISTICA 7.1 software.

Results

After controlling for the random effect of species identity, density of total aerobic mesophile bacteria estimated at the beginning of the incubation explained a significant proportion of variance in hatching success (Table 2.1). Nests that harbored higher bacterial density at the beginning of the incubation experienced lower hatching success (Fig. 2.1). No other estimates of bacterial density at the beginning of the incubation contributed significantly to explain hatching success (Table 2.1).

Table 2.1. Results of general linear models (GLMs) analyzing the effects of species identity (random factor), log_{10}-transformed eggshell bacterial loads at the beginning of the incubation and relative bacterial growth during the incubation period (total aerobic mesophiles (TSA), *Enterococcus*, (KF), *Staphylococcus* (VJ) and *Enterobacteriaceae* (HK)) on arcsin-transformed hatching success. S.E. refers to standard error. Significant results in bold.

	Beta (S.E.)	F	P	Beta (S.E.)	F	P
Models	Beginning of incubation (R^2 adjusted = 0.12; $F_{(5,152)}$= 2.03; P = 0.010)			During-incubation growth (R^2 adjusted= 0.18; $F_{(5,107)}$= 2.19; P = 0.007)		
Species identity	-	2.02	**0.016**	-	1.99	**0.025**
TSA	-0.31(0.11)	8.03	**0.005**	0.17 (0.12)	2.13	0.148
KF	-0.05 (0.11)	0.23	0.631	0.32 (0.10)	9.76	**0.002**
VJ	-0.01(0.09)	0.01	0.930	0.05 (0.10)	0.30	0.586
HK	0.18 (0.12)	2.47	0.118	-0.35 (0.12)	8.27	**0.005**

Enterococcus loads during incubation were those with the lower hatching Moreover, in a second statistical model, we detected that the during-incubation growth (increase in bacterial load between the beginning of the incubation and the

end of the incubation period) of *Enterococcus* and *Enterobacteriaceae* on the eggshells also explained significant proportion of variance of hatching success after controlling for the significant random effect of species identity (Table 2.2, Fig 2.2). Nests that decreased success. We found the opposite pattern for *Enterobacteriaceae*. Those nests that increased *Enterobacteriaceae* during incubation, experience lower hatching success (Fig 2.2).

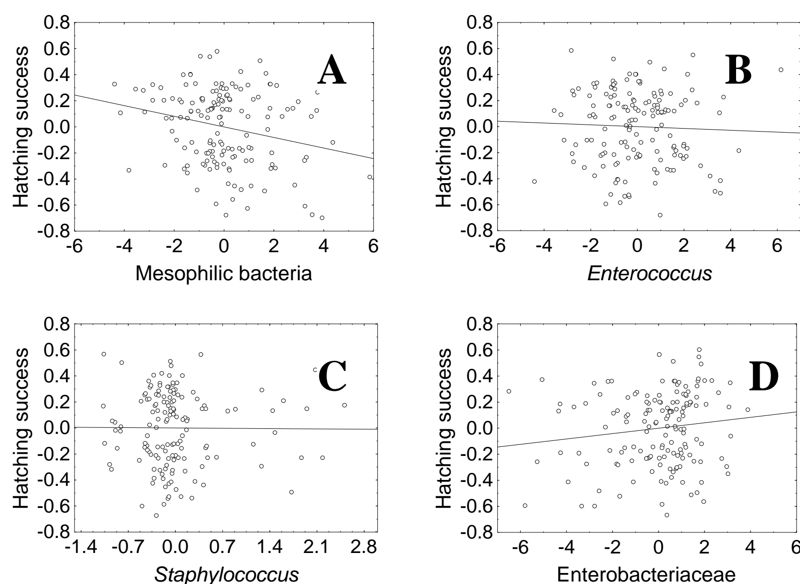

Figure 2.1. Partial correlations for residuals of arcsin-transformed hatching success and residuals of log-transformed bacterial density at the beginning of the incubation (total aerobic mesophilic bacteria (A), *Enterococcus* (B), *Staphylococcus* (C) and *Enterobacteriaceae* (D)). Each bacteria density residuals were calculated after controlling for the others bacteria densities. Slopes of the correlation lines represent partial correlation coefficients.

Finally, interactions between species identity and bacterial loads or growth estimated by the four different media did not explain significant proportions of variance of hatching success (GLM, species identity as random factor, bacterial load and bacterial growth as continuous predictors, all interactions: $P > 0.158$).

When trying to explain inter-specific differences in hatching success by inter-specific differences in eggshell bacterial loads we did not find any significant association in phylogenetic generalized least square multiple regression models (Table 2.2).

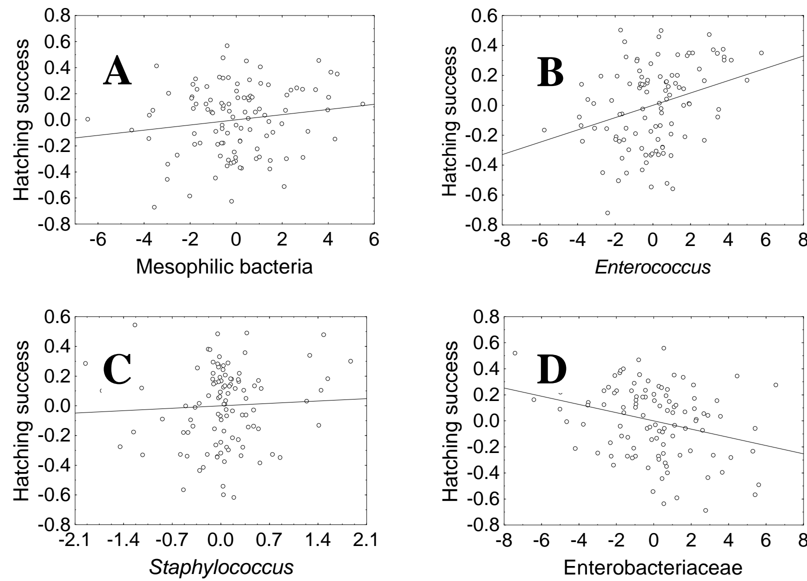

Figure 2.2. Partial correlations for residuals of arcsin-transformed hatching success and residuals of log-transformed during-incubation bacterial growth (total aerobic mesophilic bacteria (A), *Enterococcus* (B), *Staphylococcus* (C) and *Enterobacteriaceae* (D)). Each variable of during-incubation bacterial growth were calculated after controlling for the others during-incubation bacterial growth. Slopes of the correlation lines represent partial correlation coefficients.

Discussion

The main findings of this paper are (i) that among-nests variation in eggshell bacterial loads predicted among- nests variation in hatching success of wild birds; (ii) and that interspecific differences in eggshell bacterial loads and in hatching success did not covary. These results suggest on the one hand that nest bacterial environment is an important selective agent of embryo viability and, on the other hand, that different species are adapted to suffer different levels of eggshell

Table 2.2. Results of phylogenetic generalized least square regression (PGLS) analyzing the effects of species estimates mean values of log_{10}-transformed eggshell bacterial loads at the beginning of incubation and during-incubation growth during the incubation period (total aerobic mesophiles (TSA), *Enterococcus*, (KF), *Staphylococcus* (VJ) and *Enterobacteriaceae* (HK)) on species estimates mean values of hatching success. S.E. refers to standard error.

	Beta (S.E.)	t	P	Beta (S.E.)	t	P
Models	Beginning of incubation (R^2 adjusted= 0.167; $F_{(3,17)}$= 0.43; P = 0.787)			During-incubation growth (R^2 adjusted = 0.270; $F_{(3,16)}$= 0.20; P = 0.931)		
TSA	0.02 (0.03)	0.66	0.520	-0.01 (0.02)	0.12	0.902
KF	0.01 (0.02)	0.68	0.507	-0.02 (0.02)	0.81	0.437
VJ	-0.06 (0.06)	1.08	0.302	0.01 (0.06)	0.15	0.881
HK	-0.02 (0.03)	0.89	0.391	0.01 (0.02)	0.51	0.618

bacterial load that do not result in inter-specific variation in hatching success. These interpretations however depend on several methodological assumptions that, together with results, we discuss below.

The first assumption to discuss is that our estimations of eggshell bacterial loads and growth reflect the bacterial community of nest environments that established and growth on the eggshells before trans-shell infection. We have used culture-depending media to estimate eggshell bacterial community (i.e. density of four different groups of bacteria). It is known that a very low percentage of bacteria diversity can be growth with media in aerobic conditions (Amann et al. 1995; Pace 1997), and this bias may affect results and therefore conclusions. However, culture-dependent estimations of bacterial communities of eggshells have recently been validated by using molecular characterization (i.e. diversity) of communities (Cook et al. 2005a; Shawkey et al. 2009). We here explore the effect of bacterial density and growth on hatching success and find statistical support for the predicted association. Thus, independently of whether non-cultivable microorganisms were important determining probability of successful hatching, our results allow us to conclude in favor of the predicted association.

The second assumption is that the estimations of bacterial communities should reflect natural abundance of potentially pathogenic bacteria of avian eggs. We selected four different media that, apart from the heterotrophic medium, include one for *Staphylococcus*, another one for *Enterobacteriaceae*, and a third culture media for enterococci. These specific bacteria are known to live in avian eggshells but also inside the eggs and, based on extensive studies of bacteria on domestic and wild bird eggs, are considered as potentially pathogenic bacteria of avian eggs (Board & Fuller 1994; Baggott & Graeme-Cook 2002). *Staphylococcus* sp. and *Enterobacteriaceae* are saprophytic and opportunistic pathogen bacteria (Houston et al. 1997; Singleton & Harper 1998; Cook et al. 2005a) that live in skin, hair, feathers and cloacae of mammals and birds (Krieg & Holt 1984), and are pathogenic for avian embryos (Bruce & Drysdale 1994). Enterococci, the third specific analyzed group of bacteria, are also frequently found inside unhatched eggs (Bruce & Drysdale 1994; Houston et al. 1997) and,

although they are opportunistic pathogens (Franz et al. 1999), they might also have beneficial effects for embryos (Soler et al. 2008). Finally, eggshell density of these groups of potentially pathogenic bacteria are usually related to that estimated for aerobic mesophilic bacteria as it is the case here for *Enterococcus* load at the beginning of incubation ($R = 0.39$, $N = 152$, $t = 5.23$, $P < 0.001$) and for *Enterobacteriaceae* at the beginning of the incubation ($R = 0.68$, $N = 152$, $t = 11.40$, $P < 0.001$). Thus, it is likely that our estimates of bacterial density appropriately characterize communities of potential bacterial pathogens of avian eggs.

We found a negative relationship between density of aerobic mesophilic bacteria on the eggshell at the beginning of the incubation and hatching success experienced by sampled eggs. Taking into account the above discussed assumptions in general and the positive relationship between densities of aerobic mesophilic bacteria and *Enterobacteriaceae* in particular, this result suggests a link between potentially pathogenic bacteria present on the eggshells at the start of the incubation and probability of embryo death. Birds, throughout the incubation are able to control bacterial growth on the eggshell and therefore decrease probability of trans-shell bacterial colonization of egg contents (Cook et al. 2005a; Shawkey et al. 2009) which affect hatching success (Cook et al. 2003; Cook et al. 2005b). Thus, we predicted that bacterial growth during incubation should be related to hatching success. In accordance with this prediction we found that nests with the highest growth of the pathogenic *Enterobacteriaceae* were those with the lowest hatching success. These results, therefore, are in accordance with the hypothesis that bacterial environment of avian nests is an important selective agent determining hatching success of birds because, for instance, individuals better able to control bacterial community of nests at the beginning of the incubation period, or eggshell bacterial growth throughout incubation would be those with higher hatching success.

Contrary to what can be expected if enterococci detected on the eggshell were pathogenic for avian eggs we found that nests where density of this group of bacteria increased during the incubation period at the highest rates were those with the highest hatching success. Enterococci have been located inside

unhacthed eggs (Houston et al. 1997; Stewart & Rambo 2000) and, therefore, have been suggested as potentially pathogenic bacteria of eggs. Beneficial effects of some species of *Enterococcus sp.* are well known from poultry and food conservation literature (Foulquie-Moreno et al. 2006), and have also been described for avian hosts in natural conditions (Moreno et al. 2003; Soler et al. 2010). For instance, there are evidences suggesting that *Enterococcus faecalis* living in the uropygial gland of hoopoes (*Upupa epops*), or the bacteriocin-like inhibitory substances that enterococci produce, protect eggshell from pathogenic infections (Soler et al. 2008). Due to bacterial interference properties (Ji et al. 1997), the establishment and growth of relatively low pathogenic bacteria on the eggshell of birds could prevent the colonization of more pathogenic bacteria and, thus, birds favoring growth of non-pathogenic bacteria on their eggshell may experience higher hatching success than others (Soler et al. 2010). The positive relationship between enterococci growth and hatching success could therefore be explained by the relatively low pathogenesis of this group of bacteria and might suggest a role of these bacteria enhancing hatching success of wild birds that should be further explored.

All those results suggesting an important role of bacteria as environmental components of nests driving hatching success of wild birds came from exploring covariation between eggshell bacterial loads and hatching success while controlling for interspecific differences in these parameters (see results). However, when exploring covariation between eggshell bacterial load and hatching success at the interspecific level, we did not find the negative association found within species variability. Different species might optimize level of antibacterial defenses to the risk of infection that commonly experience their populations. In the case of eggs, species experiencing high risk of bacterial infections (i.e. eggshell bacterial loads and growth) may also be those laying eggs with more effective barriers against trans-shell bacterial infection. Reduce number and size of pores or increase eggshell thickness and antibiotic properties of the albumen would reduce probability of embryo infection (see Introduction) and, therefore, these defensive traits could be adapted to the expected bacterial environmental conditions of nests. Although our interspecific comparative results

are in accordance with this scenario, further work testing the predicted relationship between antibacterial defensive traits and eggshell bacterial loads is necessary to reach firm conclusions.

Summarizing, this is to our knowledge the first work showing evidence supporting the extended idea that bacteria are important selective agents for bird eggs (Board & Fuller 1994; Deeming 2002). Moreover, our results validate the use of estimates of bacteria density and growth on eggshells as variables related to probability of trans-shell bacterial infection. Finally, we have found evidence of a beneficial role of enterococci growing on the shells of bird eggs that we hope encourage further investigation on the role of this group of bacteria as symbionts of birds.

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CAPITULO III:

Number and Color Composition of Nest Lining Feathers Predict Eggshell Bacterial Community in Barn Swallow Nests: An Experimental Study

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Abstract: The use of feathers as nest lining material has traditionally been explained by the thermoregulatory properties of feathers. Feather nest lining could additionally affect nest detectability by predators, or play a role in a sexually selected context. Furthermore, feather nest lining harbors microorganisms that may influence environmental conditions where eggs and nestlings develop. Microorganisms growing on nest lining feathers could affect the bacterial load of eggshells because they occupy space and/or produce antimicrobial substances against other bacteria, including egg pathogens. Feathers of different colors are known to differ in their bacterial community (i.e. feather degrading bacteria) and, thus, color composition of nest lining feather could also affect the bacterial environment of avian nests. Here we tested this hypothesis in the barn shallow (*Hirundo rustica*) by exploring the relationship between eggshell bacterial loads and number of feathers, and the effect of experimentally modified color composition of nest lining feathers on eggshell bacterial load. In agreement with the hypothesis we found that, before treatment, the number of nest lining feathers (mainly that of unpigmented-white color) predicted eggshell bacterial load, and that, at the end of the incubation period, eggshells of experimental nests with white feathers had a lower bacterial density than those in experimental nests with black feathers. We failed to detect a relationship between bacterial load and hatching success. However, since evidence of that relationship exists for other species, these results would explain the previously detected experimental effect of color composition of nest lining feathers on hatching success of swallows. Nest design in general, and the use of nest-lining white feathers in particular, may therefore have important consequences for reproductive success of birds. The reduced eggshell bacterial loads of experimental white nests would explain preferences by barn swallows for feathers of white color.

Keywords: eggshell bacterial load, feather color, incubation, nest building, nest lining feathers.

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Introduction

Many birds use feathers as lining material (Harrison 1975; Cramp 1998; Hansell 2000) and, although it has commonly been associated with nest insulation (Møller 1984; Hilton *et al.* 2004), other hypotheses are possible. Feathers, for instance, affect nest detectability by predators (Møller 1987), but also could be a sexually selected signal of nest builders (Veiga & Polo 2005). However, most evidence is in accordance with the use of feathers as insulating nest material. For example, it has been experimentally demonstrated that in nests of barn swallows (*Hirundo rustica*) feathers affected cooling and warming rate of eggs, duration of recess periods, duration of incubation bout periods and nest attendance (Møller 1991). Therefore, an association between nest lining feathers and hatching success should exist. However, hatching success could be explained not only by thermoregulatory properties of feathers, but also by incubation behavior of adult and bacterial environmental conditions of nests (Cook *et al.* 2003; Cook *et al.* 2005a; Soler *et al.* 2009).

Nest lining feathers harbor microorganisms that in most cases are feather degrading bacteria or fungi (e.g., Pugh & Evans 1970; Shawkey, Pillai, & Hill 2003; Cristol *et al.* 2005) that could infest incubating adults or growing nestlings. These microorganisms associated with feathers could also affect the probably of trans-eggshell pathogenic infection of embryos if for instance they occupy space and/or produce antimicrobial substances against egg pathogens. Growth of feather-degrading bacteria is mainly controlled or prevented by uropygial secretions that birds spread on feathers during preening (Shawkey *et al.* 2003). However, feathers, carried to nests as lining material, are not preened and consequently microorganisms would grow more quickly in nest lining feathers.

