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Interacciones ecológicas aves-bacterias: implicaciones durante el desarrollo de los pollos en el nido

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Interacciones ecológicas aves-bacterias: Implicaciones durante el desarrollo de los pollos en el nido

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A mi familia

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ABSTRACT

The effects of bacteria on the development of birds in the nest have been barely studied under natural conditions and most of our knowledge about this topic derives from poultry as interactions between potentially pathogenic bacteria and chickens have economic and public health implications. In wild birds, there is scant evidence suggesting negative effects of gut bacteria on nestling growth. However not only gut bacteria may have effects on nestling growth as other microbiota such as those existing on the nest or on skin could affect development. Early development is a crucial determinant of fitness in many animals. In birds, in addition to some environmental factors, the body mass at fledging as well as body condition and measures of immunocompetence, have been shown to be reasonably good predictors of individual post-fledging survival. The main goal of this thesis is to increase our knowledge about the interactions between growing altricial birds and microorganisms under natural conditions from an ecological and evolutionary point of view as traditionally nestling growth has been studied without consideration of microbial effects. Furthermore the thesis explores potential adverse effects of bacterial gut and skin symbionts on nestling growth and thereby on fitness. As there may be resource-allocation trade-offs between immunity development and tissue growth, the different arms of the immune system in nestlings have also been studied.

Our study system has been the pied flycatcher (*Ficedula hypoleuca*), a model organism in avian ecology. In nestlings, we estimated the abundances of certain important types of gut bacteria (enterococci and enterobacteria) through culture-dependent techniques at two different ages, namely 7 and 13 days after hatching. Most previous studies have sampled bacterial communities in chicks only once, namely just

before fledging, which does not allow to relate adequately the development of gut microbiota with nestling growth. We found that the two types of bacteria showed different trends with respect to nestling age, so that while enterococci increased in abundance, enterobacteria decreased. This could be due to competitive interactions between different bacterial types for space and nutrient resources in the nestling gut. Competition for resources and space among intestinal microbes is a common phenomenon expressed as chemical conflict through bacteriocin production for example. These changes may be due to age-dependent variation in the diet of nestlings as food constitutes an important source of bacterial inoculation during development. The most interesting result was that the bacterial load of enterococci at the earliest age was negatively associated with the growth of the tarsus-metatarsus between the two sampling ages, a clear indication of the negative effects for nestling fitness of some gut bacteria at early ages. Although an experiment is needed to confirm this association, it suggests that bacteria detracted nutritional resources for skeletal growth either through a direct trade-off or through immunity-mediated effects.

On the other hand, several factors including climate, food, age and health state may affect the composition of the gut microbiota of individual birds. Nutrient richness in the environment, humidity and temperature have been identified as important factors affecting growth in bacterial cultures. Therefore, gut bacterial loads may be affected by weather conditions such as temperature or precipitation given that body temperature of nestlings at early ages may depend on local climate, and the strict thermal dependence of bacterial growth. As expected, loads of both bacterial types at an early age were positively associated with mean local temperature. Furthermore, loads of enterobacteria at an early age were negatively correlated with prior precipitation and hatching date. With respect to the later age, loads of enterococci were again positively correlated with

mean temperature while enterobacteria loads were not related to any weather variable but were negatively associated with hatching date. The associations of bacterial loads with breeding phenology may be related to poorer nutrition of late-hatched brood allowing fewer resources for gut bacteria. Thus, weather conditions and breeding phenology affect gut bacterial populations through nestling heterothermy at early ages and date-dependent nutrition and may in this way contribute to modulate nestling development.

Our previously mentioned studies of gut bacteria are limited to culture-dependent techniques, which imply some limitations for characterizing microbiota. The use of molecular identifications of bacteria has great advantages for certain purposes, so we have used also molecular analysis with our samples, specifically Automated Ribosomal Intergenic Spacer (ARISA), which describes bacterial communities by amplifying a sequence of the rRNA intergenic spacer 16S-23S that varies in length and sequence according to taxonomic groups. Using this technique, we obtained the OTUs, also called phylotype, which are assumed to denote bacterial lineages or species. The ARISA technique has been used mainly to characterize environmental bacterial communities, but is progressively being used to describe bacterial communities associated with wild and domestic birds. A total of 91 Operational Taxonomic Units (OTUs) were found in the gut samples at both ages. Our analyses of diversity by means of the Shannon index resulted in bacterial diversity increasing as nestlings grow while the number of OTUs was not affected by age. Possibly nestling gut microbial communities were already established at early ages while the abundance of the different bacterial types may have reached an equilibrium due to competitive interactions among bacterial strains before nestlings leave the nest.

However bacteria colonize any environment that can provide them with sufficient nutritional resources for growth. Avian nests are micro-environments very likely to be colonized by bacteria due to the presence of debris, faeces and discarded food. Furthermore, nests in cavities may offer more constant and suitable environmental conditions for bacterial growth. On the other hand, experimental studies have explored the importance of nest reuse in cavity nests for arthropod colonization of nests and for nestling health and growth but the effect of nest bacterial communities have not been hitherto explored. However, bacteria can remain dormant in nests and faeces for several months, so there may exist an increased risk of bacterial infection for birds breeding in reuse nests. Therefore we conducted an experiment, leaving a sample of nest-boxes without removing old nest materials in 2010 and compared bacterial loads of nest materials, control inert objects (a square of plastic previously sterilized with alcohol) and nestling belly skin in reused nests with those in newly constructed nests in 2011. We found that nestlings raised in reused nests had higher bacterial loads on their belly skin than those in new nests, while no difference between nest types for nest materials and control inert objects were found. There was a marginally significant tendency for wing length before fledging to be lower in reused nests. The bacterial loads of nests showed a negative association with feather growth of nestlings as expressed through wing length. These results indicate an association between nest reuse and bacterial growth on nestling skin not hitherto detected.

Like the relationships between the bacteria remaining in the reused bird nests and nestling growth, the existence of pathogenic or mutualistic associations between skin bacterial communities and nestlings has hitherto received scant attention. To remedy this, we estimated the abundance of heterotrophic bacteria on a delimited area of the naked skin of the belly of nestlings at two different ages and their association

with general growth (measured at 7 and 13 days). Bacterial loads of nestlings at 7 days were not correlated with any nestling measurement, while on day 13 they were positively associated with brood size and with nestling wing length. Larger broods develop in less hygienic conditions, especially shortly before fledging, which could explain the positive association of brood size with bacterial loads at this age. Skin bacteria may favour wing feather growth through competition with harmful bacteria or faster feather growth may facilitate bacterial growth through accumulation of remains on skin surrounding growing feathers. Only experiments can resolve the novel association found.

Finally, the immune system is highly complex and is generally classified into two main components: innate and acquired immunity. We explored the relationships among different arms of the immune system and the possible trade-offs of their development with nestling growth, although in a non-experimental setting. For that, we measured levels of natural antibodies (NAbs) and hemolysis, estimated serum immunoglobulin levels and obtained a measure of inflammation after inoculation of an antigen, Phytohemagglutinin (PHA). We found that no immune variable measured was associated with any other variable at the individual and brood levels. This indicates that different aspects of immunity are independent and difficult to integrate in a general measure of immune response capacity in altricial nestling birds. In nestling birds, some studies have shown trade-offs between the nutrients required for growth and those needed to mount an immune response. In our study, only NAbs was negatively associated with tarsus length at the age of 7 days, evidencing a resource-based trade-off between nestling growth and immunity. Nestlings are experiencing the fastest skeletal growth before 10 days of age, which possibly explains why the association with NAbs

was not found at 13 days. Our result confirms previous evidence that Nabs is the most energetically costly aspect of immunity.

In this thesis, we present descriptive results pointing out the influence of bacteria in the gut, on nests and skin on nestling growth. Further researches on the detected relationships are necessary to distinguish between causes and effects.

INTRODUCCIÓN

Antes de que los microorganismos fueran observados por primera vez, algunos autores ya sugirieron que ciertas enfermedades eran causadas por unas "criaturas vivientes invisibles" (Madigan *et al*. 2006). Sin embargo, estas "criaturas invisibles" no serían descritas hasta 1674 por el holandés Anton van Leeuwenhoek que con su simple microscopio, compuesto por dos lentes de cristal convexas montadas sobre platinas de latón, describió por primera vez los protozoos y las bacterias, a los que llamó "animalículos" (Russell & Russell 1958). Así fueron los primeros pasos de la microbiología, que años más tarde seguiría cosechando descubrimientos tan importantes como la descripción de la bacteria *Mycobacterium tuberculosis* por parte de Koch, bacteria causante de la tuberculosis (Madigan *et al*. 2006), o el aislamiento de *Thermus aquaticus*, fuente de la Taq polimerasa que es la base de la técnica de amplificación del ADN (Brock & Freeze 1969, Holland *et al*. 1991).

Por otro lado, podemos definir a la ecología como el conjunto de interacciones entre organismos y de éstos mismos con su entorno como resultado de la búsqueda y obtención de los recursos necesarios para su supervivencia y reproducción (Moreno 2008). Por consiguiente, es inevitable incluir las interacciones con las bacterias puesto que uno de los aspectos ecológicos más notables de éstas es su adaptabilidad, lo que les ha permitido colonizar un amplio elenco de hábitats, desde los ambientes relativamente benignos y ricos en nutrientes como suelos, lagos, océanos u otros organismos hasta ambientes extremos como pueden ser las profundidades del hielo antártico (Horner-Devine *et al*. 2004, Madigan *et al*. 2006). De modo que todos los organismos eucariotas están en constante contacto con las bacterias y por ende interactúan con ellas, considerándose éstas una importante fuerza selectiva que ha actuado sobre la evolución

de los mismos (Xu & Gordon 2003). Recientemente la emergencia de nuevas enfermedades infecciosas en poblaciones silvestres de aves, y consecuentemente su amenaza de transmisión a los humanos como zoonosis, está generando un interés creciente en este tema (Longbottom & Coulter 2003, Benskin *et al*. 2009). Es por ello que dichas interacciones constituyen un nuevo campo de estudio para los ecólogos en poblaciones silvestres tal y como demuestran los recientes artículos publicados principalmente durante la década pasada. Entre las relaciones con bacterias estudiadas actualmente en poblaciones naturales de aves se incluyen los efectos sobre el inicio de la incubación o el éxito de eclosión (Pinowski *et al*. 1994, Cook *et al*. 2003, Cook *et al*. 2005, Shawkey *et al*. 2009, Soler *et al*. 2011, 2012, Ruiz-de-Castañeda *et al*. 2011a, 2012b) y la degradación de las plumas (Burtt & Ichida 1999, Goldstein *et al*. 2004, Shawkey *et al*. 2007, Ruiz-de-Castañeda *et al*. 2012a), su asociación con las enfermedades de transmisión sexual (Lombardo *et al*. 1999, Poiani & Wilks 2000, Westneat & Rambo 2000, Kulkarni & Heeb 2007, White *et al*. 2010) o la relación simbiótica entre algunas aves y ciertas bacterias aisladas de la secreción de la glándula uropigial (Soler *et al*. 2008, Møller *et al*. 2009, Ruiz-Rodríguez *et al*. 2009, 2012, Martín-Vivaldi *et al*. 2010). No obstante, hay que destacar que muy pocos artículos se basan en el efecto de las bacterias sobre el crecimiento de los pollos nidícolas, crecimiento que puede tener efectos importantes sobre la eficacia biológica tanto en el nido como durante las subsiguientes etapas juvenil y adulta (Birkhead *et al.* 1999, Lindström 1999, Naguib & Gil 2005, Blount *et al.* 2006). Los escasos estudios publicados contemplan en este contexto principalmente a las bacterias intestinales (Lombardo *et al*. 1996, Mills *et al*. 1999, Potti *et al*. 2002, Moreno *et al*. 2003). Sin embargo, la información sobre las asociaciones entre el desarrollo de pollos nidícolas y

otras comunidades bacterianas como aquellas que residen en la piel o incluso en el propio nido es todavía más escasa.

Esta tesis doctoral se centra principalmente en el conocimiento de las interacciones entre aves y microorganismos en condiciones naturales desde un punto de vista ecológico y evolutivo, y más concretamente en los efectos de las bacterias sobre el crecimiento de los pollos en una especie de pájaro insectívoro forestal que nidifica en cavidades como es el papamoscas cerrojillo *Ficedula hypoleuca* Pallas. Contempla tanto las bacterias intestinales como aquellas contenidas en el material del nido o existentes sobre la piel de los pollos. El enfoque es descriptivo y pretende explorar posibles interacciones entre bacterias y pollos nidícolas que puedan dar lugar a futuros experimentos. Incluye también una aproximación a una de las principales respuestas de estos pollos a las interacciones patogénicas, como es el sistema inmunitario, cuyo desarrollo se produce precisamente durante esta etapa de la vida. Serán necesarias más investigaciones que nos permitan establecer relaciones de causa-efecto en las asociaciones que hemos detectado en esta tesis.

Las bacterias Intestinales y su relación con el crecimiento

El tracto gastrointestinal de las aves es estéril nada más eclosionar, aunque la colonización de éste comienza inmediatamente después de la eclosión del pollo, pudiéndose encontrar en 24 horas poblaciones considerables de bacterias intestinales comensales (Shapiro & Sarles 1949, Mills *et al*. 1999). Es sabido que las bacterias colonizan rápidamente cualquier nuevo hábitat que sea capaz de suministrarles nutrientes para su crecimiento y mantenimiento (Horner-Devine *et al*. 2004, Apajalahti

2005). En un estudio sobre la microflora presente en el intestino de pollos de corral a diferentes edades, se observó que *Escherichia coli* apareció a los dos días de vida mientras que los bacilos y cocos Gram-positivos aparecieron una vez que habían pasado 4 o 5 días (Rahner 1901, revisado en Shapiro & Sarles 1949). Los géneros más frecuentes que podemos encontrar en el intestino de aves de corral son *Lactobacillus*, *Enterococcus*, *Clostridium* y algunos de la familia *Enterobacteriaceae* (Shapiro & Sarles 1949). Las vías de adquisición pueden ser varias desde el mismo ambiente que los rodea, por medio de la saliva de los progenitores al ser alimentados, por sus compañeros en el nido o por el propio material del nido (Kyle & Kyle 1993, Singleton & Harper 1998, Lucas & Heeb 2005). Sin embargo, es probablemente el alimento la fuente más importante de inoculación bacteriana, y dependiendo de la comida suministrada por los padres así será la diversidad de la comunidad microbiana (Glunder 2002, Waldenström *et al.* 2002, Maul & Farris 2005). Asimismo la edad también juega un papel importante en la composición y estructura de esta comunidad puesto que como ya hemos mencionado anteriormente, según crece el individuo, se ha observado que adquiere nuevas especies bacterianas (Mills *et al.* 1999, van der Wielen *et al.* 2002, van Dongen *et al.* 2013). Estos cambios cualitativos en la composición pueden deberse a cambios fisiológicos o morfológicos en el tracto intestinal dependientes de la edad, ya que a medida que el pollo crece, aumenta el peso y longitud del intestino así como las dimensiones de las vellosidades intestinales que son el lugar de adhesión de algunos tipos bacterianos (ver **capítulo III**).

Esta microflora intestinal tiene efectos sobre la nutrición del hospedador, sobre su salud e incluso sobre el crecimiento (Barrow 1992, Stevens & Hume 1998, Torok *et al*. 2008) al interaccionar con la utilización de los nutrientes y con el desarrollo del sistema digestivo. Esta interacción es compleja, y dependiendo de las bacterias que

estén implicadas, puede tener resultados positivos o negativos para el hospedador (Yang & Choct 2009). Serían negativos en el sentido de que dichas bacterias podrían detraer recursos nutricionales (Dibner & Richards 2005, Lan *et al*. 2005), ya que una alta carga microbiana incrementará los requerimientos energéticos para su mantenimiento y por lo tanto implicará una reducción en la eficiente utilización de los nutrientes para el correcto desarrollo del hospedador. En relación con los efectos positivos, ciertas bacterias son utilizadas como probióticos en la producción avícola, como es el caso de *Bacillus subtilis*. En ocasiones los pollos de corral son inoculados con un homogeneizado de las bacterias nativas de los adultos para de esta forma evitar la colonización por parte de *Salmonella enteritidis* en lo que se ha llamado "exclusión competitiva", siendo ésta también efectiva frente a otras especies de *Salmonella* y *Campylobacter* (Rantala & Nurmi 1973, Fuller 2001, Stern *et al.* 2001).