Most bacteria detected on feathers belong to the genera *Enterococcus*, *Staphylococcus* (Parisien *et al.* 2008), *Streptomyces* (Omura *et al.* 2001) and *Bacillus* (Burtt & Ichida 1999; Gunderson 2008). These bacteria are known as producers of antibiotic substances and, therefore, if present in feathers transported to the nest they could play a role in preventing the establishment of other bacteria within the nest environment. However, mainly *Enterobacteriaceae*, but also

Staphylococcus are frequently found inside dead-in-shell eggs of ducks, waterfowl, hens and turkeys, and, therefore, might also act as pathogenic bacteria (Bruce & Drysdale 1994). Among the many strains of bacteria that degrade feathers *Bacillus licheniformis* is particularly common and abundant (Burtt *et al.* 1999; Gunderson *et al.* 2008). *B. licheniformis*, apart from its keratinolytic activity on avian feathers, is known to produce antimicrobial substances (Simlot, Specht, & Pfaender 1972) active not only against different strains belonging to the genera *Bacillus*, *Corynebacter*, *Enterococcus* and *Mycobacterium*, but also against amoebae (Galvez *et al.* 1994) and fungi (Lebbadi *et al.* 1994; Patel, Tendulkar, & Chattoo 2004). *Thus,* microorganisms from nest-lining feathers and antimicrobial chemicals producers might be transferred or migrate to the eggshell preventing colonization of pathogenic bacteria of embryos by directly coming in contact with incubated eggs. If that was the case, the use of feathers as nest lining material could at least partially be interpreted by adult birds as a growing culture of bacteria (i.e., *B. licheniformis*) that diminish the probability of embryo infection (Soler *et al.* 2009) (for a similar hypothetical effect of feathers of incubating birds in contact with eggshells see Cook *et al.* 2005a; Shawkey *et al.* 2009).

Growth of *B. licheniformis* depends on feather color (Grande, Negro, & Torres 2004; Goldstein *et al.* 2004; Gunderson *et al.* 2008). The feather color that *B. licheniformis* more easily degrades is however controversial, and although Goldstein *et al.* (2004) demonstrated that white feathers are better degraded by *B. licheniformis* (apparently because of the absence of melanin that makes feather degradation especially easy), Grande *et al.* (2004) found the opposite pattern. Gunderson *et al.* (2008) trying to resolve the apparent controversy, repeated the experiments and concluded that white feathers are more easily degraded by *B. licheniformis* than melanized feathers. However, these studies used different *B. licheniformis* strains, which could explain the contradictory results. In any case, these results indicate that feathers of different colors (i.e. white vs. black), in the absence of preening (i.e. those used for nest lining), would vary in bacterial density. Therefore, if feathers in the nest affect the bacterial community of

eggshells, it can be predicted that feather coloration should also affect the bacterial community on eggshells.

Here we tested this hypothesis by experimental transfer between nests of colored (black hereafter) and white (i.e., unpigmented) nest lining feathers between nests of barn swallows. Briefly, soon after the start of incubation, we removed all feathers of a target color (i.e. white or black) from a nest and replaced them with feathers of the other color from previously visited nests. Thus, we had experimental nests with all lining feathers of the same color. In order to estimate eggshell bacterial density, we sampled eggshells before and after (i.e. few days before hatching) manipulations, and also counted feathers of different coloration at the time of sampling. The hypothetical role of nest-lining feathers as a source of bacteria predicts a relationship between the number of feathers and estimates of bacterial density of the eggshells that could differ for feathers of different color.

Materials & methods

Field work

We performed our experiment in 2008 during the breeding season of the barn swallow at Kraghede, Denmark (57º 12’ N; 10º 00’ E). For a detailed description of the study area, see Møller (1987). We visited nests twice a week to determine laying date and clutch size, and once a clutch was complete we took a sample of bacteria from the eggshell and performed the feather experiment.

The experiment was performed 2-3 days after clutch completion and consisted of randomly removing all white or black feathers from finished nests of barn swallows after clutch completion. Briefly, we first removed and counted all white and black feathers in the nest cup and, if for instance the nest was randomly assigned to become a “white nest”, we removed all black feathers and replaced them with white feathers collected from a previously sampled nest that was assigned to the “black nests” treatment. Transfer of feathers from one nest to another, was made using single-use-sterilized paper towels to prevent further bacterial contamination. The removed black feathers were transfer to the subsequently sampled nest that was then assigned to the other experimental

treatment ("black nest"), and so on. We wore latex gloves sterilized by ethanol 70% to prevent bacterial contamination between nests. Furthermore, because barn swallow nests harbored more black than white feathers, experimental black nests had almost twice the number of feathers than in white nests. Consequently, our experiment not only modified the color composition of lining material, but also the number of feathers. A few days before hatching, we again visited the nests and counted white and black feathers present in the nest lining material. Subsequently we visited nests at the day of hatching to determine hatching success.

We have shown in another paper with this set of nests that (i) white and black nests did not differ in the number of white, black or total feathers before the experiment, (ii) that birds counteract the experimental manipulation of feather color composition, but not that of number of feathers (i.e. black nests harbored more feathers, but a similar percentage of each color than white nests), (iii) that number of feathers decreased during incubation, and (iv) that rate of feather renewal (number of feathers of the removed color found in nests close to hatching time) did not differ for experimental black and white nests (Peralta et al. submitted). Therefore, possible experimental effects on the bacterial community on eggshells could not be exclusively related to experimental feather numbers or feather color composition, but also to feather number and color composition that experimental nests harbored during incubation. Consequently, in our analyses we include treatment as a fixed factor, but number of feathers as a covariate.

Bacterial protocol

Bacterial communities on eggshells of experimental nests were sampled twice, before the experiment and one or two days before hatching. We sampled eggshells in sterile conditions mainly to prevent between nest contaminations. We wore sterilized latex gloves with ethanol and took bacterial samples by cleaning eggshells with a sterile swab slightly wet with sterile sodium phosphate buffer (0.2 M; pH 7.2). The complete clutch was cleaned with the same swab, which was preserved in an eppendorf tube at 4ºC containing the sterile buffer until lab analyses. Estimates of bacterial load were standardized to total eggshell surface sampled by taking into account number and surface of eggs in the nests. Eggshell

surface was estimated according to the formula:

$$S = 3*L^{0.771} *W^{1.229}$$ (Narushin 1997)

where S is the surface in cm^2, L the length of the egg, and W the width of the egg. Length and width of all eggs were measured with a caliper (accuracy: 0.02 mm).

Laboratory work

In the lab, samples were collected from eppendorf tubes after vigorously shaking the eppendorf in vortex (Boeco V1 Plus!) for at least three periods of 5 seconds. Serial decimal dilutions up to 10^{-6} were cultivated by spreading homogenously 100 μl of sample (measured with a micropipette) in plates containing four different sterile solid growth media (Scharlau Chemie S.A. Barcelona). We used Tryptic Soy Agar (TSA), a broadly used general medium to grow heterotrophic bacteria, and three specific media: Kenner Fecal Agar (KF) for growing bacteria belonging to the genus *Enterococcus*; Vogel-Johnsson Agar (VJ) for bacteria of the genus *Staphylococcus*; and Hecktoen Enteric Agar (HK) for Gram negative bacteria of the family *Enterobacteriaceae*. Plates were incubated at 37ºC for 72 hours, and afterwards the number of colonies on each plate was counted. Bacterial density was estimated as CFU (Colony Forming Units) per cm^2. Thus, we estimated bacterial density for first (soon after laying) and second (few days before hatching) samples. These counts are repeatable within the same clutch as we have shown previously using another set of nests of different species (For TSA: R=0.74, d.f.=1,345, F=2.45, P<0.001; For KF: R=0.79; d.f.=1,345, F=3.35, P<0.001; For VJ: R=0.66; d.f.=1,345, F=1.59, P<0.001; For HK R=0.80, d.f.=1,345, F=3.60, P<0.001).

Statistical methods

The numbers of white and black feathers and the total number of feathers approximately followed a normal distribution (Kolmogorov-Smirnov tests for continuous variables, P > 0.15). Frequencies of bacterial loads in media for heterotrophic bacteria (TSA) were approximately normally distributed after log_{10}-

log_{10}transformation (Kolmogorov-Smirnov tests for continuous variables, $P > 0.2$). Counts of bacterial colonies in specific medium for *Enterobacteriaceae* (HK) and for *Staphylococcus* (VJ) differed significantly from normality, mainly due to bimodal distributions or because bacterial growth was only detected for approximately half of the samples, respectively. We failed to transform these bacterial counts (mainly those from the second sampling (i.e., nests close to hatching) to a normally distributed variable, and in our analyses we thus used ranked values. In specific media for *Enterococcus* (KF), we only obtained colonies of bacteria from a single sample, and, consequently, we did not use this variable in subsequent analyses.

Estimates of eggshell bacterial loads at the end of the incubation period might depend on bacterial loads at the beginning of incubation, and, consequently, the effect of experimental treatment on eggshell bacterial density should in that case be corrected for estimates of eggshell bacterial load before the experiment. However, only bacterial counts in TSA ($R^2 = 0.16$, $N = 32$, $P = 0.023$), but not in VJ ($R^2 = 0.09$, $N = 32$, $P = 0.09$) or HK culture media ($R^2 = 0.07$, $N = 32$, $P = 0.14$) at the beginning and at the end of the incubation period were significantly positively related. In any case, we have performed analyses using between-sampling differences in bacterial counts to control for the effect of the experiment for eggshell bacterial density in nests before experimental treatment.

The effects of experimental treatment and covariates on normalized or ranked dependent variables were tested by using General Lineal Models. Sample sizes differed slightly for different analyses because eggs of five nests hatched before the second sampling, one nest was abandoned and two additional nests fell down from the wall before we collected the second samples. All statistical tests were two-tailed and performed with the software Statistica 8.0.

Results

Bacterial load at the start of incubation

Cultures in heterotrophic medium (TSA) of collected samples at the start of the incubation revealed the existence of bacteria in all swallow nests at the beginning

of the incubation (N = 40). Estimated bacterial densities greatly varied among nests (colonies per cm^2: minimum = 0.36; maximum = 616187.1) with a mean value (SE) of 41975. 2 (23336.8) colonies per cm^2 (median = 5.4 colonies per cm^2). Bacterial growth in specific cultures showed that *Staphylococcus* grew in 22 of the 40 samples, while *Enterococcus* did not grow in 1 of the 40 samples.

Table 3.1.- Effects of changing color composition treatment and number of total feathers (model 1) and number of white and black feathers (model 2) on load of heterotrophic bacteria (TSA), *Staphylococcus* (VJ) and *Enterobacteriaceae* (HK) on eggshells, before and after treatment (i.e., at the end of incubation).

	TSA			VJ			HK		
Before treatment	Beta (SE)	$F_{(1,37)}$	P	Beta (SE)	$F_{(1,37)}$	P	Beta (SE)	$F_{(1,37)}$	P
Number of white feathers	-0.35 (0.16)	4.85	0.034	-0.39 (0.16)	6.03	0.019	-0.46 (0.16)	8.58	0.006
Number of black feathers	-0.15 (0.16)	0.92	0.343	-0.09 (0.16)	0.35	0.558	-0.02 (0.16)	0.01	0.910
After treatment									
Model 1	Beta (SE)	$F_{(1,29)}$	P	Beta (SE)	$F_{(1,29)}$	P	Beta (SE)	$F_{(1,29)}$	P
Number of feathers	-0.34 (0.17)	4.22	0.049	-0.40 (0.18)	4.86	0.036	-0.28 (0.15)	3.46	0.07
Treatment	0.53 (0.17)	9.88	0.004	0.23 (0.18)	1.57	0.22	0.67 (0.15)	19.37	0.0001
Model 2	Beta	$F_{(1,28)}$	P	Beta	$F_{(1,28)}$	P	Beta	$F_{(1,28)}$	P
Number of white feathers	-0.14 (0.18)	0.58	0.453	-0.17 (0.19)	0.71	0.41	-0.10 (0.17)	0.38	0.544
Number of black feathers	-0.38 (0.18)	4.28	0.048	-0.44 (0.20)	4.92	0.035	-0.32 (0.17)	3.56	0.069
Treatment	0.51 (0.17)	9.02	0.006	0.21 (0.19)	1.34	0.26	0.66 (0.16)	17.88	0.0002

Estimated bacterial density of *Enterobacteriaceae* was quite high and variable (mean (SE) 49680.0 (27952.8); median = 0.4 colonies per cm^2), while those of *Staphylococcus* was quite low (mean (SE) 0.06 (0.01); median = 0.03 colonies per cm^2).

Nests of barn swallows selected for different treatments did not differ significantly in the estimates of eggshell bacterial load, in general (MANOVA, Wilks = 0.99, F = 0.07, df = 3,36, P = 0.97), or for any of the used culture media (TSA: F = 0.14, df = 1,38, P = 0.71; VJ: F = 0.10, df = 1,38, P = 0.76; HK: F = 0.01, df = 1,38, P = 0.90). These bacterial counts were negatively related to the number of feathers in the nest (MANOVA, Wilks = 0.81, F = 2.90, df = 3,36, P = 0.048; Fig. 3.1). When separately considering feathers of different colors in a multiple regression approach, we found that the number of white feathers (MANOVA, Wilks = 0.79, F = 3.18, df = 3,35, P = 0.036), but not that of black feathers (MANOVA, Wilks = 0.96, F = 0.50, df = 3,35, P = 0.68) significantly explained bacterial load of eggshells at the start of incubation independently of culture media used for estimations (Table 3.1). Finally, the percentage of white feather in nests before treatment did not significantly explain bacterial load neither in general (MANOVA, Wilks = 0.96, F = 0.52, df = 3,36, P = 0.67) nor for any of the used culture media (F < 1.44, df = 1,38, P > 0.23).

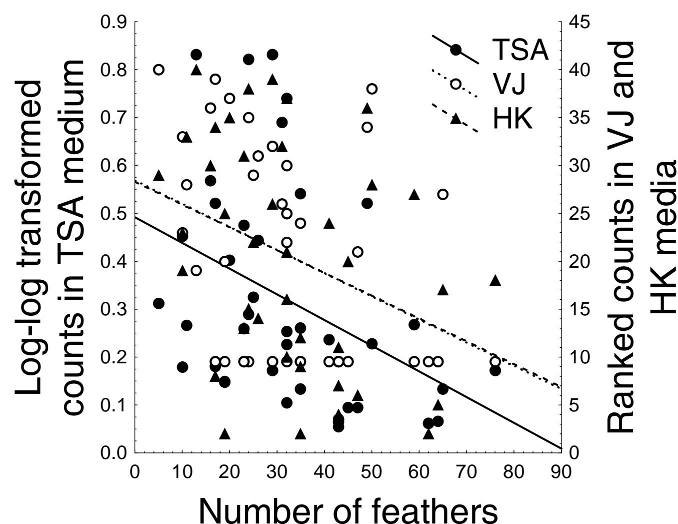

Figure 3.1. Relationships between number of feathers in nests of barn swallows at the start of incubation and eggshell density of heterotrophic bacteria (TSA), *Staphylococcus* (VJ) and *Enterobacteriaceae* (HK). Lines are regression lines.

Bacterial load at the end of the incubation

Cultures in TSA of collected samples a few days before hatching revealed the existence of bacteria in all swallow nests at the beginning of the incubation (N = 32). Estimated bacterial densities greatly varied among nests (colonies per cm^2: minimum = 0.11; maximum = 610067.9) with a mean value (SE) of 33216. 1 (20252.3) colonies per cm^2) (median = 6.8 colonies per cm^2). Bacterial growth in specific cultures revealed that *Staphylococcus* were present in 12 of the 32 samples, while *Enterococcus* did not grow in 4 of the 32 samples. Estimated bacterial density of *Enterobacteriaceae* was quite high and variable (mean (SE) = 21804.7 (15785.4); median = 0.1 colonies per cm^2), while those of *Staphylococcus* was quite low (mean (SE) 1.7 (1.4); median = 1.13 colonies per cm^2).

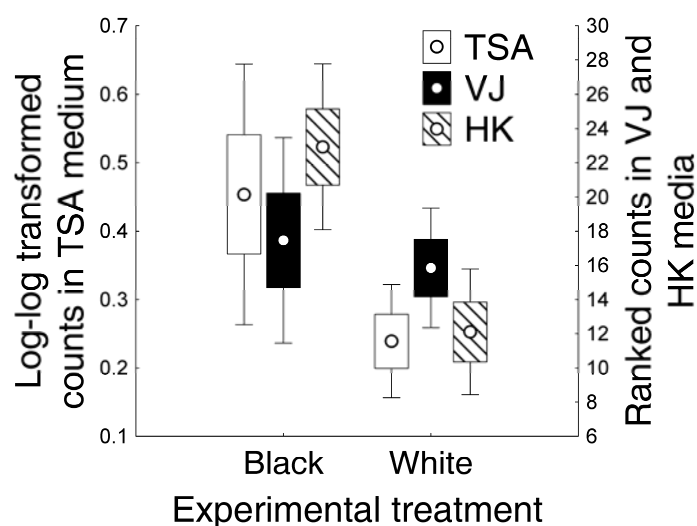

Figure 3.2. Density (means, SE, and 95% confidence intervals) of load of heterotrophic bacteria (TSA), *Staphylococcus* (VJ) and *Enterobacteriaceae* (HK) on eggshells at the end of the incubation period in experimental white and black nests of barn swallows.

A few days before hatching, experimental treatment explained a significant proportion of variance in bacterial counts in general (MANOVA, Wilks = 0.57, F = 6.82, df = 3,27, P = 0.001), or for estimates in all culture media, even after controlling for the number of feathers in the nest at the time of hatching (Table 3.1). While eggshells of the experimental white nests tend to have lower estimates of bacterial density (Fig. 3.2), the number of feathers and bacterial density

estimated for some of the culture media were negatively related (Table 3.1). The effect of number of feathers on eggshell bacterial load at the end of the incubation was however lower than that detected at the beginning of the incubation since, when the effect of the experimental treatment was removed from the model, the number of feathers failed to explain any of the dependent variables (bacterial counts) (MANOVA, Wilks = 0.87, F = 1.42, df = 3,28, P = 0.26; ANOVA tests: TSA: F = 0.91, df = 1,30, P = 0.35; VJ: F = 3.54, df = 1,30, P = 0.07; HK: F = 0.13, df = 1,30, P = 0.73).