A pesar de todo lo mencionado anteriormente, el crecimiento de las aves, fuera del mundo avícola, ha sido estudiado sin tener en consideración los posibles efectos que puedan tener las bacterias intestinales sobre el hospedador. Así en ciertas obras clave no se abordan los efectos bacterianos sobre el desarrollo en el nido (Ricklefs 1979, O'Connor 1984, Starck & Ricklefs 1998). Recientemente se han hallado en poblaciones silvestres de aves efectos positivos de las bacterias sobre el crecimiento como es el caso de los encontrados por Moreno *et al.* (2003), en los cuales se observaron asociaciones positivas entre *Enterococcus faecium* y la longitud del tarso y peso de los pollos de papamoscas cerrojillo en ausencia de *Enterococcus faecalis*. Aunque también se han detectado los efectos contrarios mediante un estudio experimental con pollos de pingüino de Magallanes *Spheniscus magellanicus*, en que se les suministró un antibiótico de amplio espectro (cefalosporina) y se observó un crecimiento más rápido que en individuos control (Potti *et al.* 2002). Incluso el mismo género o grupo

bacteriano puede afectar de diferente forma al crecimiento de ciertas partes anatómicas como son la longitud del tarso y el ala (Lombardo *et al.* 1996). Además se ha publicado que la presencia de ciertas bacterias está asociada con un alto grado de asimetría en la longitud alar, lo cual podría afectar a la supervivencia de los pollos al abandonar el nido puesto que dicha asimetría dificultaría la capacidad de vuelo (Mills *et al*. 1999). Igualmente se han encontrado relaciones significativas entre dichas comunidades bacterianas y la calidad fenotípica del pollo en relación con el reclutamiento a la población (Ruiz-Rodríguez *et al*. 2009). En relación con estas asociaciones de la microbiota intestinal con el crecimiento de los pollos, presentamos una aportación en el **capítulo I** además de tratar las posibles repercusiones que tienen ciertos factores climatológicos sobre estas bacterias en el **capítulo II**. Posteriormente en el **capítulo III** mediante técnicas moleculares estudiamos los posibles cambios en la abundancia de taxones bacterianos del tracto intestinal en relación con la edad.

Bacterias heterótrofas existentes en el nido y en la piel de los pollos y su asociación con el crecimiento

El nido de las aves tiene una estructura característica dependiendo de la especie constructora, pero también se diferencia en cuanto al material utilizado aún dentro de la misma especie, pues la composición del nido depende de la disponibilidad de distintos materiales en la zona de anidamiento (Hansell 2000, Moreno *et al*. 2009). Se ha propuesto que el nido en sí podría servir para señalizar la calidad o incluso la disposición parental por parte del individuo que lo construye, de manera que su pareja

puede invertir en un mayor esfuerzo reproductivo ateniéndose a estas actividades o a su resultado según modelos sobre "asignación diferencial" (Moreno *et al*. 1994, 2012, Soler *et al*. 1996, 1998a, 1998b, 2001, de Neve & Soler 2002).

Por otro lado, algunas especies no construyen nidos abiertos cada temporada de cría, sino que éstos son construidos en las cavidades de los árboles o en cajas-nidos si están disponibles en la zona. Sin embargo, en ocasiones sucede que, debido a una baja disponibilidad de oquedades apropiadas en la zona y como consecuencia de una fuerte competencia entre los individuos para hacerse con dichas cavidades (revisado en Mazgajski 2007a), ciertos individuos reutilizan nidos ya usados en temporadas anteriores, dándose en ocasiones incluso una preferencia por reutilizar estos nidos viejos frente a la construcción de uno completamente nuevo (Orell *et al*. 1993, Mappes *et al*. 1994, Merino & Potti 1995, Olsson & Allander 1995, Pacejka & Thompson 1996, Rendell & Verbeek 1996b). Esta reutilización de nidos conlleva beneficios puesto que reduce el tiempo de construcción, el cual puede suponer un elevado coste energético (Moreno *et al*. 2008b, 2010, Mainwaring & Hartley 2013) y de tiempo en la búsqueda de material para el individuo constructor, o bien porque provee a los nuevos ocupantes de información sobre la calidad del territorio (Orell *et al*. 1993, Olsson & Allander 1995, Mazgajski 2007, Vergara *et al*. 2010). A pesar de estos posibles beneficios, la reutilización de los nidos comporta unos costes derivados de la carga de ectoparásitos que tendrán que soportar ya que los nidos viejos suelen poseer mayor abundancia de ectoparásitos frente a los nidos nuevos (Rendell & Verbeek 1996a). Existe cierta controversia en cuanto a la reutilización de los nidos y sus efectos sobre el crecimiento, salud y mortalidad de los pollos. Mientras unos autores han encontrado efectos negativos en relación al éxito reproductor y la eficacia biológica de los pollos (Oppliger *et al*. 1994, Tomás *et al*. 2007, García-Navas *et al*. 2008, Cantarero *et al*. 2013), otros

han señalado que no encontraron efectos o éstos fueron incluso positivos (Rendell & Verbeek 1996, Allander 1998, Blem *et al*. 1999, Mazgajski 2007b, López-Arrabé *et al*. 2012).

Sin embargo no se tiene en consideración a las bacterias en este contexto, a pesar de que los nidos conforman un microambiente ideal para la colonización por parte de éstas debido a la presencia de restos de excrementos y de comida que no ha sido ingerida por parte de los pollos, así como unas condiciones microclimáticas constantes y relativamente favorables. Existen pocos estudios relativos a la existencia de bacterias en nidos y más concretamente en nidos viejos (Mehmke *et al*. 1992, Singleton & Harper 1998, Goodenough & Stallwood 2010). En un estudio realizado con nidos viejos de chochín criollo *Troglodytes aedon* se encontraron tres géneros de bacterias patógenas como son *Pseudomonas*, *Bacillus* y *Staphylococcus* (Singleton & Harper 1998). Estas bacterias existentes en los nidos viejos podrían tener efectos negativos sobre el crecimiento de los pollos, teniendo en cuenta que éstos están en continuo contacto con el material del nido existiendo como consecuencia la posibilidad de que las bacterias colonicen su piel. Además cabe destacar que las bacterias degradadoras del plumaje (*Bacillus licheniformis*) estarían encuadradas en uno de estos géneros encontrados en ciertos nidos aunque no se ha confirmado la presencia de esta especie bacteriana concreta. Consecuentemente en el **capítulo IV** de esta tesis profundizamos en el efecto de la reutilización de los nidos, desde un punto de vista microbiológico, sobre el crecimiento de pollos nidícolas.

Por otro lado, el mismo plumaje de las aves podría constituir un ambiente perfecto para amparar una compleja comunidad bacteriana, algunos de cuyos componentes son capaces de degradar la queratina de las plumas (Burtt & Ichida 1999, Lucas *et al*. 2003, Shawkey *et al*. 2003, Ruiz-de-Castañeda *et al*. 2012a). No obstante

existe poca información en la bibliografía sobre la colonización de la piel de las aves por parte de las bacterias y mucho menos sobre la interacción de éstas con el desarrollo de los individuos jóvenes. Ello es así a pesar de que, como hemos mencionado anteriormente, las bacterias presentes en el material del nido podrían asentarse sobre la piel o el plumaje de los ocupantes del nido debido al intenso contacto de éstos con aquél (Mehmke *et al*. 1992, Goodenough & Stallwood 2010). En uno de los escasos estudios, Berger *et al*. (2003) obtuvieron muestras de la piel de los pollos de estornino europeo *Sturnus vulgaris*, observando que el número de colonias bacterianas aumentaba a medida que la temporada avanzaba, aunque no detectaron una asociación de la abundancia bacteriana con el peso de los pollos o su mortalidad. Este tema será estudiado con más detenimiento en el **capítulo V** que intenta esclarecer las posibles asociaciones de dicha microbiota con el crecimiento de los pollos.

Relaciones entre el sistema inmune y el crecimiento

Un sistema inmune altamente eficiente es fundamental para la supervivencia del individuo puesto que constituye el principal mecanismo frente a los agentes patógenos (Zuk & Stoehr 2002, Davison *et al*. 2008). Su acción se suele clasificar en dos brazos diferentes: la inmunidad innata y la adquirida (Janeway & Travers 1996, Roitt *et al*. 2001). La respuesta innata provee la primera línea de protección contra los microorganismos invasores ya que está inmediatamente disponible al eclosionar si bien no conduce a una inmunidad duradera (Ricklefs 1992, Apanius 1998, Roitt *et al*. 2001, Ardia & Schat 2008, Palacios *et al*. 2009). Dicha respuesta es primordial en los pollos

nidícolas ya que su sistema inmunitario está poco desarrollado nada más eclosionar. Además, al estar constreñidos a permanecer en el nido durante el tiempo de desarrollo, probablemente tengan que enfrentarse a infecciones bacterianas o a otros patógenos, por lo que se hace necesario poseer un mecanismo de defensa eficaz ya en esta etapa de la vida. Entre los componentes efectores de dicha respuesta inmunitaria se encuentran los anticuerpos naturales que pueden reaccionar con diversos patógenos y activar el complemento (Janeway & Travers 1996). Por otro lado, la respuesta adquirida se encarga principalmente de controlar a parásitos intra y extracelulares, incluyendo a los virus y a los ectoparásitos (Roitt *et al*. 2001). Los mecanismos efectores desencadenados durante dicha respuesta están principalmente vinculados a la participación de las células del sistema inmunitario o a la producción de moléculas solubles proteicas (inmunidad humoral), destacando las inmunoglobulinas, moléculas producidas y secretadas por los linfocitos B, cuya función es el reconocimiento específico de los antígenos presentes en los agentes patógenos. Pero estas defensas adquiridas están poco desarrolladas en los primeros días de vida de los pollos nidícolas, como se deduce de que la producción propia de inmunoglobulinas no empieza en ciertos paseriformes hasta los 8-10 días de vida (Pihlaja *et al*. 2006). Por lo tanto podría existir un posible compromiso entre la inversión en los diferentes componentes del sistema inmune durante el desarrollo en el nido, sí el desarrollo de dichos componentes implica diferentes costes energéticos (Deerenberg *et al*. 1997, González *et al*. 1999, Norris & Evans 2000, Møller & Petrie 2002, Buchanan *et al*. 2003). Ello predeciría asociaciones negativas entre diferentes respuestas inmunitarias. Por otro lado, se han intentado desarrollar indicadores fiables de inmunocompetencia general en base a estudios en condiciones naturales (Adamo 2004). La inmunocompetencia general, caso de ser medible, debería poder obtenerse de diferentes medidas de respuestas inmunitarias si

existen asociaciones positivas significativas entre ellas. Es por ello de interés explorar si dichas asociaciones existen en un contexto no experimental. En el **capítulo VI** hemos abordado ambas posibilidades durante la etapa de los pollos en el nido obteniendo múltiples indicadores de respuesta para relacionarlos entre sí.

A pesar de sus beneficios en cuestión de salud y supervivencia para el individuo (Hõrak *et al*. 1999, Ardia *et al*. 2003, Hanssen *et al*. 2004), la respuesta inmunitaria también conlleva costes que pueden presentarse en forma de enfermedades autoinmunitarias (Råberg *et al*. 1998), así como una merma en términos energéticos o de recursos nutricionales que podrían ser usados para otras funciones como puede ser la reproducción (Sheldon & Verhulst 1996, Martín II *et al*. 2003). En algunos estudios se ha comprobado que existe un compromiso entre los nutrientes que se requieren para el correcto crecimiento de los pollos y los necesarios para mostrar una eficaz respuesta inmunitaria (Szép & Møller 1999, Klasing & Leshchinsky 1998, Soler *et al*. 2003, Brommer 2004, Pihlaja *et al*. 2006). En el **capítulo VI** obtuvimos diferentes medidas inmunológicas como es la respuesta inflamatoria a PHA mediada por los linfocitos T, aunque parece ser que esta respuesta también recluta componentes de la inmunidad innata (veáse Martin II *et al.* 2006). Otra de las medidas fue la respuesta humoral obtenida a través de la cuantificación total de inmunoglobulinas en suero (Martínez *et al.* 2003). Por último cuantificamos la inmunidad innata a través de las respuestas de aglutinación (anticuerpos naturales) y lisis (activación del complemento). Con estas medidas, intentamos profundizar en el posible compromiso energético que subyace entre el crecimiento y el desarrollo del sistema inmune en esta fase de la vida de las aves. En los pollos nidícolas el crecimiento se caracteriza por su rapidez (Ricklefs 1979, O'Connor 1984, Starck & Ricklefs 1998) lo que posibilita un rápido abandono del nido para evitar la depredación. Al mismo tiempo estos pollos necesitan invertir en el

desarrollo de un sistema inmune eficiente para poder luchar contra los patógenos que existan en el entorno inmediato. Por consiguiente el estudio de este posible compromiso es de vital importancia ya que tanto la condición del individuo como el sistema inmune son factores determinantes para la supervivencia del individuo una vez que abandona el nido (Smith *et al.* 1989, Ringsby *et al.* 1998, Hõrak *et al.* 1999, Møller & Saino 2004, Moreno *et al.* 2005, Lobato *et al.* 2008, Naef-Daenzer & Gruebler 2008)

MATERIAL Y MÉTODOS

I. ZONAS DE ESTUDIO

La zona de estudio principal es un bosque caducifolio situado en los Montes de Valsaín (40º 53' 74N, 4º 01' O, 1200 m.s.n.m.). Dichos montes se localizan en la vertiente noroeste de la Sierra de Guadarrama (Sistema Central), en la provincia de Segovia. El bosque está compuesto principalmente por pies jóvenes de roble melojo *Quercus pyrenaica,* aunque existen también ejemplares aislados de pino silvestre *Pinus silvestris* y fresno *Fraxinus angustifolia*. En cuanto al sustrato arbustivo, está dominado por jaras *Cistus laurifolius* (Figura 1).

En esta zona existen un total de 300 cajas nido de madera, protegidas por malla metálica y con un tubo de plástico para evitar la depredación por parte de pequeños carnívoros y de pícidos (véase Lambrechts *et al*. 2010 para más información). Estas cajas suelen ser utilizadas principalmente por el herrerillo común *Cyanistes caeruleus,* papamoscas cerrojillo *Ficedula hypoleuca*, carbonero común *Parus major* y trepador

azul *Sitta europeae,* siendo claramente menor la ocupación por estas dos últimas especies.

La segunda zona de estudio utilizada, también es un bosque de roble melojo *Quercus pyrenaica* situado en Lozoya, Madrid (40º 58’ N, 3º 48’ W) a 1400 m.s.n.m., aunque a diferencia de los Montes de Valsaín, en esta zona no encontramos jaras en el sotobosque. En esta zona existen 100 cajas nido con las mismas características que en Valsaín, siendo colonizadas por las mismas especies que en la primera zona.

Figura 1. Fotografías de una de las zonas de estudio (Valsaín). En la fotografía de la derecha se puede observar una de las cajas-nido que existen en la zona.