Our experimental approach resulted in experimental black nests harboring almost twice as many feathers as experimental white nests, and, therefore, the effect of feather-color treatment could be confounded by the effect of experimental feather number. However, several pieces of information suggest that this is not the case. First, in the model that included the experimental treatment (fixed factor), number of experimental feathers (those included in the nests at the time of performing the experiment), and number of feathers in nests at the end of the incubation (covariates), only the fixed factor (MANOVA, Wilks = 0.65, F = 4.60, df = 3,26, P = 0.01), but none of the feather counts (MANOVA, Wilks > 0.90, F < 1.00, df = 3,26, P > 0.40), explained a significant proportion of variance in eggshell bacterial density. Second, when removing the effect of experimental treatment from the previous model, none of the feather counts explained bacterial counts in general (MANOVA, Wilks > 0.86, F < 1.48, df = 3,27, P > 0.24). Third, when we separately analyzed the effect of treatment (white *vs.* black nests) and that of experimental number of feathers on eggshell bacterial loads at the end of the incubation period, the former (MANOVA, Wilks = 0.59, F = 6.36, df = 3,28, P = 0.002), but not the latter (MANOVA, Wilks = 0.85, F = 1.61, df = 3,28, P = 0.21) factor explained a significant proportion of variance. Furthermore, when the model exclusively included these two associated factors, the results did not change (Treatment: MANOVA, Wilks = 0.63, F = 5.12, df = 3,27, P = 0.006; Feather number: MANOVA, Wilks = 0.91, F = 0.84, df = 3,27, P = 0.48). Finally, we tested for robustness of results related to experimental treatment by sequentially excluding from the analyses pairs of cases with the most extreme (i.e. positive and negative) values, and estimating effect sizes (partial-eta squared)

associated with the treatment effect in a model that included number of feathers at hatching. Since effect sizes do not depend on sample sizes, a negative relationship between effect sizes and degrees of freedom should appear if differences in number of feathers between experimental white and black nests were important explaining detected effect size of experimental treatment. However, estimates of effect sizes of the 10 first models (i.e. reducing degrees of freedom from 28 to 8) were not associated with degrees of freedom ($R = -0.02$, $N = 10$, $P = 0.953$). Therefore, all these analyses suggest that the detected experimental treatment effect on bacterial density was independent of the larger number of feathers in experimental black compared to white nests.

The effect of experimental treatment was still significant in explaining bacterial load on eggshells, even when separately considering the number of white and black feathers in the close-to-hatching nests of barn swallows (Table 3.1). In addition, the number of black (MANOVA, Wilks = 0.82, $F = 1.86$, $df = 3,26$, $P = 0.16$), but not that of white (MANOVA, Wilks = 0.97, $F = 0.26$, $df = 3,26$, $P = 0.85$) feathers explained an additional proportion of variance in bacterial counts (Table 3.1). The numbers of white and black feathers in nests at the time of the second sampling were significantly negatively related (Beta (SE) = -0.37 (0.16), $F = 5.68$, $df = 1,35$, $P = 0.023$). When removing the number of black feathers in the nest from the model, the effect of number of white feathers on bacterial counts were still far from significant ($F < 0.08$, $df = 1,29$, $P > 0.78$). Therefore, the detected negative effects of the number of black feathers on bacterial density were not due to its association with the number of white feathers. Finally, the percentage of white feathers in close-to-hatching nests did not explain bacterial load neither in general (MANOVA, Wilks = 0.95, $F = 0.47$, $df = 3,27$, $P = 0.70$) nor for any of the used culture media ($F < 1.40$, $df = 1,29$, $P > 0.24$).

Differences in eggshell bacterial loads between the two sampling periods

In accordance with the detected effects of the experimental treatment on eggshell bacterial load, we found that after controlling for the effect of differences between the two sampling periods in number of feathers present in the nest, detected differences in heterotrophic (TSA; $F = 5.56$, $df = 1,29$, $P = 0.\ 025$), but not

Enterobacteriaceae (HK; F = 3.41, df = 1,29, P = 0.075) or *Staphylococcus* (VJ; F = 1.50, df = 1,29, P = 0.23) was explained by the effect of experimental treatment.

Bacterial load and hatching success

None of the variables describing eggshell bacterial loads at the beginning or at the end of incubation, or differences in eggshell bacterial loads between the two sampling periods, explained the probability of hatching failures in nests of barn swallows (Generalized Lineal Models, Binomial distribution and log-link function, univariate analyses; Wald Statistic (minimum - maximum) = 0.007 - 0.76, df = 1, P > 0.38). However, when including all three kinds of bacterial counts in the same multivariate models, heterotrophic (TSA: Wald Statistic = 3.19, P = 0.074) and *Enterobacteriaceae* (HK: Wald Statistic = 3.00, P = 0.083) counts, but not that of *Staphylococcus* VJ: Wald Statistic = 0.02, P = 0.88) tended to explain the probability of hatching failures. Swallow nests with higher density of heterotrophic bacteria (TSA; partial regression coefficient (SE) = -4.01 (2.47)) but lower density of *Enterobacteriaceae* (HK; partial regression coefficient (SE) = 0.09 (0.05)) tended to experience higher risk of hatching failures. Multiple regression analyses including bacterial loads at the end of incubation, or differences in eggshell bacterial loads between the two sampling period, did not show any tendency explaining the probability of hatching failure (Wald Statistic (minimum - maximum) = 0.03 - 0.75, df = 1, P > 0.41).

Discussion

We have shown an important role of feathers explaining bacterial density on the eggshells of barn swallows. The main results suggesting such an association are (i) a negative relationship between number of feathers and eggshell bacterial load at the start of the incubation period that was mainly explained by the number of white feathers ; (ii) a significant effect of experimental modification of feather color composition of nests of barn swallows at the beginning of incubation on eggshell bacterial loads estimated at the end of incubation; and (iii) the effect of number of feathers on eggshell bacterial load was weaker at the end of incubation

and, contrary to that detected at the beginning of incubation, was mainly related to number of black feathers in the nests. Below we discuss these results in relation to the hypothetical function of feathers in controlling bacterial infection of eggshells.

Several possible scenarios predict a relationship between feather nest lining and bacterial density of eggshells of birds (see Introduction). Feathers could be a source of bacteria transferred to eggshells and, therefore, increase the probability of eggshell bacterial colonization. Furthermore, some bacteria growing on feathers (i.e. keratinolitic bacteria) are antibiotic producing microorganisms that when in contact with eggs could transfer chemicals to eggshells thereby preventing establishment of other bacteria. These two possible effects (colonization, or chemical transfer to eggshells) would result in an increased probability of successful hatching if it affects probability of eggshell colonization by pathogenic bacteria for embryos (see Soler *et al.* 2009). We found that the number of nest lining feathers at the beginning or the end of incubation was negatively related to bacterial loads of eggs at these stages of the breeding cycle, which therefore could suggest that antibiotics produced by bacteria or other microorganisms living in nest lining feathers could explain these negative relationships.

Another possible explanation for the negative relationship is that nest lining feathers are nest material with a reduced density of microorganisms capable of growing on the eggshells. Therefore, a larger number of feathers would contribute to a more sterile nest environment. However, this explanation is unlikely because keratinolitic bacteria, but also other sometimes pathogenic bacteria, are commonly found growing on feathers (Shawkey *et al.* 2003; Gunderson 2008). Moreover, birds prevent nest feather contamination of microorganisms by preening (Shawkey *et al.* 2003; Soler *et al.* 2008), but nest lining feathers are unprotected, which predicts an even larger bacterial load of nest lining feathers in comparison with active feathers on birds. Finally, it is also possible that the negative relationship between number of feathers and eggshell bacterial load was not due directly to feathers or microorganisms living on them, but to the antimicrobial chemicals of the uropygial secretion that nest lining feathers probably included. However, birds preen feathers several times per day

and, thus, a long-term effect of uropygial secretions preventing bacterial growth is unlikely. Consequently, preen secretions on nest lining feathers would in any case have a limited effect on eggshell bacterial density. Therefore, we believe that the more likely hypothesis explaining the association between number of feathers and eggshell bacterial load is related to the beneficial effect of bacteria living in nest lining feathers. However, detailed studies of the bacterial community of nest lining feathers and their antimicrobial properties in relation to bacterial load detected in the eggshell are necessary before reaching firm conclusions.

There is evidence suggesting that bacterial growth differs for feathers of different colors (see Introduction). To test the hypothesis that nest lining feathers of different colors affected eggshell bacterial load differentially, we first related the number of nest lining feathers of different color to eggshell bacterial load. Furthermore, we experimentally modified feather color composition of nests of barn swallows and explored the effect on eggshell bacterial load at the end of incubation. In accordance with the hypothesis, we found that the number of white, but not of black feathers explained eggshell bacterial load at the beginning of incubation. Furthermore, we found a significant treatment effect that was in accordance with the negative relationship between number of white feathers and bacterial load previously mentioned because at the end of incubation eggshells of experimental white nests harbored lower bacterial density than that of experimental black nests. Nests having the black treatment also received a larger number of feathers than experimental white nests (see Materials and Methods) and, thus, the detected treatment effect could be due to between nest differences in number of feathers rather that to experimental modification of feather color composition. However, when statistically correcting for the number of experimental feathers each nest received, as well as the number of nest lining feathers found in nests of barn swallows at the end of incubation (see Results), these variables did not significantly explain bacterial load while the effect of experimental treatment did have such an effect (see Results). Therefore, experimental feather color composition, but not experimental number of feathers was the cause of the detected experimental treatment.

In addition to the detected treatment effect explaining eggshell bacterial

load, we found that the number of black, rather than the number of white feathers explained a significant proportion of variance in bacterial load at the end of the incubation. This result, together with previous ones, could suggest a beneficial effect of feathers of black color when present in nests at the end of incubation, while feathers of white color would be more beneficial during laying and at the start of incubation. We lack a robust hypothesis that could explain these differences, but because shells of unincubated eggs are more prone to infection than incubated ones (Cook *et al.* 2005a; Shawkey *et al.* 2009), the hypothetical protection of nest lining feathers should be more important for early than for late incubation. In accordance with this possibility, the significant effect of feathers on eggshells bacterial density at the end of incubation disappeared when treatment was not included in the model (see Results). Consequently, it is likely that the effect of feathers preventing eggshell infections was more important for white than for black feathers.

White feathers from live chickens were more rapidly degraded *in vitro* by *B. licheniformis* than black feathers and they supported higher bacteria growth (Goldstein *et al.* 2004; Gunderson *et al.* 2008). It has been suggested that this variation could be explained by the effect of melanin that could blind keratin and make feathers more difficult to attack by bacterial keratinases (Goldstein *et al.* 2004; Gunderson *et al.* 2008), or by the direct negative effect of melanin on bacterial growth (Suh & Lee 2001; Goldstein *et al.* 2004). However, Grande et al. (2004) found the opposite pattern with black feathers being more degraded by *B. licheniformis* than white ones. Different *Bacillus* strains could therefore be adapted to grow better on feathers of different colors (Grande *et al.* 2004). Black and white feathers may then harbor different bacterial loads and/or communities that could be transmitted to the eggshell directly, or just the antimicrobial chemical, therefore explaining differences in bacterial load of eggshells in nests that varied in color composition of lining feathers. In any case, further work is necessary to explore antimicrobial properties of the bacterial community of white and black feathers to know the underlying causes of the detected effect of the experiment of color composition of nest lining feathers.

The results presented here may have important consequences. We estimated bacterial density of heterotrophic bacterial in general, but also of *Staphylococcus* and *Enterobacteriaceae*; two groups of bacteria that are mainly considered egg pathogens (Bruce *et al.* 1994) (but see Introduction). Bacterial load on eggshells increase the risk of trans-shell infection and egg viability (Cook *et al.* 2003; Cook *et al.* 2005a; Cook *et al.* 2005b). *Staphylococcus* sp. and *Enterobacteriaceae*, as the commonest bacteria found in unhatched eggs are saprophytic and opportunistic bacteria (Singleton & Harper 1998; Houston, Saunders, & Crawford 1997; Cook *et al.* 2005a) that live in skin, hair and feathers of mammals and birds (Krieg & Holt 1984), and they are known to be pathogenic for avian embryos (Bruce *et al.* 1994). Our results showed a negative association between density of these opportunistic and/or pathogenic bacteria and the number of feathers in the nest as well as experimental treatment. This scenario predicts a relationship between nest lining feathers and hatching success that should be mediated by differential bacterial load of eggshells.

In a previous paper (Peralta *et al.* submitted) we analyzed the effect of our experimental treatment and number of feathers of different colors on hatching success and found support for the predicted relationship. Experimental white nests with larger number of added white feathers experienced a lowest probability of hatching failures. In that article we did not explore the effect of eggshell bacterial load on egg hatchability, but directly the effect of the experiment, together with information on the number of white feathers experimentally removed (or added) from swallow nests. Here, however, we failed to find a close relationship between probability of hatching failure and eggshell bacterial load, and, consequently, we cannot conclude that the previously detected effect of feathers on hatching success is mediated by the relationship between nest-lining feathers and eggshell bacterial load. In any case, the detected negative effect of feathers on bacterial load of eggshells of swallows suggests that it should be the case. We estimated bacterial density for only four groups of bacteria, but nest lining feathers could be associated with other groups of bacteria more closely related to the probability of hatching failure. Therefore, information from a concise study of bacterial load on feathers used as lining material, as well as the antimicrobial properties of these

bacteria, is necessary before discussing the expected association between nest lining feathers, nest bacterial environment, and hatching success.

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CAPÍTULO IV:

Colour Composition of Nest Lining Feathers Affects Hatching Success of Barn Swallows, *Hirundo rustica* (Passeriformes: Hirundinidae)

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Abstract: Many bird species use feathers as lining material and its functionality has traditionally been linked to nest insulation. However, nest lining feathers may also influence nest detectability by predators, differentially affect reproductive investment of mates in a post-mating sexual selection process, and affect the bacterial community of the nest environment. Most of these functions of nest lining feathers could affect hatching success but the effect might vary depending on feathers colouration (i.e. pigmented vs. white feathers). That would be the case if colouration is related to (i) thermoregulatory properties, (ii) attractiveness of feathers in the nest for mates, and (iii) eggshell bacterial density. All these hypothetical scenarios predict (1) that feathers of different colours would differentially affect hatching success of birds, and (2) that birds should preferentially chose the most beneficial feather-colour for lining their nests. Results from two different experiments performed in a Danish barn swallow, *Hirundo rustica*, population were in accordance with these predictions. First, barn swallows preferentially selected white experimentally offered feathers for lining their nests. Secondly, experimental manipulation of feather colour composition of nests of barn swallows had a significant effect on hatching success. Experimental nests with more white feathers added at the beginning of incubation had lower probability of hatching failures suggesting differential beneficial effects of nest lining feathers of this colour. We discuss relative importance of hypothetical functional scenarios that predicted the detected associations, including those related to sexual selection or community of micro-organisms associated to feathers of different colours.

Keywords: bacteria-feather colour-hatching success-lining material-nest building-sexual selection

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Introduction

Many birds use feathers as nest lining material (Harrison 1975; Hansell 2000), and their function has commonly been assumed to arise from nest insulation. Among the nest lining materials tested for insulating properties, down were the best at reducing egg cooling rates in artificial nests (Hilton et al. 2004). Comparative evidence consistent with the insulation function of feathers as nest lining material includes that early breeding species of European passerines are more likely to use feathers than later breeders, and that smaller species use feathers in their nests more frequently than large species (Møller 1984). There also exists strong experimental support for this function because feather removal from nests of barn swallows (*Hirundo rustica* L.) and tree swallows (*Tachycineta bicolor*) caused increased rate of heat loss from eggs, increased incubation effort, and resulted in longer nestling periods (Møller 1991; Winkler 1993; Lombardo et al. 1995). The colour of feathers could also be related to their thermal properties, and, as for other biological structures, influence thermoregulation of animals (e.g. Endler 1978; Stuart-Fox & Moussalli 2009). When used as nest lining material, feathers of different colour could therefore affect thermoregulation of the nest environment and consequently incubation behaviour and hatchability of eggs. Feathers in the nest cup of birds might also act as a barrier between nest parasites and nestlings (Lombardo et al. 1995), but the detected association between experimental removal of feathers and parasite load of nestlings could more readily be related to changes in temperature of the nest environment (Winkler 1993; Lombardo et al. 1995).

Colour composition of nest lining feathers may also affect reproductive success of birds (i.e., hatching success). Nest building behaviour is known to be an energetically costly activity (Hansell 2000) that in some species is sexually selected because of the relationship between nest building effort and differential reproductive investment of the individual pair (Palomino et al. 1998; Soler et al. 1998). In particular, the use of feathers as nest lining material has recently been suggested as a potential female signal in the spotless starling (*Sturnus unicolor*) (Veiga & Polo 2005) that females adjust to male sexual display (Polo & Veiga

2006). This hypothetical function of feathers as sexual display for females highlights the importance of feathers colouration, suggesting that feathers are arranged to maximize their conspicuousness within the nest (Veiga & Polo 2005). Thus, feathers of different colour would affect reproductive investment of males and females, as clutch size, incubation effort and hence hatching success, in a scenario of sexual selection.

Nest size in general (Slagsvold 1989), and nest lining feathers in particular (Møller 1987a), affect nest conspicuousness for predators. Feathers of different colouration could differentially affect nest crypsis and consequently nest detectability by predators. Because nest conspicuousness likely affects time spent in the nest by incubating individuals (Kreisinger & Albrecht 2008), it is possible that in this scenario feathers of different colour influence incubation behaviour and, therefore, egg hatchability.

The use of feathers as lining material of nests might also affect the abundance of microorganisms (Peralta-Sanchez et al. 2010). Most of these would be feather degrading bacteria or fungi (Pugh & Evans 1970; e.g. Shawkey et al. 2003; Cristol et al. 2005) that could infect incubating adults or growing nestlings. These microorganisms associated with feather material could also affect the probability of trans-eggshell pathogenic infection of embryos if pathogens occur on feathers. Likewise, non-pathogenic microorganisms associated with feathers could have beneficial effects if, for instance, they occupy space and prevent establishment of pathogens on the eggshell and/or produce antimicrobial substances against egg pathogens (Soler et al. 2010). Interestingly, feather colour affects bacterial growth and the bacterial community on feathers (Grande et al. 2004; Goldstein et al. 2004; Gunderson et al. 2008). Therefore, the colour of feathers used to line nests could influence the bacterial community of the nest environment (i.e. eggshells) (Peralta-Sanchez et al. 2010) , which simultaneously could affect hatching success (Soler et al. 2010).