II. Especies de estudio

Esta tesis se centra en el estudio de las relaciones entre una especie de ave, el papamoscas cerrojillo, y ciertos tipos bacterianos asociados al sistema digestivo, y a la piel de los pollos durante su estancia en el nido. La especie aviar y los tipos bacterianos involucrados en los estudios son presentados a continuación, excepto para las bacterias

del nido y de la piel puesto que éstas se cultivaron en un medio que no nos permite seleccionar a nivel de familia o género, es decir, se estimaron las abundancias globales de bacterias heterótrofas cultivables en dicho medio general.

II.1. Papamoscas cerrojillo (*Ficedula hypoleuca*)

El papamoscas cerrojillo *Ficedula hypoleuca* es un paseriforme de pequeño tamaño (longitud: 12-13 cm, envergadura: 21.5-24 cm, peso: 12-13 g). En la Península Ibérica encontramos la subespecie *Ficedula hypoleuca iberiae* (Morales 2012).

Es una especie migradora transahariana cuyos cuarteles de invierno se encuentran en regiones tropicales del continente africano. En primavera, migra hacia las zonas de cría situadas en el norte de África y en Europa. Los machos suelen llegar a partir de mediados de abril, siendo los más tempranos los individuos con mayor éxito reproductor (Potti & Montalvo 1991, Lobato *et al*. 2010), mientras que las hembras llegan 7-14 días más tarde.

El nido es construido principalmente por la hembra aunque recientemente se ha observado que hay machos que también aportan material al nido, poniendo las hembras de estas parejas huevos de mayor tamaño (Martínez-de la Puente *et al*. 2009). El nido consiste en una base de material vegetal, la cual varía dependiendo de su disponibilidad en el hábitat, pudiendo estar constituida por cortezas de jara, por hojas de roble y hierba seca o por cortecillas de pino en zonas con pinos (Moreno et al. 2009). Por último se habilita un cuenco bien definido y compacto, pudiendo éste incluir algunos pelos de mamíferos residentes en la zona, siendo principalmente crines de caballo en la primera zona de estudio (Valsaín).

La puesta del primer huevo suele ocurrir en la segunda quincena de mayo (Potti *et al*. 1987, Moreno *et al.* 2006). El tamaño de la puesta oscila entre 4 y 7 huevos, con

una media de 5.7 huevos (Moreno & Sanz 1994, Sanz 1997, Moreno *et al.* 2006). Una vez que eclosionan los huevos, lo cual sucede durante la primera quincena de junio en nuestra población, la hembra empolla con intensidad decreciente durante 7 días aportando el macho la mayor parte del alimento a los pollos (Sanz & Moreno 1995, Moreno *et al.* 2011). Este tiempo que pasa la hembra con los pollos es importante debido a que los pollos a edades tempranas no son capaces de mantener una temperatura corporal constante. La capacidad homeotérmica de los pollos aumenta con la edad hasta ser prácticamente total durante la segunda semana en el nido. Los pollos son alimentados por ambos progenitores y permanecen en el nido durante unos 15-16 días, produciéndose el abandono en la segunda quincena de junio. Nuestro estudio se ciñe exclusivamente a esta etapa de residencia de los pollos en el nido, durante la cual hemos obtenido las medidas biométricas y las muestras bacterianas a una o distintas edades según las necesidades del estudio. Principalmente los pollos han sido medidos a los 7 y 13 días ó únicamente a los 13 días desde su eclosión (día 1), siendo las medidas obtenidas el peso estimado con un dinamómetro Pesola (precisión 0.25g), la longitud del tarso medido mediante un calibre digital (0.01mm) y finalmente la longitud alar medida con una regla con tope (1mm). Si era necesaria la medida de los pollos a día 7, entonces éstos eran anillados en este día para su posterior reconocimiento a día 13, pero si dicha medida no era necesaria los pollos eran anillados a la edad más tardía.

II.2. Enterococos

El género *Enterococcus* como tal no aparece en la primera edición del Manual de Sistemática Bacteriológica de Bergey ya que sus especies fueron incluidas en el género *Streptococcus* en un principio. En 1984 el género *Enterococcus* fue propuesto por Schleifer y Kilpper-Bälz para incluir en él a las especies *Streptococcus faecalis* y

Streptococcus faecium (Holt 1994). Estudios quimiotaxonómicos posteriores incluyeron a otras especies en este género como *E. avium*, *E. casseliflavus*, *E. durans* y *E. gallinarum* (Collins *et al*. 1984), casi todos ellos encontrados en muestras cloacales de hembras de papamoscas cerrojillo (Ruiz-de-Castañeda et al. 2011). Con el paso de los años se han descrito nuevas especies, destacando *E. phoeniculicola* aislada en la glándula uropigial de la abubilla arbórea verde *Phoeniculus purpureus* (Law-Brown & Meyers, 2003).

Los enterococos son cocos Gram-positivos que se agrupan en cadenas cortas o por parejas. Son anaerobios facultativos, no forman endosporas y su temperatura óptima de crecimiento es de 37ºC con un rango de presencia de 10 a 45ºC (Figura 2). Ampliamente distribuidos, se localizan habitualmente en el intestino de animales (Holt 1994).

Figura 2. De izquierda a derecha: medio selectivo para enterococos y el medio con unidades formadoras de colonias (CFU) de dicho género.

II.3. Enterobacterias

La familia *Enterobacteriaceae* es la más ampliamente estudiada dentro del mundo de los microorganismos. Tiene una amplia distribución aunque la mayoría de las especies pueden aislarse del intestino de los animales o del hombre, por lo cual recibe su nombre griego del intestino *entéron* (Breed *et al*. 1957). Por este motivo, su presencia en las aguas es utilizada como indicador de contaminación fecal.

Dentro de esta familia se encuentran bacterias tan patógenas como la responsable de la fiebre tifoidea (*Salmonella tiphy*), la peste (*Yersinia pestis*) o la disentería bacilar (*Shigella dysenteriae*). Sin duda la especie más conocida de esta familia es *Escherichia coli*, descrita por primera vez por Theodore von Escherich quién la denominó como *Bacterium coli*, pero que posteriormente pasó a ser denominada *Escherichia coli* en honor a su descubridor. Esta especie bacteriana encuentra su hábitat natural en el intestino de animales y humanos, actuando como comensal y ayudando así a la absorción de nutrientes. Sin embargo, existen cepas de esta especie capaces de causar diarreas pudiendo ser dichas diarreas de carácter hemorrágico.

Se caracterizan por ser mayoritariamente bacilos Gram-negativos, oxidasa negativa, pudiendo ser fermentadores de lactosa y al igual que los enterococos no forman endosporas. Su rango de temperatura de crecimiento oscila desde 22ºC a 37ºC (Breed *et al*. 1957).

III. Protocolo en el campo y procesamiento en el laboratorio.

En este apartado se describen los aspectos más generales de la metodología utilizada, centrándose en los protocolos de muestreo de bacterias en condiciones naturales y de análisis en el laboratorio.

III.1. Protocolo de muestreo bacteriano en bolsas fecales (**Capítulos I y II**).

En la temporada de 2009 en la zona de Valsaín, a día 7 de edad (día de eclosión = 1), los pollos fueron anillados para su posterior identificación a día 13, pesados y medidos (longitud del tarso y ala) además de obtener las bolsas fecales de dos pollos escogidos al azar por nido. Debido al pequeño tamaño de los pollos no se pudo insertar el hisopo en su cloaca por lo que se muestrearon bolsas fecales producidas en el momento del muestreo. Dichas bolsas eran recogidas en tubos Eppendorf esterilizados para evitar cualquier contaminación. Los tubos con las muestras eran transportados en una nevera portátil hasta su llegada al laboratorio. Una vez allí, en condiciones de esterilidad, se impregnaba con la bolsa fecal un hisopo con medio de transporte Amies (MEUS S.r.l. Piove di Sacco, Italia) que permite la supervivencia de los microorganismos. Posteriormente se almacenaban en cámara fría (4ºC) durante 20 días hasta su procesamiento debido a las dificultades logísticas para poder procesarlas en los días inmediatamente posteriores a la recogida. Este mismo proceso se realizaba cuando los pollos alcanzaban la edad de 13 días, además de obtener sus medidas biométricas (peso, longitud alar y de tarso).

Concluido el trabajo de campo, las muestras eran procesadas en un plazo de 20 días exactos desde de su obtención. De esta manera todas las muestras estaban en las mismas condiciones y por lo tanto nos evitábamos el posible efecto temporal en las estimas de abundancias bacterianas. El hisopo con la muestra fecal era introducido en un tubo Eppendorf con 1 mililitro (ml) de tampón fosfato salino (PBS, pH = 7.2; Química Clínica Aplicada, Tarragona, España) para a continuación realizar diluciones sucesivas en solución salina (0.85%

NaCl). Finalmente, se sembraban 100 microlitros (µl) de cada dilución en placas con los medios selectivos MacConkey (enterobacterias) y DCO (enterococos), siendo estas placas incubadas a 37 ± 1ºC durante 48 ± 1h. Los conteos se realizaban en la placa correspondiente a la dilución, en la cual se podían contar de 30 a 300 unidades formadoras de colonia (CFU) con un contador de colonias (Suntex Instruments Co. Ltd., Taipei County, Taiwan; Herbert 1990).

III. 2. Protocolo de extracción de ADN de muestras fecales y obtención de las Unidades Operativas Taxonómicas (Operational Taxonomic Units, OTU) (**CAPÍTULO III**).

Para este protocolo, utilizamos una parte de las muestras fecales obtenidas en el apartado III.1. Estas muestras eran obtenidas a los 7 y 13 días. Una vez desarrollado el protocolo del apartado III.1, 500 µl de la dilución original, es decir la que contenía 1 ml de PBS y el hisopo con la materia fecal, fueron traspasados a un tubo de rosca de 1.5 ml que contenía aproximadamente 1 ml de medio de congelación (el cual estaba compuesto por 20 g de leche desnatada (Difco, Laboratories, Detroit, MI, USA), 30g de Triptona (Pronadisa, CondaLab, Torrejón de Ardoz, Madrid), 8 ml de Glicerol (Panreac Química, s. l., Catellar del Vallés, Barcelona) y 1000 ml de agua destilada). Posteriormente fueron congelados a -80ºC hasta su procesamiento para extraer el ADN bacteriano.

Para la extracción de ADN seguimos el protocolo desarrollado por Martín-Platero *et al.* (2007). Antes de empezar con la extracción, 500 µl de nuestra muestra contenida en el medio de congelación fueron lavados dos veces

con 500 µl de PBS y centrifugados con el objetivo de limpiar la muestra de posibles restos fecales. A continuación el pellet obtenido del paso anterior fue resuspendido en 100 µl de buffer TES, centrifugado durante 1 minuto (min) e incubado por 30 min a 37ºC. Después las células fueron lisadas, añadiéndose 600 µl de buffer de lisis e incubándose durante 15 min a temperatura ambiente. El lisado fue tratado con 10 µl de proteinasa K e incubado por 15 min a 37ºC, incrementando así la pureza del ADN extraído. Posteriormente el tubo se incubó a 80ºC durante 5 min, seguido de un enfriamiento de 10 min a temperatura ambiente. Se añadieron 200 µl de acetato sódico y se mezcló todo con vortex durante 15 seg, enfriándose con hielo durante 15 min y centrifugándose otra vez durante 10 min. El sobrenadante de este último paso fue traspasado a otro tubo limpio, pues dicho sobrenadante contenía los ácidos nucleicos. Éstos fueron precipitados con 600 µl de isopropanol y centrifugados durante 5 min. Se obtuvo así un pellet de ADN, el cuál fue lavado con 1 ml de etanol al 70 % y secado a temperatura ambiente. Finalmente, el ADN fue resuspendido en 200 µl de 0.5 x buffer TE. Como medida de comprobación para verificar que la extracción había sido satisfactoria, 9 µl de la extracción fueron analizados en un gel de agarosa al 0.7%.

Una vez comprobado que la extracción era correcta, se procedió a realizar una reacción en cadena de la polimerasa (PCR) para amplificar dicho ADN. Para ello se utilizaron los primers 72 F (5'- TGCGGCTGGATCTCCTT-3') y 38 R (5'-[6HEX] CCGGGTTTCCCCATTCGG- 3') marcado fluorescentemente con HEX (6-carboxyhexafluorescein) (Ranjard *et al.* 2001). La PCR fue llevada a cabo con un volumen final de 50 µl formado por 3 µl de ADN (50 nanogramos), 1x buffer de PCR (QIAGEN), 6 µl de $MgCl_2$ (25 mM),

5 μl de cada primer (10 pmol/ μl), 1 μl de Deoxinucleosido trifosfato (dNTPs, 10 mM), 0.3 μl de Taq polimerasa (QIAGEN) y por último agua milliQ hasta completar el volumen de 50 μl. El proceso de la PCR consistió en una desnaturalización inicial a 94ºC durante 3 min, seguido de 1 min a 94ºC, 30 seg a 55ºC, y 1 min a 72ºC, lo que conllevó 30 ciclos desde la desnaturalización inicial hasta éste último paso. Por último se realizó una extensión final a 72ºC durante 5 min. A continuación, estos productos fueron corridos en un gel de agarosa al 1% para determinar que la amplificación había sido correcta.

Posteriormente a dicha verificación, 5 μl de cada producto de PCR diluido 15 veces en agua milliQ fue analizado en el secuenciador 3130 Genetic Analyzer (Applied Biosystems) en el Centro de Instrumentación Científica (CIC) de la Universidad de Granada.

El secuenciador detectó fragmentos comprendidos entre los 100 y 1000 pares de bases (bp), los cuales fueron analizados con el programa Peak Scanner versión 1.0 (Applied Biosystems). Una vez obtenidos los picos así como sus respectivos tamaños y áreas, fijamos el tamaño de la ventana (bin size) y la variación (shift) siguiendo el artículo de Ramette (2009), dando como resultado los OTUs correspondientes, mientras que la intensidad de la señal detectada se consideró un indicador de la abundancia de cada OTU al igual que en otros trabajos (Yannarell & Triplett 2005, Mennerat *et al.* 2009, White *et al.* 2010, 2011).

III.3. Protocolo de muestreo bacteriano en la piel y nido (**Capítulos IV y V**)

En la temporada de 2011 se trabajó en dos zonas diferentes: Lozoya, en la cual se realizó un experimento para estudiar el posible efecto de las bacterias residentes en nidos reutilizados en el crecimiento de los pollos, y Valsaín, dónde se muestrearon las bacterias de la piel de los pollos y se estudió su posible cambio con la edad así como el efecto sobre el crecimiento de los pollos. Por consiguiente durante esta temporada se llevaron a cabo dos protocolos diferentes, los cuales se detallan a continuación.

III.3.1. Protocolo en la zona de Lozoya (**CAPÍTULO IV**)

Durante la temporada 2010, al terminar la época de reproducción una parte de las cajas-nido (aproximadamente 50) se dejaron sin limpiar mientras que del resto se eliminó el nido construido durante dicha temporada. Por lo tanto tenemos dos tipos de cajas-nido (vacías y con nido viejo) que fueron ocupadas por las parejas que criaron durante 2011. El mismo día que eclosionaron los pollos (día 1), se insertó entre el material una lámina de plástico amarillo y rugoso en forma de cuadrado siendo la superficie total de 3 cm^2 si contamos ambos lados. Antes de su colocación fue esterilizada con alcohol por ambas caras para evitar la posible colonización por parte de bacterias ambientales que no pertenecieran al material del nido o al ambiente de la caja-nido. Además para evitar la contaminación del objeto por parte del investigador, éste llevaba guantes de látex esterilizados con alcohol. Dicho objeto nos

servía para descartar que las bacterias pudieran tener la misma predilección por un objeto inerte frente a uno orgánico como es el pollo (Figura 3a).