The above scenarios predict that feather colour composition in barn swallows would affect hatching success. Here, we tested this prediction in a Danish population of barn swallows. In a first experimental approach we determine colour preferences of nest-lining feather by swallows. In a second

experiment, by manipulating colour composition of nest lining feathers we explore the effect on hatching success.

Material & Methods

Field work

The fieldwork was carried out in May-June 1982 and 2008, during the breeding season of the barn swallow at Kraghede, Denmark (57º 12' N, 10º 00' E). For a detailed description of the study area, see Møller (1987a). In 1982, our experiment was performed in one farm while the observational data were collected in non-experimental farms in the study area. In 2008, we used four farms for the experiment including that used in 1982.

Most nest-lining feathers in natural nests of barn swallows in 1982 were from chickens (> 70%), and, thus, we selected feathers from this species to perform the colour preference experiment. In 2008, only one of nine farms in the Kraghede area had chickens (Plymouth Rock), and, consequently, chicken feathers were unavailable for most barn swallows (i.e., we did not find any chicken feathers in barn swallow nests checked in 2008). Furthermore, due to absence of chicken in the farms, the availability of white feathers of appropriated size greatly decreased in 2008. In contrast to 1982, more than 90% of all feathers in barn swallow nests in 2008 were body feathers of gulls *Larus* spp., with additional pigmented feathers from partridge *Perdix perdix*, pheasant *Phasianus colchicus*, blackbird *Turdus merula* and several other species (A. P. Møller *et al.*, unpublished data). Feathers from gulls were either pigmented feathers from juvenile birds, or white feathers from adults. Feather colour composition between years highlight the importance of performing experiments that allow the study of both feather colour preference and differential effects of feather colour on hatching success.

1982 feather supplementation study

The experiment consisted of offering white and pigmented feathers at a similar relative frequency (50%) to determine feather colour preference. Feathers for the

experiment were collected from a single mixed chicken farm with a mixture of White Leghorn and Plymouth Rock and sorted into two categories of white and pigmented (i.e. mainly brownish) feathers, respectively, with all feathers longer than 4 cm being excluded because barn swallows prefer smaller feathers for nest lining. Subsequently, 250 feathers of each of the two categories were dyed black at the base of the shaft with water resistant ink to allow recognition of experimental feathers from other feathers in the environment. On 22 May 1982, APM put out all 500 feathers in the farm yard in the morning at 7.30 a.m., at a time when barn swallows typically are engaged in nest building. On 1 June 1982 APM removed all feathers from the 26 occupied barn swallow nests on the farm, counting the number of white and pigmented feathers with a black shaft. APM also recorded nest lining feathers in 56 non-experimental nests in non-experimental farms during incubation in 1982 by removing all feathers from the nest lining and simply recording the number of white feathers and the number of feathers of other colours. All feathers were subsequently returned to the nests.

We tested the null hypothesis of no colour preference by testing the actual proportion of unpigmented feathers observed in the nests against the null hypothesis of 50% unpigmented feathers.

2008 feather replacement experiment

During May-June 2008, we visited nests twice weekly to determine laying date and clutch size. Once the clutch was complete, we counted feathers of the two colours in each nest. The experiment consists in removing all pigmented feathers, leaving the nest with only white feathers in experimental white nests. The pigmented removed feathers were added in the subsequently sampled black experimental nest from which white feathers were removed keep for using in subsequent experimental white nest. Thus, we alternated experimental treatment among sampled nests. After excluding nests that hatched before second sampling (6) and those that failed to hatch because of predation (1), desertion (1) or because the nest fell down (1), we collected data from 22 experimental “white nests” and 14 experimental “pigmented nests”.

To prevent bacterial contamination between nests, we wore new latex

gloves sterilized with ethanol 70% in each nest. Because pigmented feathers are more numerous than white feathers in nests of swallows (see Results), this experimental approach implies that not only feather colour, but also the number of feathers differed between treatments (Fig. 1). Thus, in addition to experimental treatment, we took into account the experimental change in number of feathers in the nests of barn swallows (number of feathers of different colour that were experimentally added (positive values) or removed (negative values) from experimental nests). Barn swallows were randomly chosen for each treatment and our experiment was blind with respect to parental quality, clutch size and nest size. A few days before hatching, we again visited the nests and counted the number of white and pigmented feathers present in the nest lining material. Subsequently we visited nests at the day of hatching to determine hatching success as the percentage of successfully hatched eggs relative to clutch size.

Statistical methods

Hatching success did not approach a normal distribution, and we used the information as a dichotomous variable (i.e., nests with or without hatching failure). The numbers of white, pigmented and total feathers were approximately normally distributed (Kolmogorov-Smirnov tests for continuous variables, $P > 0.15$). Proportions were square-root arcsine-transformed before tests, reaching normality. Therefore, we used parametric statistical tests in our analyses of these variables.

For exploring the effect of experimental treatment on within-nest variation we used repeated-measures ANOVAs with experimental treatment as between-factor and effect of feather number or feather colour, as within-factors. To explore the effect of number of feathers and experimental treatment on probability of hatching failure we used Generalized Linear Models with binomial error and logistic link function. Akaike's information criterion was used for selecting best models that explained hatching success. This best model was selected from the smallest AICc and higher Akaike weight, ω_i (Table 1). Statistics associated with each term in the best model were estimated in a generalized linear model.

Reported values were means (SE) and statistical tests were two-tailed. Statistica 7.1 package program were used for all performed analyses.

Results

Feature-colour preference

In 1982, the mean (standard error, SE) number of feathers found in experimental nests at the beginning of incubation was 28.0 (2.0) (N = 26). In natural non-experimental barn swallow nests white feathers were more common than pigmented feathers (white feathers 73.0% (2.0), N = 56). In 2008 barn swallow nests on average harboured 31.3 (2.7) (N = 36) feathers with a larger proportion of pigmented feathers (white feathers 42.3% (3.3), N = 36).

All experimental nests in 1982 had more than the expected 50% white experimental feathers, ranging from 56 to 90%. The percentage of white feathers in nests derived from the experiment was 79.0 % (2.0), differing significantly from the random expectation of 50% (one-sample t-test, t_{25} = 59.43, P < 0.0001). Therefore, swallows preferred white feathers for lining their nests.

Hatching success, number and colour of nest lining feathers, and experimental treatment

Before the experiment, nests with different experimental treatments did not differ significantly in total number of feathers or number of pigmented and white feathers (t_{35} < 1.6, P > 0.12). However, before the experiment and for completely random reasons, experimental pigmented nests harboured a larger proportion of feathers of pigmented colour than experimental white nests as revealed from within nest comparisons (Fig. 1; repeated-measures ANOVA, experimental treatment (between factor): $F_{1,35}$ = 0.40, P = 0.53, feather colour (within factor): $F_{1,35}$ = 20.06, P < 0.0001; interaction colour x treatment: $F_{1,35}$ = 7.19, P = 0.011).

Our experimental change in colour composition of feathers in barn swallow nests did not significantly affect the probability of hatching failure (i.e. nests with or without hatching failure) after controlling for the non-significant effects of number of feathers before treatment, number of experimentally added

Table 1: Comparisons of number of white and pigmented feathers in barn swallow nests that did and did not experience hatching failures. All models included experimental treatment as a term. The term "No. experimentally added feathers" (Exp. added feathers) refers to the number of feathers after treatment minus that before treatment. The term "No. added feathers" refers to the number of feathers of the experimentally removed colour that were found in nests of barn swallows at hatching. Results are from generalized linear models with binomial error and logistic link function.

	With hatching failures (N = 13) *Mean (SE)*	Without hatching failures (N = 23) *Mean (SE)*	*Wald statistics*	*P*
Model I				
No. feathers at laying	36.46 (5.08)	28.39 (3.02)	0.21	0.646
No. exp. added feathers	-1.00 (5.68)	1.30 (2.51)	1.65	0.199
No. added feathers	13.77 (1.66)	9.78 (1.38)	3.46	0.063
No. feathers at hatching	25.85 (3.20)	22.04 (2.75)	0.01	0.921
Treatment			1.65	0.198
Model II				
No pigmented feathers at laying	21.92 (3.65)	17.52 (2.53)	1.56	0.211
No. exp. pigmented added feathers	1.00 (7.83)	-2.39 (4.44)	0.18	0.671
No. added feathers	13.77 (1.66)	9.78 (1.38)	2.19	0.139
No. pigmented feathers at hatching	10.38 (3.20)	13.96 (2.95)	1.49	0.222
Treatment			0.07	0.790
Model III				
No. white feathers at laying	14.54 (2.23)	10.87 (1.16)	0.04	0.840
No. exp. white added feathers	-2.00 (2.75)	3.70 (2.44)	4.46	0.035
No. added feathers	13.77 (1.66)	9.78 (1.38)	2.13	0.145
No. white feathers at hatching	15.46 (2.00)	8.09 (1.22)	5.60	0.018
Treatment			3.96	0.047
Model IV (best model)				
No. exp. white added feathers	-2.00 (2.75)	3.70 (2.44)	6.53	0.011
No. white feathers at hatching	15.46 (2.00)	8.09 (1.22)	5.05	0.025
Treatment			4.11	0.043

feathers, and number of feathers at hatching time (Table 1). Subsequently, we analyzed separately effect of the number of white (model III in Table 1) and pigmented feathers (model II in table II) on the probability of hatching failures. The effect of experimental treatment, as well as number of white feathers that were experimentally added and those found in nests at hatching, were significantly related to the probability of hatching failure (Table 1). Experimental white nests where at least one egg failed to hatch, had lower hatching failures than nests receiving the pigmented treatment (42.9%, N = 14, of pigmented nests and 31.8%, N = 22, of white nests had). Nests with a larger number of experimentally added white feathers on one hand, and with a smaller number of white feathers at hatching on the other experienced a lower probability of hatching failure (Table

1). These results were independent of inclusion of information on both white and pigmented feathers, or exclusion of non-significant terms in the model (results not shown).

Table 2. Best model explaining hatching success in barn swallows. Results are based on the Akaike Information Criterion where hatching success where used as dependent factor and treatment as explanatory categorical factors. "No. experimentally added feathers" (No. exp. added feathers) refers to the number of feathers after treatment minus that before treatment. "No. added feathers" refers to the number of feathers of the experimentally removed colour that were found in nests of barn swallows at hatching. "No. feathers at laying" and "No. feathers at hatching" refer number of feathers of each colour at those moments in the incubation period. Models were run without interactions terms. AIC weights (ω) were calculated from the 10 best models.

Models					AICc	Weight	Evidence ratio
No. exp. white added feathers	No. white feathers at hatching	Treatment			38.32	21.1	1.00
No. exp. white added feathers	No. white feathers at hatching	No. added feathers	Treatment		38.46	19.6	1.07
No. exp. white added feathers	No. exp. pigmented added feathers	No. white feathers at hatching	No. added feathers	Treatment	39.60	11.1	1.89
No. exp. white added feathers	No. exp. pigmented added feathers	No. white feathers at hatching	Treatment		40.01	9.0	2.33
No. exp. white added feathers	No. exp. pigmented added feathers	No. white feathers at hatching			40.10	8.7	2.43
No. exp. white added feathers	No. exp. pigmented added feathers	No. white feathers at hatching	No. added feathers		40.29	7.9	2.68
No. white feathers at laying	No. exp. white added feathers	No. white feathers at hatching	Treatment		40.82	6.0	3.49
No. exp. white added feathers	No. white feathers at hatching	No. pigmented feathers at hatching	Treatment		40.84	6.0	3.52
No. exp. white added feathers	No. white feathers at hatching	No. pigmented feathers at hatching	No. added feathers	Treatment	41.05	5.4	3.92
No. pigmented feathers at laying	No. exp. white added feathers	No. white feathers at hatching	Treatment		41.08	5.3	

When including information on the number of feathers of different colour in these analyses, the best model explaining probability of hatching failure (Table 2) included treatment and the number of white feathers at hatching plus information on the number of white feathers experimentally added (Table 2). All these results in combination suggest a direct effect of feather colour on hatching success of barn swallows.

Our experimental approach resulted in experimental pigmented nests harbouring almost twice as many feathers as experimental white nests, and, therefore, the detected effect of feather-colour treatment on hatching failures could be confounded by the effect of experimental feather number. However, several pieces of information suggest that this is not the case. First experimentally added feathers did not explain probability of hatching failures (model 1 in Table 2). The effect of treatment reached statistical significance only in the case that information of number of white feathers was included in the models (models III and IV in Table 1) and appeared even after controlling for the effect of total number of feather added to experimental nests (model IV in Table 1).

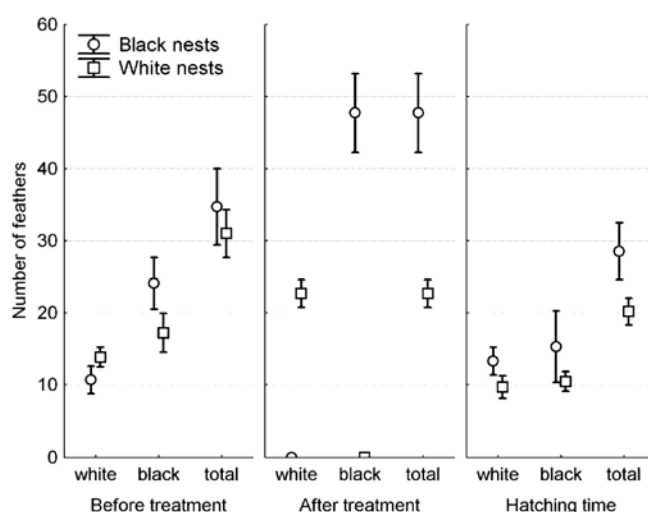

Fig. 1. Average number (± SE) of white, pigmented and total number of feathers before and after treatment and at the end of incubation in barn swallow nests.

Finally, we tested the robustness of results related to experimental treatment by sequentially excluding from the analyses pairs of cases with the most

extreme (i.e. positive and negative) values of number of feathers in the nest after treatment and calculating effect sizes associated with treatment effect in the best model explaining probability of hatching failure (model IV, Table 1). Since effect sizes do not dependent on sample size, we will expect to find a negative relationship between effect size and degrees of freedom if differences in number of feathers between experimental pigmented and white nests were important explaining the detected effect size of experimental treatment. However, estimates of effect size of the 9 first models (reducing degrees of freedom from 32 to 16) were not significantly related with degrees of freedom (R = 0.001, N = 20, P = 0.997). Therefore, all these analyses suggest that the relationship between hatching success and treatment was independent of the larger amount of feathers that pigmented nests received compared with white ones.

Variation in the number of feathers and their colour composition in barn swallow nests along the incubation period

At the time of hatching the experimental effect on feather colour composition (Fig. 1: percentage of white feathers: $t_{35} = 0.88$, $P = 0.38$; number of pigmented feathers: $t_{35} = 1.10$, $P = 0.28$; number of white feathers: $t_{35} = 1.45$, $P = 0.16$), but not that of total number of feathers (Fig. 1; $t_{35} = 2.11$, $P = 0.042$) disappeared, suggesting that nest maintenance activity partially counteracted the experiment. The number of feathers of the removed colour in experimental nests at hatching did not differ significantly between white and pigmented experimental nests (pigmented nests: *mean (SE)* = 13.3 (1.9); white nests: *mean (SE)* = 10.4 (1.3); $t_{35} = 1.23$, $P = 0.23$), suggesting that neither feather colour composition nor the number of feathers significantly affected rate of feather addition to nests.

The total number of feathers in experimental nests decreased from laying to hatching when considering either the number of natural (Fig. 1; repeated-measures ANOVA, effect of time: $F_{1,35} = 13.4$, $P = 0.0008$) or experimental feathers at the beginning of incubation (repeated-measures ANOVA, effect of time: $F_{1,35} = 29.3$, $P < 0.0001$). However, the experimentally manipulated number of feathers decreased at a larger rate in pigmented than in white experimental nests (Fig. 1; repeated-measures ANOVA, interaction treatment x time: $F_{1,35}$ =

13.44, $P = 0.0008$).

Discussion

Barn swallow females, like many other birds, spend considerable amounts of time searching for feathers to line their nests during a period of several weeks before and during laying and incubation (Møller 1987b). Females transporting feathers are chased by other nest owners, and by males seeking extra-pair copulations, suggesting that this behaviour may entail costs.

Experimental change in feather composition may have implications for thermal insulation, nest detectability by predators, attractiveness of nests for mates, or change the nest-bacterial community including pathogenic or beneficial microorganisms. Because these potential effects could affect egg hatchability (see Introduction), we predicted that our experiment on nest lining feathers could affect hatching success. In accordance with this prediction we found that experimental white nests with a larger number of experimentally added white feathers experienced lower probability of hatching failure than experimental pigmented nests. Furthermore, our experiment in 1982 indicated that barn swallows preferred white feathers for nest lining. These results suggest beneficial effects of white feathers at the beginning of incubation and an adaptive preference by barn swallows for white feathers.

Experimental manipulation of the number of feathers in nests of birds has previously been performed (Peralta-Sanchez et al. 2010). Feather removal experiments caused an increase in the rate of heat loss from eggs and an increase in incubation effort, but a direct effect on hatching success had never previously been detected (Møller 1991; Lombardo et al. 1995). Here we manipulated the number of feathers in barn swallow nests and, similarly to previous works, failed to show an effect of the number of feathers on egg hatchability. However, when separately considering white and pigmented feathers, we recorded a statistically significant effect and found that experimental white, but not pigmented nest lining feathers, affected hatching success of barn swallows.