El investigador llevaba guantes durante dichas manipulaciones los cuales eran continuamente lavados con alcohol entre cada muestreo y cambiados de un nido a otro para evitar posibles contaminaciones entre muestras. A día 13 de los pollos, dos pollos fueron escogidos al azar por cada nido para muestrearles 3 cm^2 de la parte del vientre que está desprovista de plumas y por consiguiente obtener así una muestra de las bacterias existentes sobre la piel del pollo, utilizando para ello una plantilla de plástico rígido transparente en el que previamente se había recortado un rectángulo con esta medida (3 cm^2). Esta plantilla fue lavada con alcohol a modo de esterilización antes de colocarla en contacto con la piel del pollo. Una vez colocada la plantilla sobre el pollo, se pasó un hisopo con medio de transporte Amies impregnado en PBS estéril durante 30 seg por la superficie de piel enmarcada por la plantilla, evitando al máximo el posible contacto con las plumas (Figura 3b). Los pollos fueron anillados, medidos (longitud del tarso y ala) y pesados en este día después de haber obtenido la muestra.

También se muestreó el cuenco del nido para obtener una cuantificación de las bacterias heterótrofas existentes en el material del nido que está en contacto con los pollos, utilizando para ello igualmente una plantilla del mismo material que la de los pollos y esterilizada con alcohol. Se colocó la plantilla sobre las paredes del cuenco, evitando los excrementos que pudiera haber, pasándose a continuación el hisopo con medio de

transporte Amies impregnado en PBS por la superficie de material enmarcada por la plantilla durante 30 seg (Figura 3c).

Figura 3. Muestreo bacteriano. a) objeto control inerte introducido entre el material del nido, b)muestreo de la piel de la parte ventral del pollo a través de una plantilla de material plástico rígido (las plumas eran separadas de la zona de muestreo antes de pasar el hisopo por la piel), c) muestreo del cuenco del nido de la misma forma que el realizado para los pollos.

Asimismo se recuperó de entre el material del nido el objeto control, recogiéndolo con unas pinzas previamente esterilizadas (limpiadas con alcohol y flameadas) para evitar cualquier posible contaminación por parte del investigador. Para la recogida de bacterias que colonizaron el objeto, se pasó un hisopo con medio de transporte Amies impregnado en PBS por ambas caras del objeto.

Todos los hisopos fueron transportados en una nevera portátil. Una vez en el laboratorio en condiciones de esterilidad óptimas, fueron

introducidos en tubos de rosca de 1.5 ml que contenían medio de congelación (ver apartado III. 2 para una composición más detallada de este medio) y congelados a -80ºC hasta su posterior procesamiento, de tal forma que las bacterias permanecían viables para su posterior cuantificación. El procesamiento consistió en hacer diluciones seriadas, sembrando 100 µl de estas diluciones en un medio general utilizado para cuantificar o aislar bacterias aeróbicas como es el medio de Agar Triptona Soja (TSA, Scharlau, Barcelona) para posteriormente incubarlas a 25 ± 1ºC durante 48 ± 1h. Transcurrido este tiempo se cuantificó el nº de colonias existentes en las muestras.

III.3.2. Protocolo en la zona de Valsaín (**CAPÍTULO V**)

En Valsaín los pollos fueron muestreados de la misma manera que en el apartado anterior, pero a diferencia del protocolo que se siguió en la zona de Lozoya, se realizó el muestreo tanto a día 7 como a día 13 de edad con el fin de observar un cambio en la abundancia bacteriana residente en la piel. Para distinguir los pollos, éstos fueron anillados a día 7 de tal manera que en el segundo muestreo se les pudiera identificar fácilmente.

Otra diferencia radica en que se metieron dos objetos control el mismo día de eclosión, uno para que fuera el control del día 7 (muestra 1) que era recogido y muestreado en el mismo día que los pollos, y otro para el muestreo del día 13 (muestra 2). Como hemos mencionado anteriormente, este objeto nos serviría a posteriori para verificar que las

bacterias no tienen la misma predilección por una superficie inorgánica que por la piel de los pollos (Figura 3a y b). Además se obtuvieron las medidas biométricas (peso, longitud del tarso y del ala) de los pollos a ambas edades. El nido no fue muestreado pues sólo se quería estimar la abundancia de bacterias sobre la piel de los pollos y no la influencia del material del nido sobre dicha abundancia.

Al igual que en el apartado anterior, los hisopos fueron transportados en una nevera portátil e introducidos en el laboratorio en tubos de rosca que contenían medio de congelación (véase apartado III.2 para una composición más detallada), y almacenados a -80ºC. Posteriormente se hicieron diluciones, sembrándose 100 µl en medio TSA e incubándose a 25 ± 1ºC durante 48 ± 1h para después contar las colonias.

III.4. Protocolo para la recogida de muestras de inmunidad (**CAPÍTULO VI**)

Durante la temporada de 2009, los mismos pollos de los que se obtuvo muestra fecal, fueron inyectados intradérmicamente en el patagio a día 12 con 0.02 mg de fitohemaglutinina (PHA, Sigma aldrich) disuelta en 0.02 ml de PBS (Figura 4a; Moreno *et al.* 2005). La diferencia entre el grosor inicial y la respuesta inflamatoria 24 horas más tarde se utilizó como la estimación de la respuesta inmune celular (Figura 4b).

De estos mismos pollos, se obtuvo una muestra sanguínea (aproximadamente 2 capilares) a los 13 días de edad (Figura 4c) para estimar las inmunoglobulinas en suero y la inmunidad innata. Las muestras de sangre fueron traspasadas de los capilares a tubos eppendorf, los cuales fueron transportados en una nevera

portátil hasta el laboratorio durante el día de muestreo. Una vez en el laboratorio las muestras fueron centrifugadas para separar la fracción celular del suero y almacenadas a -20ºC. A continuación, describiremos brevemente las técnicas para cuantificar inmunoglobulinas en suero así como el protocolo seguido para obtener las medidas de inmunidad innata.