Following the hypothetical scenarios that allowed us to predict that

feathers of different colour may differentially affect hatching success, the detected beneficial effect of white feathers suggest that feathers of this colour have superior thermoregulatory ability that could enhance incubation and/or could be more attractive for mates than pigmented feathers. Furthermore, white feathers in barn swallow nests might be less conspicuous for predators and, thus, females would be able to incubate more efficiently due to lower risk of predation. Finally, white feathers could affect the probability of embryo infection by pathogenic microorganisms (Peralta-Sanchez et al. 2010). Our experiment do not allow to distinguish between these different scenarios, although we can exclude the possibility that attraction of nest predators plays a role, because nest predation is virtually absent in the barn swallow (Møller 1994). These hypothetical scenarios are in any case not mutually exclusive, and it is possible that differential effects of feathers of different colour associated with each of the proposed scenarios have additive effects explaining the associations detected here. Further research is therefore necessary for exploring the function of white nest-lining feathers preventing hatching failures.

The positive relationship between white nest-lining feathers and hatching success predicts that individuals should preferentially select white feathers for their nests. In accordance with this hypothesis, we found that most barn swallows preferred experimentally offered white feathers over those of other colours. Experiments of feather-colour selection by barn swallows for lining their nests were performed 26 years before the experiment of colour composition of nest lining feathers. During this period, availability of feathers of different colour also changed and thus natural colour composition of nest lining feathers of barn swallow nests. Results from 1982 were, however, experimental and we have no reason to believe that barn swallow preference for white feathers has changed during this period.

Adults counteracted our experimental manipulation in 2008, and at hatching the percentage of white feathers did not differ from that found before the experiment. This counteracting behaviour by nest owners did not impaired our experimental approach because the effect of the experimental number of feathers was still present at the end of incubation. Anyway, and apparently in contradiction

to the detected experimental beneficial effects, we found that the number of white feathers at the end of the incubation was positively related to the probability of hatching failure. Composition of nest lining feathers at the end of the incubation is not caused by the experiment, and that paired statistical tests suggested that colour composition at this stage did not differ from that before the experiment. Thus, any factor that covaries with particular colour composition of nest lining feathers, and that also affects probability of hatching failure, would explain the association. For instance, it is possible that birds adjusted the number of beneficial white feathers to the environmental conditions of the nest that predicted hatching success. In this case, and taking into account the experimentally demonstrated beneficial effect of white feathers, barn swallows should increase the number of white and scarce feathers in nests in risky environments (i.e. relatively high probability of hatching failures). In 2008 white feathers were rarer than pigmented feathers, suggesting a significant effort on the part of barn swallows to restore feather composition, especially important when they have to differentially increase the number of white feathers. In any case, this result is not experimental and consequently more difficult to interpret. Feather proportion during the incubation period is an interesting question needing further studies.

In conclusion, we have shown that experimental manipulation of the number and colour composition of nest lining feathers at the beginning of incubation influenced hatching success of barn swallows, suggesting that nest design may have important consequences for reproductive success of birds. The experimental addition of white nest-lining feathers at the beginning of incubation enhanced hatching success, and barn swallows preferred feathers of this colour for nest lining. The experimentally detected effects of feather colour on hatching success and preferences for white feathers can be explained by different ecological scenarios that should be further investigated before conclusions about the different functions of white feathers can be reached.

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CAPÍTULO V:

Nest Visiting by Parasites and Bacterial Environmental Conditions of Avian Nests. The Case of the Brood Parasite Great Spotted Cuckoo and its Magpie Host

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Abstract: Factors related to bacterial environment of nests are of prime interest for understanding the causes of embryo infection and the evolution of antimicrobial defensive traits of birds. Nest visitors such as parasites could act as vectors of bacteria and/or affect hygienic conditions of nests and, therefore, influence nest bacterial environment. We tested this hypothetical scenario in the great spotted cuckoo (*Clamator glandarius*) – magpie (*Pica pica*) system of brood parasitism. Great spotted cuckoos visit the nests of their magpie hosts and, frequently, damage some of the host eggs during the eggs laying or in subsequent visits. Therefore, it is a good system for testing the effect of nest visitors on the bacterial environment of nests. In accordance with this hypothesis, we found that bacterial load of magpie eggshells was greater in parasitized nests. Because we found evidence supporting the assumption that bacterial load of magpie eggshells is related to probability of embryo infection, these results suggest that brood parasitism increases the probability of bacterial infection of magpie eggs. Moreover, comparisons of bacterial loads of cuckoo and magpie eggs revealed that (i) cuckoo eggshells harboured lower bacterial density than those of their magpie hosts in the same nests and (ii) prevalence of bacteria inside unhatched eggs was higher for magpies than for great spotted cuckoos. These interspecific differences were predicted because brood parasitic eggs, but not host eggs always experience the bacterial environments of parasitized nests and, therefore, our results suggest that parasitic eggs are better adapted to environments with high risk of bacterial contamination than those of their magpie hosts.

Keywords: Coevolution, Eggshell bacterial load, Embryo infection, *Enterobacteriaceae*, *Staphylococcus*.

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Introduction

Bacterial environment of avian nests is traditionally considered an important selective agent acting on embryo viability (Baggott & Graeme-Cook 2002) and, therefore, factors related to bacterial environment of nests are of prime interest for understanding the causes underlining probability of embryo infection and the evolution of antimicrobial defensive traits of birds. Temperature, humidity, and hygienic conditions in the nest are known to determine bacterial growth on the eggshells and thus the trans-shell bacterial infection of embryos (Bruce & Drysdale 1994). Moreover, apart from physical and chemical antimicrobial barriers of avian eggs (Board et al. 1994), behaviours such as those related to the nest cleaning (Bruce & Drysdale 1991) or the use of nest materials with antimicrobial properties (Clark & Mason 1985; Clark & Mason 1988; Mennerat et al. 2009) also affect bacterial environment of nests. In addition, others behaviours such as the nest site selection (Godard et al. 2007) or the early start of incubation (Cook et al. 2003; Cook et al. 2005a; Shawkey et al. 2009) would affect temperature and humidity of eggshells and therefore eggshell bacterial load, which is considered a good predictor of trans-shell embryo infection (Bruce *et al.* 1994; Cook *et al.* 2003; Cook *et al.* 2005b). Activity of nest visitors such as ecto- and brood parasites might also influence bacterial environment. They for instance could directly act as vectors of some potentially pathogenic bacterial strains. Otherwise, their activity such as blood sucking, defecation, or breakage of host eggs could affect hygienic conditions of nests and enhance bacterial growth within the nest environment. This hypothetical influence of nest visitors on the bacterial environmental conditions of nests could imply a possible additional cost of parasitism for host species that, as far we know, has never been investigated.

Avian brood parasitism is a reproductive strategy by which parasites lay their eggs in the nests of other species, the hosts, which incubate and take care of parasitic offspring. Brood parasitism often drastically reduces breeding success of their hosts, and it selects for adaptive host responses. When effective defences against brood parasites spread in the host population, counter-defences will rapidly be selected in the brood parasite population, which again selects for more

refined host defences in a coevolutionary arms race between brood parasites and their hosts (Rothstein 1990; Davies 2000; Soler & Soler 2000). Brood parasites inflict usually severe fitness costs on their hosts by, for instance, reducing the number or even eliminate host offspring in parasitized nests. This reduction can be the result of direct adult behaviour when female parasites eat or break some of the host eggs while laying and/or in possible subsequent visits, or due to parasite nestlings evicting or outcompeting host nestlings (see examples in Davies 2000). Interestingly, host-egg damage, either as result of rapid laying from the rim of the nest, or from active pecking of host eggs by brood parasites could deteriorate the hygienic conditions in host nests. It is because nest lining material and eggs could become smudgy with yolk and egg white from damaged eggs which increase nutrient availability for bacterial growth on the eggshell (Stadelman 1994). In addition, even without egg destruction, parasitic eggs or visits to the nest by adult parasites could result in new bacteria from the brood parasite species colonizing host nests (see, Ruiz-Rodriguez et al. 2009) and influencing the bacterial community of host nests. Great spotted cuckoos (*Clamator glandarius*) usually break some of the magpie (*Pica pica*) eggs in the nests (Soler, Soler & Martínez 1997), multi-parasitism is relatively common in this system (Martínez et al. 1998) and we have evidences that cuckoos can visit the magpie nests in several occasions during the egg incubation (Soler *et al.* 1995b). Consequently, there are good reasons to predict that brood parasitism by the great spotted cuckoos would affect bacterial environment of magpie nests.

Here, we explore several predictions of the hypothetical influence of brood parasitism on bacterial environments of host nests by estimating eggshell bacterial loads of magpie and great spotted cuckoo eggs. If brood parasitism affects bacterial environment of magpie nests, we should find that bacterial loads of magpie eggs should be higher in parasitized nests. Evidence of a positive relationship between bacterial load of magpie eggshells and probability of hatching failures would further support the interpretation of the predicted effects as additional costs of brood parasitism. Because the effect of bacteria on hatching failures can be confounded with the effect of parasitism, we explored such as

relationship in a separate magpie population sporadically parasitized by the great spotted cuckoo.

Interestingly, selection pressures of such hypothetical changes in environmental conditions caused by brood parasitism should be asymmetric for brood parasites and hosts (i.e. rare enemy effect (Dawkins & Krebs 1979)). That is, while brood parasitic eggs will frequently experience contaminated nest environments (i.e., with broken eggs or with bacteria from both host and brood parasitic species), that will not be the case for host eggs (i.e., non-parasitized nests). Because the probability of brood parasitism greatly vary in space and time (e.g, Brooke, Davies & Noble 1998; Soler *et al.* 2001; Stokke *et al.* 2008), parasitic eggs, in comparison with host eggs, should have been selected for developing in nest environments with relatively higher risk of infection by microorganisms. This scenario predicts that when brood parasitic and host eggs share identical bacterial environment, probability of embryo infection (i.e., prevalence of bacteria of eggs) would be lower for parasite than for host eggs. We explore this possibility by analysing interspecific differences in eggshell bacterial loads and in probability of trans-shell bacterial contamination of magpie and great spotted cuckoo eggs that did not hatch for unknown reasons.

Material and methods

Study area

The study was performed during the breeding seasons of 2006-2008 in southeast Spain, in the Hoya de Guadix (37º18’N, 3º11’W) (N = 94) a high altitude plateau (1000 m a. s. l.), dominated by a semi-arid climate. The typical vegetation in the areas is cultivated crops, olive and almond plantations, sparse holm oaks remaining from the original Mediterranean forest, small shrubs in abandoned fields, and deciduous trees in seasonal streams and villages. Probability of brood parasitism of magpie nests by the great spotted cuckoo is quite high, but temporally and spatially variable at the small geographic scale of the study area (Soler *et al.* 1999; Soler *et al.* 2000; Martin-Galvez *et al.* 2007).

Field work

Magpie territories from previous years were visited once a week since the 15th of March to detect new nests. Once we found a new nest, it was visited twice a week, which allowed us to know laying date and to detect brood parasitism. During the incubation period, eggshells were sampled twice for bacteria. First samples were taken 2-4 days after clutch completion, which assured that all sampled eggs were incubated. Second samples were collected 2-3 days before hatching. In accordance with previous studies (Soler *et al.* 1995a), magpie clutch sizes of parasitized and non-parasitized nests did not differ (unparasitized: 6.8(0.12), parasitized: 6.4(0.16); $t = 1.78$, $df = 83$, $P = 0.08$) and thus number of days that sampled eggs stayed in the nests before first sampling did not differ between groups of magpie nests. Samples were taken in the field from eggshells attempting to keep conditions as aseptic as possible. New latex gloves sterilized with 96º ethanol were used for each nest to prevent inter-nest contamination. Once gloves were dry, we gently handled and sampled eggs by rubbing the complete eggshell with a sterile rayon swab (EUROTUBO® DeltaLab) slightly wet with sterile sodium phosphate buffer (0.2 M; pH = 7.2). We sampled all the eggs of the same species in the nest with a single swab that, after cleaning the complete egg surface, was introduced in a rubber-sealed microfuge tube with a known volume of sterile phosphate solution and transported in a portable refrigerator at 4-6ºC. Crushed eggs in the nests were not sampled. Samples were stored at 4ºC until being processed in the laboratory. Estimates of bacterial load were standardized to number of colonies (CFU's, Colonies Forming Units) per cm^2 (i.e. eggshell bacterial density, see below) by taking into account total eggshell surface and number of eggs sampled in each nest. Eggshell surface was estimated according to the formula proposed by Narushin (1997):

$$S = 3 \times L^{0.771} \times W^{1.229}$$

where S is the surface in cm^2, L the length of the egg, and W the width of the egg. Length and width of each sampled egg were measured with a caliper (accuracy: 0.02 mm).

Laboratory work

Samples stored in microfuge tubes were shaken in a vortex (Boeco V1 Plus!) for at least three periods of 5 seconds. Afterwards, the solution containing bacteria was used for cultivation. Bacteriology was performed by spreading homogenously 100 µl of sample of each serial dilution onto Petri dishes of four different agar media (Scharlau Chemie S.A. Barcelona). We used Tryptic Soy Agar (TSA), a broadly used general medium to grow aerobic mesophilic bacteria, and three specific media: Kenner Fecal Agar (KF) for *Enterococcus*; Vogel-Johnsson Agar (VJ) for *Staphylococcus*; and Hektoen Enteric Agar (HK) for *Enterobacteriaceae*. The plates were incubated aerobically at 32ºC and colonies were counted at 72h after inoculation. Bacterial density was estimated as number of CFU (Colony Forming Units) per cm^2. We estimated eggshell bacterial density for first samples (soon after laying) and second samples (few days before hatching) for each growth bacterial medium used.

Unhatched eggs were sampled and cultured to detect both internal and external bacterial contamination. Eggshells of each egg was also entirely swabbed using a sterile swab which was aseptically transferred to a sterile tube with 10 ml of PBS; from this tube 1 ml was extracted and used for serial dilutions. Total aerobic mesophilic bacteria were enumerated by duplicate plating of 100 µl aliquots onto TSA (bioMérieux España, S.A.). Plates were incubated at 32ºC for 24-48h. Afterward, colonies were counted and eggshell bacterial density estimated. After disinfection of the eggshells surface with ethanol 70%, unhatched eggs were broken and yolk and egg white homogeneously mixed. Embryos were separated before mixture and in no case were longer than 5 mm (i.e. embryos dead during the first few days of incubation). An aliquot of the content was surface plated onto MacConkey agar and Columbia blood agar (bioMérieux España, S.A.). MacConkey agar is a selective medium for growth of Gram negative bacteria, and Columbia blood agar is a media used to isolate pathogenic organisms and detect hemolytic activity. Plates were incubated for 48 h at 37°C under aerobic and anaerobic conditions. One colony of each sample yielding growth was selected as representative isolate and sub-cultured for further identification. The bacteria were biochemically identified by using commercial

systems (bioMérieux España S.A.). Bacterial analyses were also performed for detection of *Salmonella* contamination. External and internal samples were pre-enriched with buffered peptone water (bioMérieux España, S.A.). After 16 to 20 h at 37°C, two methods of enrichment were used for 18 to 24 h. The first method consisted of inoculation of three drops of sample in a circle close to the periphery of a Rappaport-Vassiliadis (MSRV) plate (Difco). We verified the presence of the halos of growth after 24 h of incubation at 42ºC. Suspected positive growth were surface plated onto Xylose lysine deoxycholate (XLD) and *Salmonella* detection and identification (SMID) agars (bioMérieux España, S.A.), followed by incubation at 37°C for 24 h. The second method was a selective enrichment in Muller Kauffmann with tetrathionate and novobiocin (MKTTn) broth (bioMérieux España, S.A.), which was incubated at 37ºC for 24 h and plated on the above selective solid mediums. A portion of the suspected colonies from XLD and SMID agars were confirmed by the Enterotube system (Difco).

Culture-based techniques do not characterize microbial communities as well as molecular techniques do since only around 1% of micro-organisms are cultivable (Amann, Ludwig & Schleifer 1995). However, culture-independent methods also have limitations of bias and errors (Qiu et al. 2001; Speksnijder et al. 2001; Shawkey et al. 2005). Interestingly, both methodologies yielded identical conclusions when exploring the effects of incubation on eggshell bacterial community (Cook et al. 2005a; Shawkey et al. 2009). Moreover, apart from the general medium for aerobic mesophiles, we have selected specific media for most common groups of bacteria known to live in avian eggshells and known to reduce embryo viability based on extensive studies of bacteria on domestic and wild bird eggs (Board & Tranter 1986; Kozlowski *et al.* 1989; Bruce *et al.* 1991; 1994; Houston, Saunders & Crawford 1997; Cook *et al.* 2003; 2005a; 2005b; Soler *et al.* 2008; Shawkey *et al.* 2009; Peralta-Sánchez *et al.* 2010). Used together, these media should adequately characterize the relative load of bacterial groups living on the avian eggshell that are known to produce pathogenic infection of embryos.

Sample sizes and statistics

Bacterial loads did not approach normal distributions even after log_{10}-transformed

(Kolmogorov-Smirnov tests for continuous variables, $P < 0.05$). Thus, we used rank values rather than $\log_{10}$-transformed bacterial counts in our analyses. In figures, and just to show information on eggshell bacterial density, we show $\log_{10}$-transformed data.

Year or its interaction with species identity did not significantly explain eggshell bacterial load either, at the beginning (MANOVA; dependent variables: TSA, KF, VJ, and HK culture media; independent variables: species identity and year of sample: effect of year: Wilks = 0.92, $F = 2.27$, $df = 4, 109$, $P = 0.07$; univariate results: $F < 2.76$, $df = 1, 112$, $P > 0.10$; year and species identity interaction: Wilks = 0.97, $F = 0.97$, $df = 4, 109$, $P = 0.43$; univariate results: $F < 1.23$, $df = 1, 109$, $P > 0.27$), or at the end of the incubation period (identical MANOVA model: effect of year: Wilks = 0.96, $F = 0.67$, $df = 4, 68$, $P = 0.61$; univariate results: $F < 1.97$, $df = 1, 71$, $P > 0.16$; year and species identity interaction: Wilks = 0.99, $F = 0.21$, $df = 4, 68$, $P = 0.93$; univariate results: $F < 0.24$, $df = 1, 71$, $P > 0.62$). Therefore, we did not include year and its interaction in subsequent analyses.

MANOVAs with eggshell bacterial densities in TSA, KF, VJ and HK as dependent variables and nest status (parasitized *vs*. non-parasitized) or species identity (magpie and great spotted cuckoo in parasitized nests) as independent factors were used respectively to test for the effect of parasitism and species on eggshell bacterial loads. For within-nest comparisons we used Repeated Measures MANOVAs with media for bacterial growth as dependent variables and species identity (magpie *vs* great spotted cuckoo eggs) or stage of incubation (at the beginning or at the end of incubation) as within factor.

We collected information from 33 parasitized and 51 non-parasitized magpie nests, but sample sizes differ for first and second sampling of parasitized and non-parasitized nests (Table 5.1) for several reasons. Swabs from nests that were found after clutch completion (first samples) were not used except for nests that hatched 2-3 days after swabbing the eggs (second samples). Furthermore, we lost a first sample of a great spotted cuckoo egg, and some of the sampled nests at the beginning of the incubation were depredated before the second sampling.

The effect of eggshell bacterial load on probability of hatching failures

was tested in 2008 in a magpie subpopulation of our study area rarely parasitized by the great spotted cuckoo (only two of 64 magpie nests were parasitized). In our analyses we did not use parasitized nests or those with magpie eggs damaged (five additional nests). We avoid the use of parasitized nests in this analysis because parasitism by the great spotted cuckoo may results in micro-fissures of magpie eggs (Soler et al. 1997) that are difficult to detect and that may affect gas-exchange of eggs and therefore hatchability. Thus, results from this test should be interpreted as showing the relationship between bacterial environment of magpie nests and hatchability of magpie eggs, but not of the influence of bacteria from brood parasitism by the great spotted cuckoos. After excluding nests depredated before sampling, we collected information from 49 non-parasitized magpie nests. The hypothesysed association between probability of hatching failure (nest with or without hatching failures) and eggshell bacterial density were tested by mean of logistic multiple regression. Non-significant terms in the models were sequentially removed up to find significant differences in deviance (-2*log(Likelihood)) of two consecutive models.

Table 5.1: Number of parasitized and unparasitized nests sampled at the beginning and at the end of the incubation period. Number of sampled nests containing eggs of both species, as well as those sampled twice during the incubation period is also shown.

	Species	Early incubation	Late incubation	Both times
Parasitized				
	Magpie	33	19	18
	Great spotted cuckoo	32	11	9
	Both species	32	10	9
Unparasitized				
	Magpie	51	45	36

Unhatched, but incubated eggs of great spotted cuckoos (N = 9, from 3 different nests) and magpies (N = 13, from 11 different nests) (only from one nest we collected eggs from both species) were collected during field work in 2008 and 2009 five to seven days after the last hatching event in the nests. Within nest variation in eggshell bacterial counts was smaller than the among nest variation for magpie (One-way ANOVA, F = 58.06, df = 10,2, P = 0.017), but not for cuckoo unhatched-eggs (One-way ANOVA, F = 0.37, df = 2,6, P = 0.70). Thus, we used two types of analyses, using either eggs or nests as independent data

points. Unhatched eggs were visually and carefully inspected for fissures or narrow cracks on the eggshells, and we only collected those without such traces. These eggs were stored in individual and sterile tubes at 4ºC until processing in the lab. Finding bacteria inside eggs that failed to hatch cannot be interpreted as these bacteria being the cause of hatching failure since, for instance, trans-shell infection by bacteria may have occurred after death of embryos. Thus, following previous studies, we used unhatched eggs for exploring interspecific differences in probability of trans-shell contamination of eggs (see examples in Bruce *et al.* 1994).

All analyses were two-tailed and conducted with STATISTICA 9.0.

Results

<u>Eggshell bacterial loads and hatching success of eggs in an unparasitized magpie subpopulation</u>

Hatching failures (13 out of 49 nests) occurs more frequently in magpie nests with eggs that harboured high density of *Staphylococcus* at the beginning of the incubation (Logistic regression; -2*log(Likelihood) = 25.8; χ^2 = 5.12, df = 1 , P = 0.024, Fig. 5.1; the second best model included also *Enterobacteriaceae*; difference in deviance = 2.63, P = 0.10). Eggshell bacterial density of *Enterobacteriaceae* at the end of the incubation explained significantly the probability of hatching failures (Logistic regression; -2*log(Likelihood) = 25.8; χ^2 = 5.01, df = 1 , P = 0.025, Fig. 5.1; the second best model included also *Staphylococcus*; difference in deviance = 1.79, P = 0.18) (Fig. 5.1). Furthermore, estimates of eggshell bacterial load from the four culture media were significantly related to each other at the beginning (R > 0.33, N = 49, P < 0.019) and at the end of the incubation (R > 0.46, N = 49, P < 0.001, except for *Staphylococcus* counts (R < 0.21, N = 49, P > 0.14)).

When comparing bacterial loads of magpie eggshells in parasitized and non-parasitized nests we found statistically significant differences for load estimates at the beginning of the incubation period, with a greater bacterial load on eggs of parasitized nests, but not at the end of the incubation period (Table 5.2,

Fig. 5.2). In parasitized nests, magpie eggs harbored higher bacterial density on their shells than eggs of the great spotted cuckoo, but this difference completely disappeared at the end of the incubation period (among nests comparisons, Table 5.2, Fig. 5.2). Results from Repeated Measures ANOVAs (i.e., within nest comparisons, Table 5.2) again resulted in significant differences between cuckoo and magpie eggs at the beginning of the incubation period although at the end of incubation, shells of magpies eggs showed a non-significant trend to harbor *Staphylococcus* (i.e. VJ) at a higher density than great spotted cuckoo eggs in the same nests (Table 5.2, Fig. 5.2).

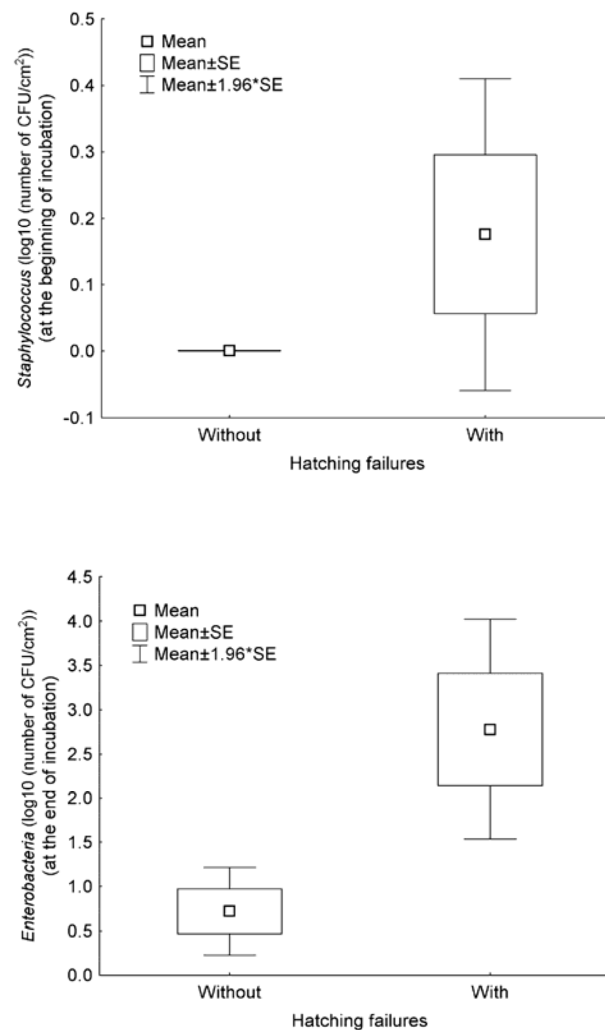

Fig. 5.1: Means (± SE (bars) and confidence intervals (whiskers)) of log10-transformed estimates of eggshells bacterial loads of magpies in

nests with and without hatching failures at the beginning (*Staphylococcus* sp.) and the end (*Enterobacteriaceae*) of the incubation period.

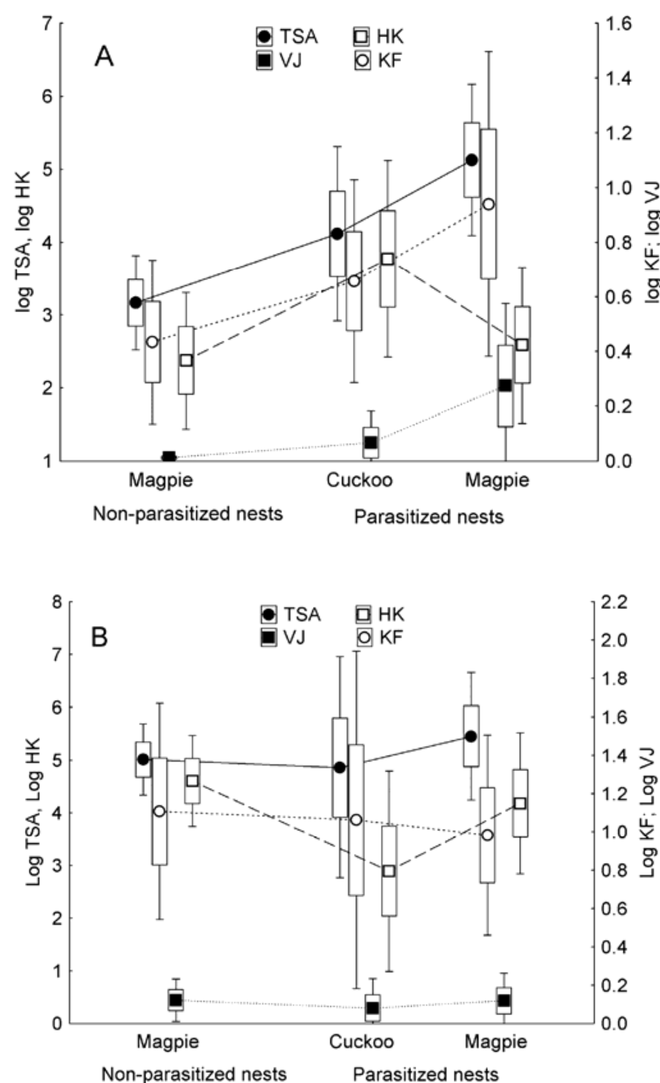

Fig 5.2: Means (± SE (bars) and confidence intervals (whiskers)) of log10-transformed estimates of eggshells bacterial loads of great spotted cuckoos and magpies in parasitized and non-parasitized nests at the beginning (A) and the end (B) of the incubation period. Estimates were performed from cultures in non-specific medium (TSA), as well as in specific media for *Enterococcus* sp. (KF), *Staphylococcus* sp. (VJ) and *Enterobacteriaceae* (HK).

Eggshell bacterial loads and brood parasitism

Finally, from unhatched eggs (i.e. those collected to study the bacterial load inside the eggs), estimates of density of total aerobic mesophiles were higher on magpie than on great spotted cuckoo eggshells, both when considering nests as independent data points (Mann-Whitney U Test; magpies: N = 11, Rank Sum =

Table 5.2. Multivariate comparisons of eggshell bacterial loads of magpie and great spotted cuckoo eggs estimated in parasitized and non-parasitized nests, at the beginning and the end of the incubation period. Estimates from the four different culture media (TSA, KF, VJ, and HK) were included as dependent variables and parasitism status (parasitized vs. non-parasitized), or species identity (magpie vs. cuckoo eggs) as between or within (i.e. Repeated Measures) independent factors. Univariate results are also shown.

		MANOVA TESTS				UNIVARIATE RESULTS								
		Wilks	F	df	P		TSA		KF		VJ		HK	
						df	F	P	F	P	F	P	F	P
Among nests comparisons														
Parasitized vs. non-parasitized nests	Laying	0.81	4.61	4,79	0.002	1,82	15.68	0.0002	3.94	0.05	1.95	0.17	0.16	0.69
	Hatching	0.90	1.73	4,59	0.15	1,62	1.04	0.31	4.07	0.05	0.39	0.53	0.24	0.62
Magpie *vs.* cuckoo eggs	Laying	0.81	3.59	4,60	0.011	1,63	1.06	0. 31	0.23	0.63	1.46	0.23	1.82	0.18
	Hatching	0.91	0.66	4,25	0.63	1,28	0.19	0.57	0.01	0.98	0.04	0.84	1.91	0.18
Within nests comparisons														
Magpie *vs.* cuckoo eggs	Laying	0.69	3.18	4,28	0.028	1,31	1.28	0.27	0.52	0.48	1.52	0.23	3.24	0.08
	Hatching	0.53	1.33	4,6	0.36	1,9	1.06	0.30	0.22	0.65	3.79	0.08	2.30	0.16

96; cuckoo: N = 3, Rank Sum = 9; Z = 2.10, P = 0.038, Table 5.3) and when using eggs as independent data points (Mann-Whitney U Test; magpies: N = 13, Rank Sum = 204; cuckoo: N = 9, Rank Sum = 49; Z = 3.64, P = 0.0003, Table 5.3).

Table 5.3: Log-transformed eggshell bacterial loads (number of CFU (Colony Forming Units) per cm^2) of unhatched magpies and great spotted cuckoo eggs. Bacteria species detected inside each analysed eggs are also shown.

Species	Nest (egg)	Eggshell bacterial loads	Species of bacteria detected within eggs
Magpie			
	1	6.696	*Enterococcus faecium, Enterococcus faecalis*
	2	7.112	*Staphylococcus auricularis, Enterococcus faecium*
	3	7.604	*Pseudomonas fluorescens*
	4 (1)	6.088	*Enterobacter cancerogenus*
	4 (2)	7.424	*Enterococcus faecium, Enterococcus faecalis*
	5	6.792	*Enterococcus faecium*
	6 (1)	7.394	*Enterococcus casseliflavus, Enterococcus faecium*
	6 (2)	7.119	*Enterococcus faecium, Serratia marcescens*
	7	8.548	*Enterococcus faecium*
	8	8.035	None
	9	6.148	*Aerococcus viridans, Staphylococcus xylosus*
	10	3.760	*Stomacoccus mucilaginosus*
	11	5.720	*Salmonella spp.*
Great spotted cuckoo	11	4.681	*Salmonella spp.*
	12 (1)	4.886	None
	12 (2)	3.942	None
	12 (3)	2.845	None
	13 (1)	2.763	*Staphylococcus xylosus*
	13 (2)	2.767	None
	13 (3)	4.869	*Enterococcus faecium*
	13 (4)	3.658	None
	13 (5)	2.839	None

Variation of eggshell bacterial loads along the incubation period

In non-parasitized nests, bacterial load of magpie eggshells increased throughout the incubation period (Repeated Measures MANOVA, time of sampling (i.e. beginning or end of incubation) as within factor, Wilks = 0.67, F = 4.02, df = 4, 32, P = 0.009; univariate results: Total aerobic mesophilic bacteria (TSA) (F = 6.77, P = 0.01); *Enteroccoccus* (KF) (F = 5.59, P = 0.023); *Staphylococcus* (VJ) (F = 2.03, P = 0.16); and *Enterobacteriaceae* (HK) (F = 7.07, P = 0.01); all df = 1, 35). This tendency was not detected in parasitized nests (Repeated Measures MANOVA, time of sampling as within factor: for cuckoo eggs, Wilks = 0.25, F = 3.65, df = 4, 5, P = 0.09; for magpie eggs, Wilks = 0.63, F = 2.10, df = 4, 14, P = 0.14) (Fig. 5.2). However, when looking at the univariate results, *Enterococcus*

(Repeated Measures ANOVAs; magpie eggs in non-parasitized nests: F = 6.72, df = 1, 17, P = 0.019) and *Enterobacteriaceae* (Repeated Measures ANOVAs; F = 5.61, df = 1, 17, P = 0.03) increased during incubation in magpie eggs in parasitized nests. Throughout the incubation period, eggshell bacterial load of cuckoo eggs did change with the exception of *Enterococcus* (Repeated Measures ANOVAs; F = 13.16, df = 1, 8, P = 0.006, Fig. 5.2).

Bacterial loads inside eggs

We detected bacteria inside unhatched eggs of both great spotted cuckoos and magpies (Table 5.3). However, magpie and great spotted cuckoo eggs differed in probability of trans-eggshell infection (12 infected out of 13 magpie eggs and 3 infected out of 9 cuckoo eggs, Fisher exact test, P = 0.007). Bacterial diversity inside of infected magpie eggs were also higher than that of cuckoo eggs: 11 bacterial species were characterized for magpie eggs while only three bacteria species were detected in unhatched eggs of great spotted cuckoos (Table 5.3). Furthermore, while a single bacterial species was detected per infected cuckoo eggs (N = 3), half of the infected magpie eggs harboured more than a single bacterial species (average = 1.5 species per infected egg (N = 12)). As mentioned above, the presence of bacteria inside unhatched eggs should not be interpreted as evidence of bacteria being the cause of embryo death (see above), but in terms of eggshell permeability to different bacteria. Accordingly, these results suggest that eggshells of magpie eggs were apparently more permeable to more bacterial species that eggshells of cuckoo eggs.

Discussion

We quantified density of *Enterobacteriaceae*, *Staphylococcus* and *Enterococcus* on the eggshells of magpies and their brood parasite, great spotted cuckoos, at the beginning and at the end of the incubation period. *Enterobacteriaceae* and *Staphylococcus* sp. are saprophytic and opportunistic bacteria (Singleton & Harper 1998; Houston et al. 1997; Cook et al. 2005a) that live in skin, hair and feathers of mammals and birds (Krieg & Holt 1984). They commonly appear on

avian eggshells and are known to be pathogenic for avian embryos (Bruce *et al.* 1994). Enterococci, the third analysed group of bacteria, are also frequently found inside unhatched eggs (Bruce *et al.* 1994), including those of magpie and great spotted cuckoo (see Table 5.3). Although Enteroccocci are opportunistic pathogens (Franz, Holzapfel & Stiles 1999), they might also have beneficial effects for embryos (Soler et al. 2008). Most of these bacteria are able to penetrate eggshells (Board et al. 1994; Cook et al. 2003) and accordingly, we identified some of them inside unhatched eggs (see results). In addition, we also quantified total eggshell bacterial load able to grow in aerobic heterotrophic medium, which is positively related to probability of embryo infection (Bruce *et al.* 1994; Cook *et al.* 2003; Cook *et al.* 2005b), and therefore our estimations of eggshell bacterial load of great spotted cuckoo and magpie eggs is likely reflecting probability of embryo infection experienced by eggs of both species. In agreement with this assumption, we found that density of *Staphylococcus* and that of *Enterobacteriaceae* on the eggshell of magpies estimated at the beginning and at the end of the incubation, respectively, explained probability of hatching failures of magpie eggs in a subpopulation scarcely parasitized by the great spotted cuckoo. Moreover, estimates of eggshell bacterial density from four different culture media were positively related to each other, which support our estimates as a proxy of bacteria selection pressure on the eggs of magpies and great spotted cuckoos.

Our analyses showed that at the beginning of incubation magpie eggshells in parasitized nests harboured higher bacterial density than those of non-parasitized nests, and these differences disappeared at the end of incubation. A second group of results showed that bacterial density on eggshells of great spotted cuckoo eggs was lower than that estimated for magpie eggs, even when considering within nest variation. Therefore, these results suggest that brood parasitism could increase the probability of bacterial infection of magpie eggs, and that parasitic eggs may be better adapted to environments with high risk of bacterial contamination than host eggs. This interpretation is supported from bacteriologic analyses of eggs that failed to hatch because trans-shell colonization of eggs was more frequent for magpie than for cuckoo eggs. Below we discuss

these interpretations and alternative scenarios that could explain our results.

Most embryo mortality occurs at the beginning of incubation (Beissinger, Cook & Arendt 2005), and bacterial loads at that stage seems to be the key factor predicting embryo bacterial infections (Bruce *et al.* 1994; Shawkey *et al.* 2009). Therefore, the higher eggshell bacterial loads detected in parasitized magpie nests at the beginning of incubation may result in lower hatching success and imply an extra cost of parasitism by the great spotted cuckoo. Hatching success in parasitized magpie nests is known to be lower than that in non-parasitized nests (Soler, Martínez & Soler 1996), which has been interpreted to be a consequence of host eggs breakage due to brood parasitism. Number of magpie eggs broken as a result of brood parasitism in our study area was however quantified in another article (1.49 per clutch, (Soler et al. 1997)) and, estimates of hatching success of intact eggs in parasitized nests (32.2%) were still lower than those in non-parasitized nests (71.8%) (Soler et al. 1996; Soler et al. 1997)). Thus, it is possible that variation in hatching success between parasitized and non-parasitized magpie nests was at least partially explained by differences in eggshell bacterial load detected here. This hypothesis is difficult to test with empirical data in host populations heavily parasitized by great spotted cuckoos because parasitism might result in undetected damage of host eggs that could affect hatching failures. Trying to detect the predicted association between eggshell bacterial loads and hatching failures in non-parasitized magpie nests, in 2008, we studied a magpie subpopulation very rarely parasitized by the great spotted cuckoo (3.1% of magpie nest were parasitized). In accordance with the assumption, we found that density of *Staphylococcus* and *Enterobacteriaceae* predicted probability of hatching failures in unparasitized magpie nests. Thus, because bacterial densities estimated from different specific media are related to each other (see results), our results support the use of eggshell bacterial loads as a proxy of probability of trans-shell embryo infection and hatching failure in magpie nests.

Causes explaining detected differences in bacterial load of magpie eggshells could be related to particular environmental conditions related with brood parasitic activity at host nests that enhance bacterial growth. For instance, at the time of parasitism, but also during subsequent nest visits, great spotted

cuckoos can damage some magpie eggs (Soler et al. 1997; Soler & Martínez 2000), and sometimes magpie (and cuckoo) eggshells became partially covered with yolk and egg white from damaged eggs (Soler et al. 1997). Such remains of damaged eggs are in fact prime nutrients for bacterial growth (Stadelman 1994), and we think they could be the cause of the detected higher bacterial load in parasitized nests. However, we have so far no experimental data to test this hypothesis. An alternative hypothesis explaining the higher bacterial load of magpie eggshells in parasitized nests is that bacteria living in the oviduct and/or cloacae of great spotted cuckoos could colonize the host eggshells in parasitized nests. Intestinal microbiota of great spotted cuckoos and magpies likely differ (Ruiz-Rodriguez et al. 2009), and eggshells in parasitized, but not in non-parasitized nests, can harbour bacteria from the intestinal tract (i.e. cloacae) of both parasitic and host females.

Our data are non-experimental and consequently we cannot exclude any of these alternative explanations, which otherwise should be considered complementary causes underlining the hypothesis that nest visiting of brood parasites would affect the bacterial environment of nests. Because of the correlative nature of our results, an alternative non-functional explanation would imply that great spotted cuckoos selectively parasitize nests with high bacterial load. However, great spotted cuckoos select foster parents of a higher than random phenotypic quality (Soler *et al.* 1995a), and, as far as we know, a positive relationship between eggshell bacterial load and phenotypic quality seems improbable. On the other hand, the larger number of eggs in parasitized nests (host plus parasitic eggs) might also be the cause of the detected higher bacterial load; however, we estimated bacterial load per cm^2 of eggshell, and thus our estimates were statistically controlled by clutch size. Thus, our results suggest an additional direct cost of brood parasitism for magpies that may affect coevolutionary processes between great spotted cuckoos and magpies in particular, but also those between other host species and their brood parasites.

Egg incubation reduces eggshell bacterial load and therefore probability of embryo infection (Cook et al. 2005a; Shawkey et al. 2009). Thus, although the mechanism is not clear (Cook et al. 2005a), incubation should reduce the effect of

brood parasitism on bacterial load of magpie eggs. In accordance with this prediction, we found no difference in bacterial load of magpie eggs in parasitized and non-parasitized nests at the end of incubation. However, bacterial load of magpie eggshells increases rather than decrease during incubation. This increase in bacterial load was mainly detected in non-parasitized nests, those with lower eggshell bacterial density, suggesting that the effect of incubation on bacterial growth depended on bacterial load of eggshell at the beginning of the of incubation. These results would also indicate that magpies through incubation behaviour are able to control a runaway growth of bacteria established at a high density on the eggshell of parasitized nests. Another alternative explanation is that the bacterial carrying capacity of eggshells in parasitized nests was close to the maximum at the beginning of incubation, and that bacterial density could only increase in eggs that at the beginning of incubation harboured low bacterial density (i.e. those from non-parasitized nests). Our non-experimental results, however, do not allow distinguishing between these alternative explanations, and thus, further experimental approaches are needed.

The detected higher eggshell bacterial load in parasitized nests should have consequences for the evolution of host and parasite strategies that reduce probability of bacterial colonization and growth on the eggshell if it affects hatching failures due to embryo bacterial infection (Bruce *et al.* 1994; Beissinger *et al.* 2005; Shawkey *et al.* 2009). We have found a positive relationship between bacterial load of eggshells in unparasitized magpie nests and probability of hatching failure. Thus, if we assume a similar relationship for parasitized nests, where bacterial densities are higher, all parasitic eggs, but only some host eggs will develop in nests with high probability of eggshell bacterial contamination (i.e. parasitized nests), and consequently the former are under stronger selection than the latter. Therefore, this evolutionary scenario predicts that parasitic eggs should be better adapted to develop in nest environments with elevated probability of bacterial colonizing and/or crossing the eggshell. Several findings suggest that certain characteristics of the eggshells of the great spotted cuckoo may function to reduce eggshell bacterial load and growth during the incubation period. First, we have found that at the beginning of incubation eggshell bacterial density of great

spotted cuckoos is lower than that of magpie eggshells. That was the case even when comparing bacterial load of parasitic and host eggs within the same nest (see Results). However, interspecific differences tended to disappear at the end of incubation, and, therefore, it could be argued that detected interspecific differences in the bacterial community of eggshells were due to species-specific traits (i.e., variation in cloacae bacterial community of great spotted cuckoo and magpie females). Several reasons make this explanation unlikely. First, the eggshells of both species were sampled some days after egg-laying and, therefore, the identical environmental conditions that eggs of both species shared for several days should help diluting hypothetical initial interspecific differences. Secondly, at the end of incubation, we found that density of *Staphylococcus* isolates from magpie eggshells was higher than that of great spotted cuckoo eggs. Finally, we found interspecific differences in eggshell bacterial load of eggs that failed to hatch and that were kept in the nests for five-seven days after incubation finished, which cannot be explained by interspecific differences in the bacterial community of adult females.

The second group of results suggesting that great spotted cuckoo eggshells are better adapted than those of magpies to an environment of high probability of bacterial infection of eggs came from the analyses of bacterial community inside unhatched eggs of both species. Trans-shell bacterial contamination occurs in nature at a quite low rate, and it is thought to be consequence of the very efficient antibacterial defences of eggs (Board et al. 1994). Therefore, sample sizes necessary to detect interspecific differences of embryo infection in natural conditions with viable eggs would be enormous, but also ethically unacceptable. Bacteria are more frequently found inside eggs that fail to hatch independently of whether or not embryo death was due to bacterial infection. This is because physical and chemical barriers deteriorate with time (Stadelman 1994), but also because eggshell bacterial load should increase when incubation cease (see above). Thus, by assuming a depreciable rate of bacterial infection during egg formation (Baggott *et al.* 2002), unhatched eggs are commonly used for exploring bacteria able to penetrate the eggshells or for detecting differential probability of trans-shell contamination of eggs in relation to different environmental conditions

(see examples in Bruce *et al.* 1994). Accordingly, we tested the null hypothesis of no interspecific differences in probability of trans-shell bacterial infection with cuckoo and magpie eggs that failed to hatch. We found higher bacterial prevalence and a more diverse bacterial community in eggs of magpies than in those of the great spotted cuckoo, which suggests that specific characteristics of eggs of great spotted cuckoos restrict bacterial infection. It is known that brood parasitic species lay eggs with shells that are thicker, denser, more rounded and, hence, stronger than those of both their hosts and their non-parasitic relatives (e.g., Rahn, CurranEverett & Booth 1988; Brooker & Brooker 1991; Picman & Pribil 1997). Interspecific differences in eggshell properties (availability of appropriate space for bacterial growth (i.e., porosity), antibacterial properties of cuticle, etc.) should therefore explain differences in eggshell bacterial density of cuckoo and magpie eggs sharing environmental conditions of parasitized magpie eggs. Support of this hypothesis would need further research, including detailed microscopy of eggshells of these species and lab experiments exploring bacterial permeability to bacteria of eggshells varying in thickness, pore density, and pore size. It should be noted here that this suggestion is based on results from low sample size and therefore should be cautiously interpreted.

To summarize, our results suggest that changes in magpie nest environments associated with brood parasitism by great spotted cuckoos elevate bacterial density on eggshells. Furthermore, because parasitic eggs more frequently experience environments with elevated bacterial density, while host eggs do not, we predicted and found support to the hypothesis that great spotted cuckoo eggs should be better adapted to that environment. Further work is however necessary to determine the causes explaining the association between parasitism and eggshell bacterial load, as well as to determine what cuckoo egg traits that reduce the bacterial load of their eggshells.

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CAPÍTULO VI

Chelex-based DNA isolation procedure for the identification of microbial communities of eggshell surfaces

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Abstract: Microbial communities present on eggshell surfaces of wild birds are weakly studied, especially their influence in embryo infection, thus egg viability. Bacterial density of wild bird eggshells is very low and most DNA-extraction protocols are frequently unsuccessful. We have efficiently adapted a chelex-based DNA isolation method for 16S ribosomal gene amplification from the total communities of eggshell surfaces from six avian species.

Keywords: microbial communities; eggshell; DNA; PCR

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The eggshell microbiota has mainly been studied on farm eggs using traditional culturing microbiological methods (Bruce & Drysdale 1994). The emergence of culture independent methods in microbial diversity studies and their functionality within complex ecosystems explores both cultivable and non-cultivable bacteria, i.e. a much higher number and diversity (Rappe & Giovannoni 2003, Schleifer 2004). The first culture independent study for these communities has been recently published by Shawkey and coworkers (2009) on eggs of the avian species *Margarops fuscatus*. DNA analysis is the basis for most of these techniques; consequently, we aimed to achieve a DNA isolation procedure suitable for a high number of samples, and total community DNA suitable for further analyses. To this end we adapted a chelex-based DNA isolation method for studying microbial community from eggshells. Chelex is a chelating resin that has high affinity for metals ions, protecting the DNA and improving PCR (Scheu et al. 1998). The advantages of using this method are based on the simplicity of the method, its low cost, the avoidance from using hazardous reagents, and the successful PCR from micro amounts of DNA (Huang & Huang 2008, Tomasek et al. 2008). Isolation of DNA by a chelex method has been applied to samples of different origin including bacteria (Lamballerie et al. 1992, Giraffa et al. 2000), but has limited application to study complex communities. In the curret work we have successfully applied a modified chelex method for DNA isolation from eggshells which could be used to study the microbial diversity from this environment.

Samples from 25 clutches (Table 6.1) were collected in the field with sterile gloves as follows. The eggs were completely cleaned with a sterile swab slightly wet with sterile phosphate buffer and stocked up in a microcentrifuge tube with 1.2 ml of sterile phosphate solution. Samples were stored at 4 ºC until processing. Then 10 µl of each sample was 10:1 serially diluted in sterile saline solution (0.8% NaCl) up to 10^{-6} and a 100 µl aliquot of each dilution was spread on agar plates for bacterial enumeration on general and selective media: Tryptic Soy Agar (TSA), Hecktoen Enteric Agar (HK), Vogel-Johnsson Agar (VJ), and Kenner Faecal Agar (KF). Plates were incubated at 37 ºC for 3 days.

The remaining volume of each sample was retained for total genomic DNA isolation. Bacterial cells were harvested by several centrifugation steps

Table 6.1. Microbial counts and DNA extraction efficiency from eggshell surfaces.

		Microbial counts[a] (Log CFU/clutch)					16S PCR DNA dilution			16S PCR (plus BSA) DNA dilution		
Sample	Avian species	TSA	HK	KF	VJ	Eggs/clutch	0	1/10	1/100	0	1/10	1/100
Cast. Quem.2	*Upupa epops*	7.151	7.084	NG[b]	4.623	4	-	-	+	-	+	+
H789.1	*Upupa epops*	7.955	7.532	5.441	NG	8	+	+	+	+	+	+
C.44.1	*Upupa epops*	5.155	4.812	3.429	2.193	7	+	+	+	+	+	+
H851	*Upupa epops*	8.121	7.924	7.096	NG	7	+	+	+	+	+	+
H.HV14.1	*Upupa epops*	8.121	7.857	7.136	3.183	7	+	+	+	+	+	+
TEJ 25.1	*Parus major*	6.158	6.960	1.857	1.380	8	+	+	+	+	+	+
CA51.2	*Parus major*	5.778	4.477	3.158	NG	10	+	+	-	+	+	+
C18.1	*Parus major*	3.778	2.742	1.079	NG	7	+	+	-	+	+	-
CA61.1	*Parus major*	2.121	1.857	NG	NG	4	-	+	-	-	+	-
CA51.1	*Parus major*	7.885	7.635	3.799	NG	10	+	+	+	+	+	+
BEC 15.1	*Athene noctua*	3.778	2.742	1.079	NG	3	+	+	+	+	+	+
769.1	*Athene noctua*	8.310	8.193	6.692	1.079	3	+	+	+	-	+	+
133.1	*Athene noctua*	7.376	5.426	3.320	5.523	3	+	+	+	-	+	+
842.1	*Athene noctua*	7.730	6.225	5.792	NG	5	+	+	+	+	+	+
55.1	*Athene noctua*	7.955	8.121	3.788	NG	5	+	+	+	+	+	+
P25.2	*Sturnus unicolor*	4.769	3.294	1.778	1.380	5	+	+	-	+	+	+
HUE 1.1	*Sturnus unicolor*	8.121	7.677	8.598	NG	6	+	+	+	-	+	+
HUE 31.1	*Sturnus unicolor*	5.487	5.317	2.732	1.079	4	-	+	+	-	+	-
HUE 27.1	*Sturnus unicolor*	7.459	7.196	4.334	1.380	6	+	+	+	+	+	+
HUE 39.1	*Sturnus unicolor*	9.576	8.401	3.291	NG	5	+	+	+	+	+	+
N29.1	*Pica pica*	7.009	6.659	NG	NG	8	-	+	+	-	+	+
N7.1	*Pica pica*	5.304	2.905	NG	NG	7	-	+	-	-	+	-
N32.1	*Pica pica*	8.494	7.694	3.412	1.079	6	+	+	+	+	+	+
N3.1	*Pica pica*	8.611	8.477	NG	NG	7	+	+	+	+	+	+
N32.1c	*Clamator glandarius*	4.225	2.494	NG	NG	2	-	+	+	-	+	-

[a] TSA for total aerobic mesophilic bacteria, HK for enterobacteria, KF for enterococci and VJ for staphylococci
[b]NG - No growth

(Fig. 6.1.A) to maximize cell recovery. The swab was placed into a 0.5 ml microcentrifuge tube (top and bottom nicked), which was placed in a 1.5 ml microcentrifuge tube (top nicked) and centrifuged for 5 min at 14,000 rpm. The swab was then discarded, and after cell resuspension, the filtrate was returned to the original tube with the remaining sample. Once the cells from the swab have

been recovered, the phosphate solution containing the bacterial cells was centrifuged during 5 min at 14,000 rpm. The supernatant was discarded and the cell pellet submitted to DNA extraction.

DNA isolation (Fig. 6.1.B) consisted in suspending the bacterial pellet in 100 µl 0.1 × TE buffer with 10 mg/ml lysozyme and incubating for 45 min at 37 ºC. After this period, 1 µl of 10 mg/ml proteinase K and 1 µl of 10% SDS were added and incubated for a further 30 min at 37 ºC. Then, 100 µl of 10% chelex 100 (200-400 mesh, Bio-Rad) was added, gently mixed, and incubated for 30 min at 56 ºC. Subsequently, samples were vortex during 10 s and incubated for 10 min at 100 ºC. Finally, samples were centrifuged during 5 min at 14,000 rpm and the supernatant transferred to a new microcentrifuge tube.

DNA quality was tested by the PCR amplification of the 16S ribosomal DNA gene and TTGE analysis according to Ogier and co-workers (2002). A negative control was included and consisted of an sterile swab stocked up in a microcentrifuge tube with 1.2 ml of sterile phosphate solution, which did not produce any PCR amplification.

Samples showed bacterial counts for total aerobic mesophilic bacteria from 1.3×10^2 to 3.8×10^9 CFU/clutch. Enterobacteriaceae were often at lower levels, with greater heterogeneity counts for enterococci and staphylococci (Table 6.1). The microbiological groups detected are within the bacterial groups described on eggshells of other avian species (Bruce & Drysdale 1994, Cook et al 2003), with densities that differed for different species of birds (Peralta-Sánchez et al., unpublished).

The DNA isolated by the chelex based protocol was suitable for PCR amplification (Table 6.1, Fig. 6.1.C-6.1.D). Chelex does not eliminate all PCR inhibitors but an easy way to remove them is to dilute the DNA solution 10 or 100 fold (Sheu 1998, Ishii & Loynachan 2004), so we used diluted DNA as template getting the best results in DNA diluted 1:10 (24 positives). Moreover, and because of the risk of high protein contents in the DNA extracted due to the simplicity of et al 2004). In this way, we could amplify the 16S gene of the microbial communities of all the sample studied using the isolated DNA diluted 1:10 (Table 6.1; Fig. 6.1.C). A TTGE analysis as measure of microbial diversity from eggshell

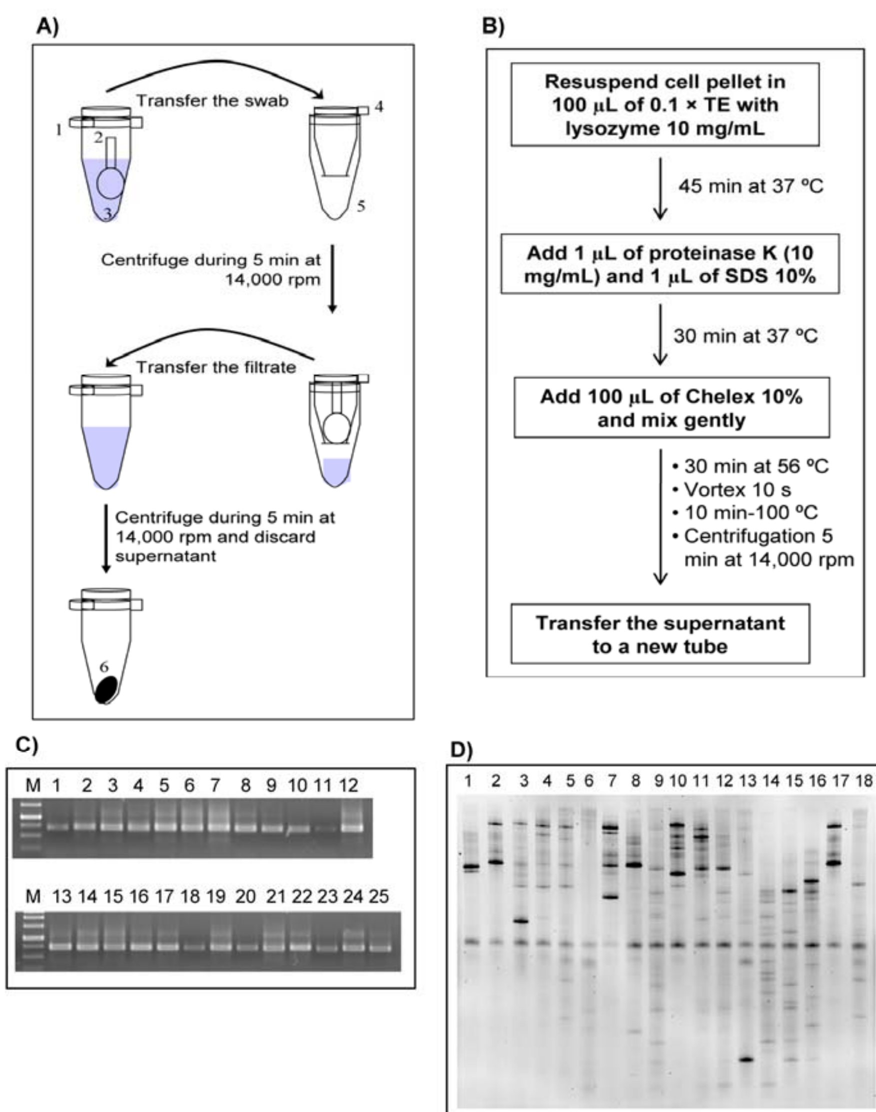

Fig. 6.1. DNA isolation flow chart. A) Cell recovery from samples. 1, microcentrifuge tube of 1.5 mL; 2, swab (most of the stick nicked); 3, phosphate buffer; 4, microcentrifuge tube of 0.5 mL with top and bottom nicked; 5, microcentrifuge tube of 1.5 mL with top nicked; 6, cell pellet. B) DNA isolation procedure. C) Eubacterial 16S ribosomal RNA (rRNA) gene amplification. M, 1 Kb ladder (Biotools); 1, P25.2; 2, Cast. Quem.2; 3, H789.1; 4, C.44.1; 5, H851; 6, BEC 15.1; 7, H.HV14.1; 8, TEJ 25.1; 9, CA51.2; 10, C18.1; 11, CA61.1; 12, 769.1; 13, 133.1; 14, CA51.1; 15, 842.1; 16, 55.1; 17, N29.1; 18, N32.1c; 19, HUE 1.1; 20, HUE 31.1; 21, HUE 27.1; 22, HUE 39.1; 23, N7.1; 24, N32.1; 25, N3.1. D) TTGE profiles of V3 16S rDNA fragments. 1, Cast. Quem.2; 2, H789.1; 3, H851; 4, H.HV14.1; 5, TEJ 25.1; 6, CA51.2; 7, CA51.1; 8, BEC 15.1; 9, 769.1; 10, 133.1; 11, 842.1; 12, HUE 1.1; 13, HUE 27.1; 14, HUE 39.1; 15, N29.1; 16, N32.1; 17, N3.1; 18, N32.1c.

the method, we repeated the 16S amplification including bovine serum albumin (BSA, 200 ng/µL) in PCR reaction mixture to protect the Taq polymerase from the possible presence of protease and PCR inhibitors (Sheu 1998, Radström is

presented to test this method (Fig. 6.1.D). When different kits or traditional techniques were used to purify DNA from these communities, just 20% of samples provided a PCR product for 16S ribosomal DNA (results not shown).

In conclusion, we have successfully tested the analyses of microbial communities of eggshell from six avian species with a high variation in their microbiological levels using this simple, low-cost and rapid method, which ideally can be applied to a high numbers of samples.

Abbreviations used
PCR: polymerase chain reaction; TSA: Tryptic Soy Agar; HK: Hecktoen Enteric Agar; VJ: Vogel–Johnsson Agar; KF: Kenner Fecal Agar; SDS: sodium dodecyl sulfate; TTGE: temporal temperature gradient gel electrophoresis; CFU: colony-forming units; BSA: bovine serum albumin.

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Apéndices

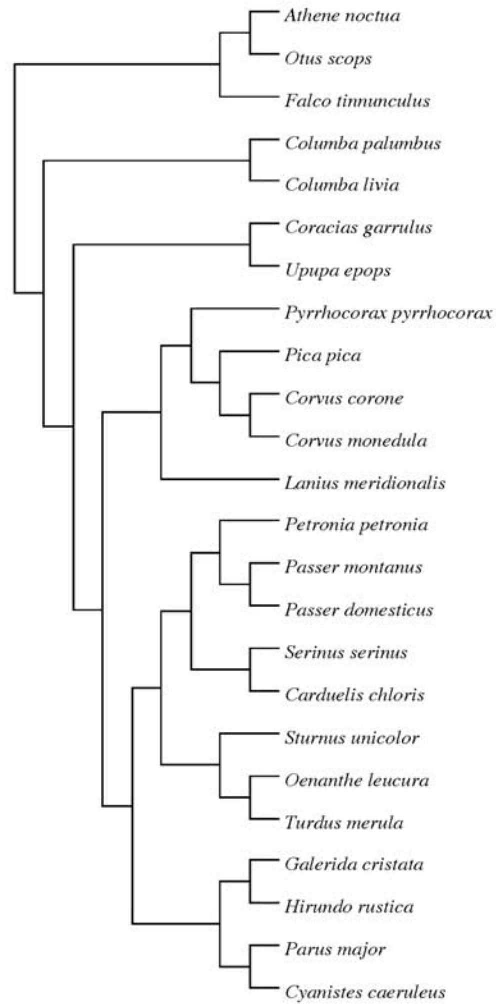
Appendix 1. Standarized log-transformed values of the variables used as estimates of bacterial density on the eggshells for the regressions analyses in Chapter I. TSA, KF, VJ and HK are number of colonies (adjusted to one cm2 of sampled eggshells) that grew in specific media for heterotrophic, enterococci, staphylococci, and enterobacteria, respectively, at the beginning of the incubation (1) and their relative growth (RG) period.

Species	N at the beggining of the incubation 2006	TSA 1 2006	KF 1 2006	VJ 1 2006	HK 1 2006	N at the beginning of the incubation 2007	TSA 1 2007	KF 1 2007	VJ 1 2007	HK 1 2007	N Relative Growth 2006	RG TSA 2006	RG KF 2006	RG VJ 2006	RG HK 2006
Athene noctua	9	0.69	1.59	-0.36	0.74	11	0.47	0.99	-0.66	0.39	5	-0.47	-2.20	-0.69	-2.05
Carduelis chloris	4	-1.86	-0.81	-0.32	-0.66										
Columba livia	25	-0.32	0.87	1.15	-0.14	16	-1.75	-0.93	1.04	-1.88	6	1.26	0.23	0.71	2.44
Columba palumbus	5	0.19	-0.88	-0.48	-0.01	6	-0.09	-0.84	-0.76	0.37					
Coracias garrulus	13	-1.02	0.71	-0.67	0.06	10	0.40	1.38	-0.09	0.67	8	-0.09	-0.66	3.24	-0.15
Corvus corone	16	-0.03	0.28	-0.64	-1.20	4	-2.75	-0.37	-0.86	-2.26	14	1.04	-0.84	0.14	0.87
Corvus monedula	7	0.96	-0.34	-0.32	2.14	8	0.16	0.09	-0.38	0.15	6	0.59	2.70	-0.58	0.27
Cyanistes caeruleus	4	0.60	-0.70	-0.80	-0.50										
Falco tinnunculus	12	0.80	1.79	0.47	1.27	9	0.98	0.92	-0.38	1.04	8	-0.32	0.81	-0.10	0.01
Galerida cristata						3	0.09	-1.34	-0.15	0.08					
Hirundo rustica	18	-1.38	-0.95	-0.59	-1.47	12	0.26	-0.44	0.19	0.32	16	0.15	0.21	-0.37	-0.10
Lanius meridionalis	3	0.93	-0.95	-0.43	-1.51	6	-0.02	-1.01	-0.83	0.12	3	-2.61	-0.01	0.02	0.62
Oenanthe leucura	9	-1.14	-0.92	-0.54	-1.29	7	0.98	-0.54	-0.83	-0.44	6	-1.11	-0.01	-0.51	-0.05
Otus stops	15	-0.27	0.54	-0.57	0.33	14	0.08	1.07	-0.84	0.61	9	-1.29	-0.91	0.24	-1.66
Parus major	15	0.74	-0.38	0.53	0.55	13	0.32	0.78	0.57	1.06	8	0.58	0.05	-1.02	0.29
Passer domesticus	3	0.24	-0.81	-0.72	-0.53	6	0.94	1.69	0.71	1.36					
Passer montanus	23	0.46	-0.68	-0.27	-0.08	12	0.56	-0.77	-0.12	0.68	10	0.46	0.13	0.62	-0.50
Petronia petronia	3	1.26	0.92	2.18	0.99	5	1.23	0.83	-0.67	0.13					
Pica pica	52	-0.44	-0.44	0.06	-0.12	54	-2.04	-1.01	-0.80	-1.75	30	0.75	0.30	-0.37	0.38
Pyrrhocorax pyrrhocorax						4	-0.22	-0.81	2.85	-1.28					
Serinus serinus						10	-0.56	-0.91	-0.64	-0.42					
Sturnus unicolor	15	0.83	-0.12	-0.20	0.93	19	0.00	0.30	-0.02	0.26	6	-0.07	0.21	-0.17	-0.56
Turdus merula	6	-2.17	-0.94	-0.54	-0.83	35	0.19	-0.83	0.40	-0.35					
Upupa epops	32	0.92	2.19	3.04	1.33	28	0.75	1.75	2.26	1.14	21	0.86	-0.04	-1.04	0.12

Appendix 1. (Continuation)

Species	N Relative Growth 2007	RG TSA 2007	RG KF 2007	RG VJ 2007	RG HK 007	Nest type	Plants	Feathers	Incubation
Athene noctua	8	1.11	1.77	-0.09	2.41	hole	no	no	asynchronous
Carduelis chloris						open	yes	yes	synchronous
Columba livia	4	4.96	0.17	0.01	5.19	hole	no	no	asynchronous
Columba palumbus						open	yes	no	synchronous
Coracias garrulus	8	1.13	-2.01	-0.28	0.27	hole	no	no	asynchronous
Corvus corone	4	6.33	-1.17	0.08	5.69	open	yes	yes	asynchronous
Corvus monedula	7	0.50	-0.35	-0.04	1.67	hole	yes	yes	asynchronous
Cyanistes caeruleus						hole	yes	yes	asynchronous
Falco tinnunculus	5	-0.48	1.31	-0.20	0.32	hole	no	no	asynchronous
Galerida cristata	19	0.11	-0.55	-0.08	0.35	open	yes	no	asynchronous
Hirundo rustica	7	-2.05	-2.07	0.47	-3.08	hole	no	yes	synchronous
Lanius meridionalis	6	1.71	-0.22	-0.02	1.10	open	yes	yes	asynchronous
Oenanthe leucura	7	0.17	-0.92	-0.03	3.69	hole	no	yes	asynchronous
Otus scops	14	-0.17	-0.57	-0.02	-0.18	hole	no	no	asynchronous
Parus major	9	0.74	-1.63	-0.20	-0.24	hole	no	yes	asynchronous
Passer domesticus						hole	yes	yes	asynchronous
Passer montanus	5	0.25	-0.72	-0.29	-0.72	hole	yes	yes	synchronous
Petronia petronia						hole	yes	yes	asynchronous
Pica pica	40	1.22	0.46	0.15	2.20	open	yes	no	asynchronous
Pyrrhocorax pyrrhocorax	3	-1.00	0.82	-1.42	1.89	hole	yes	no	asynchronous
Serinus serinus						open	yes	yes	synchronous
Sturnus unicolor	13	1.55	-1.30	-0.01	0.34	hole	yes	yes	synchronous
Turdus merula						open	yes	no	asynchronous
Upupa epops	10	0.51	0.20	-0.22	-0.12	hole	no	no	asynchronous

Appendix 2. Phylogenetic relationships among species included in the analyses of Chapter I.



Appendix 3. Hatching success and log-transformed values of the geometric mean of bacterial density on the eggshells of different species used in Chapter II. TSA, KF, VJ and HK are number of colonies per cm2 of eggshell surface that grew in specific media for heterotrophic, enterococci, staphylococci, and enterobacteria, respectively, at the beginning of the incubation (1) and their relative growth (RG).

Species	N 1	TSA	KF	VJ	HK	N RG	RG TSA	RG KF	RG VJ	RG HK	N Hatching
Athene noctua	8	6.77	4.35	0.02	5.75	5	0.35	1.01	-0.04	0.88	10
Otus scops	9	5.29	3.16	0.02	4.62	9	-0.01	-0.28	-0.02	0.58	9
Falco tinnunculus	5	7.30	1.73	0.28	5.86	4	-0.52	1.56	-0.25	0.27	7
Columba livia	9	3.84	1.19	0.96	1.75	3	4.84	-0.07	0.02	4.67	9
Coracias garrulus	10	6.29	4.05	0.25	5.32	8	1.13	-2.01	-0.28	0.27	12
Upupa epops	17	6.78	4.40	1.04	6.03	14	-0.06	-0.52	-0.27	0.05	24
Pyrrhocorax pyrrhocorax	4	5.43	0.92	1.12	2.11	3	-1.00	0.82	-1.42	1.89	8
Pica pica	8	2.11	0.25	0.05	0.33	7	4.60	2.18	0.25	6.08	12
Corvus monedula	8	5.96	2.20	0.16	4.47	7	0.50	-0.35	-0.04	1.67	8
Lanius meridionalis	5	5.43	0.75	0.03	3.97	5	2.09	-0.32	-0.02	1.36	5
Passer montanus	7	6.08	1.30	0.26	5.58	5	0.25	-0.72	-0.29	-0.72	8
Serinus serinus	5	4.91	0.33	0.06	2.40						5
Sturnus unicolor	13	5.80	2.40	0.37	4.45	11	1.23	-1.14	-0.04	0.02	15
Oenanthe leucura	6	6.64	1.44	0.00	2.91	6	0.86	-1.05	0.00	4.26	7
Turdus merula	20	5.72	0.43	0.14	3.88	6	1.17	0.96	0.07	0.44	20
Hirundo rustica	9	5.85	1.00	0.44	4.22	6	-1.97	-1.20	0.55	-2.49	12
Parus major	10	6.60	3.63	0.39	5.79	8	0.69	-1.33	-0.25	-0.47	11

Appendix 4. Phylogenetic relationships among species included in the analyses of Chapter II.