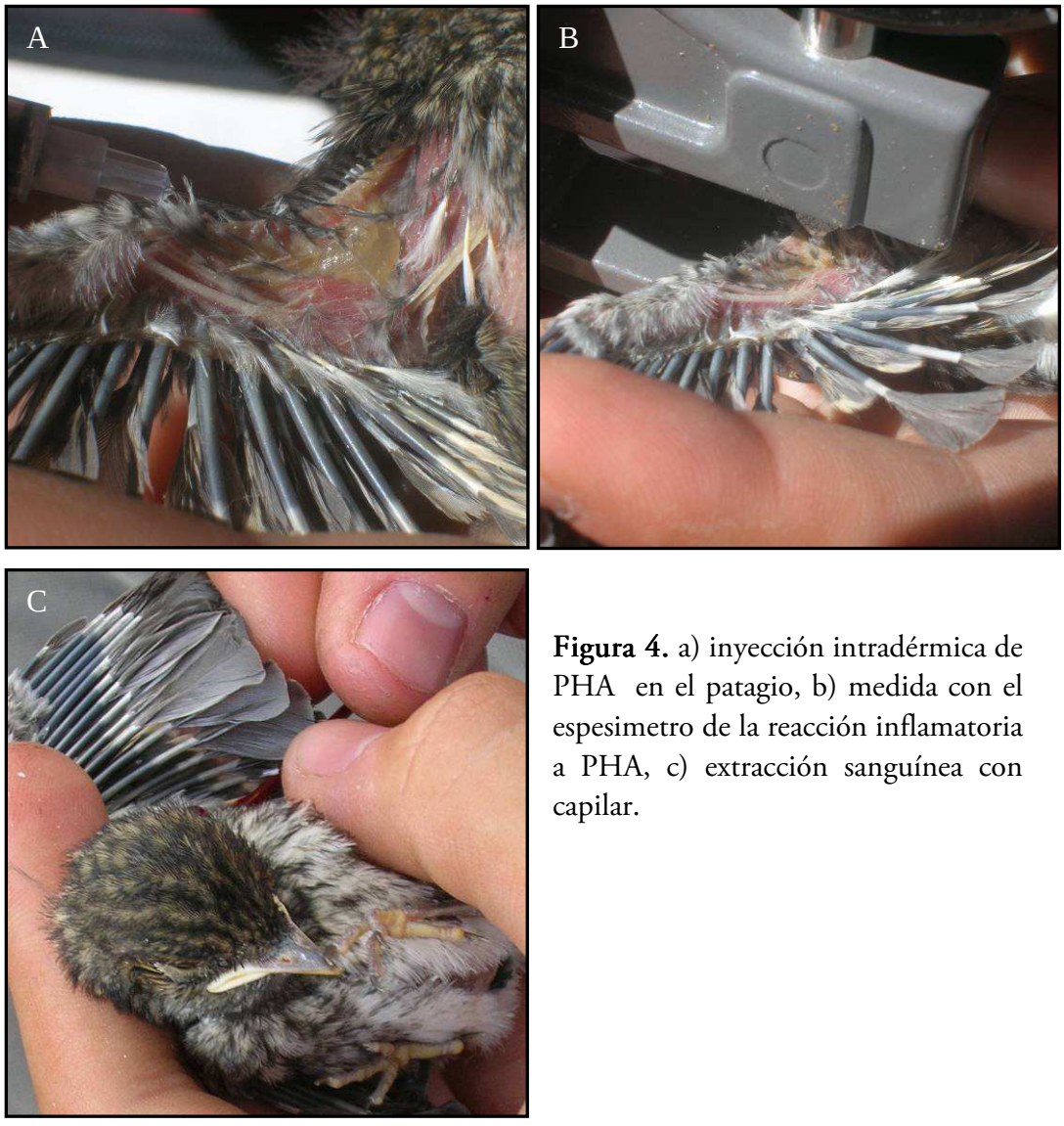

Figura 4. a) inyección intradérmica de PHA en el patagio, b) medida con el espesimetro de la reacción inflamatoria a PHA, c) extracción sanguínea con capilar.

III.4.1. Protocolo para la medición de inmunoglobulinas en suero.

Para estimar las inmunoglobulinas a partir de suero congelado, seguimos el protocolo desarrollado por Martínez *et al.* (2003). El primer paso consistió en obtener con un pool de muestras elegidas al azar el rango lineal de la curva y por ende la dilución óptima para nuestras muestras, que sería la dilución (suero diluido en tampón carbonato/bicarbonato) más cercana al centro de dicho rango. Una vez obtenida la dilución idónea, 100 μl de ésta se cargó en placas ELISA (Maxi-sorp, Nunc, Rochester, NY, USA) y se incubó durante 1 h a 37ºC para luego dejarlo durante toda la noche a 4ºC. Al día siguiente, los pocillos eran bloqueados con leche en polvo diluida en PBS-Tween (100 μl) durante 1 h a 37ºC. Pasado este tiempo, se le añadió el anticuerpo conjugado (Sigma A-9046, MO, USA) diluído en PBS-Tween, incubándolo por 2 horas a 37ºC (100 μl). Después del tiempo de incubación, se añadió la solución reveladora (100 μl) y se volvió a incubar durante 1 h a 37ºC. Finalmente, se leyeron las absorbancias a λ= 405 nm usando para ello un espectrofotómetro.

III.4.2. Protocolo inmunidad innata.

Para obtener ambas medidas de inmunidad innata (aglutinación y lisis), hemos seguido el protocolo desarrollado por Matson *et al.* (2005), el cuál describiremos brevemente en este apartado. Primero se obtuvo una solución al 1% de eritrocitos de conejo a partir de sangre fresca de conejo con anticoagulante Alsever (HemoStat Laboratories, Dixon,

USA). Esta solución fue guardada en la nevera (4ºC) hasta su utilización en el mismo día.

Las muestras de plasma fueron homogenizadas mediante vortex, para posteriormente pipetear 25 µl de ésta en las dos primeras columnas de una placa de poliestireno con fondo redondeado. Seguidamente se añadieron 25 µl de 0.01 M de PBS en todos los pocillos, excepto en la primera columna. A partir del contenido de la segunda columna se hicieron diluciones seriadas hasta la columna 11, quedando la última columna como control negativo al contener solamente PBS y la dilución de eritrocitos de conejo, que se añadió a todos los pocillos (25 µl). Las placas cubiertas con una pegatina y agitadas por 10 seg fueron incubadas durante 90 min en un baño a 37ºC. Una vez transcurrido este tiempo, las placas fueron sacadas del baño y colocadas con una inclinación de 45º a temperatura ambiente durante 20 min para su posterior lectura con un scanner. Después de esta primera lectura, se volvieron a colocar de la forma antes descrita durante un periodo de 70 min para realizar una segunda lectura, en la cual se obtenía la máxima actividad lítica.

OBJETIVOS

- CAPÍTULO I: Estimar la abundancia de dos tipos bacterianos comunes de la flora intestinal (enterococos y enterobacterias) durante el desarrollo de los pollos en el nido así como observar su posible asociación con el crecimiento de éstos.

- CAPÍTULO II: Estudiar el efecto de las variables climatológicas (temperatura, precipitación) así como parámetros fenológicos (fecha de eclosión) sobre la abundancia de ciertos tipos bacterianos que forman parte de la microflora intestinal.

- CAPÍTULO III: Estudiar el posible cambio en la composición de la comunidad microbiana residente en el intestino con respecto a la edad.

- CAPÍTULO IV: Comprobar los posibles costes de la reutilización de nidos viejos, asociados a las bacterias existentes en el material del nido, para el crecimiento de los pollos.

- CAPÍTULO V: Estimar la abundancia de bacterias heterótrofas en la piel de los pollos y su asociación con el crecimiento.

- CAPÍTULO VI: Investigar si existen asociaciones negativas (compromiso) o positivas (inmunocompetencia) entre los diferentes brazos del sistema inmune y el posible compromiso de la intensidad de las respuestas con el crecimiento de los pollos.

RESULTADOS PRINCIPALES

BLOQUE I:

En dicho bloque se agrupan los **capítulos I**, **II** y **III**, centrándose en la microflora intestinal de los pollos de papamoscas cerrojillo. Concretamente los dos primeros capítulos abordan dos tipos concretos de bacterias, enterococos y enterobacterias, las cuales son bastante comunes en los tractos intestinales de las aves mientras que en el tercero utilizamos técnicas moleculares. El **capítulo I** se centra en la posible asociación de las bacterias intestinales con el crecimiento de los pollos, mientras que en el **capítulo II** se estudian los posibles efectos ambientales que pueden afectar al crecimiento de dichas bacterias. Finalmente en el **capítulo III** estudiamos los posibles cambios cualitativos en la composición bacteriana a medida que el pollo va creciendo. A continuación, se hará una breve descripción de los resultados más relevantes de estos tres capítulos.

CAPÍTULO I:

En este primer capítulo se estimó la abundancia de dos tipos bacterianos comunes en el intestino, enterococos y enterobacterias, a diferentes edades de los pollos (7 y 13 días desde su eclosión) y las posibles asociaciones de estas abundancias con el crecimiento de ciertas estructuras (tarso y ala) así como con el peso en pollos de papamoscas cerrojillo.

Se observó que la abundancia de estas bacterias difería en función de la edad del pollo. Así, mientras unas aumentaban su abundancia en el transcurso del crecimiento (enterococos), las otras disminuían durante el mismo período (enterobacterias) (Figura

5). Posiblemente estos diferentes patrones se deban a las interacciones competitivas factibles entre dichos grupos bacterianos por los recursos y el espacio en el intestino, para lo que podrían servirse de ciertas moléculas antimicrobianas llamadas bacteriocinas que son producidas bajo condiciones de estrés y que dan como resultado la rápida eliminación de las bacterias vecinas que no son inmunes o resistentes a su efecto (Balciunas *et al*. 2013, Klaenhammer 1988, Moreno *et al*. 2003, Riley & Wertz 2002, Soler *et al*. 2009). Complementariamente, estos cambios cuantitativos en la abundancia de ambos tipos bacterianos podrían deberse a posibles cambios en la alimentación aportada por los adultos puesto que el alimento puede constituir la principal fuente de colonización bacteriana del intestino (Waldenström *et al*. 2002, Maul & Farris 2005).

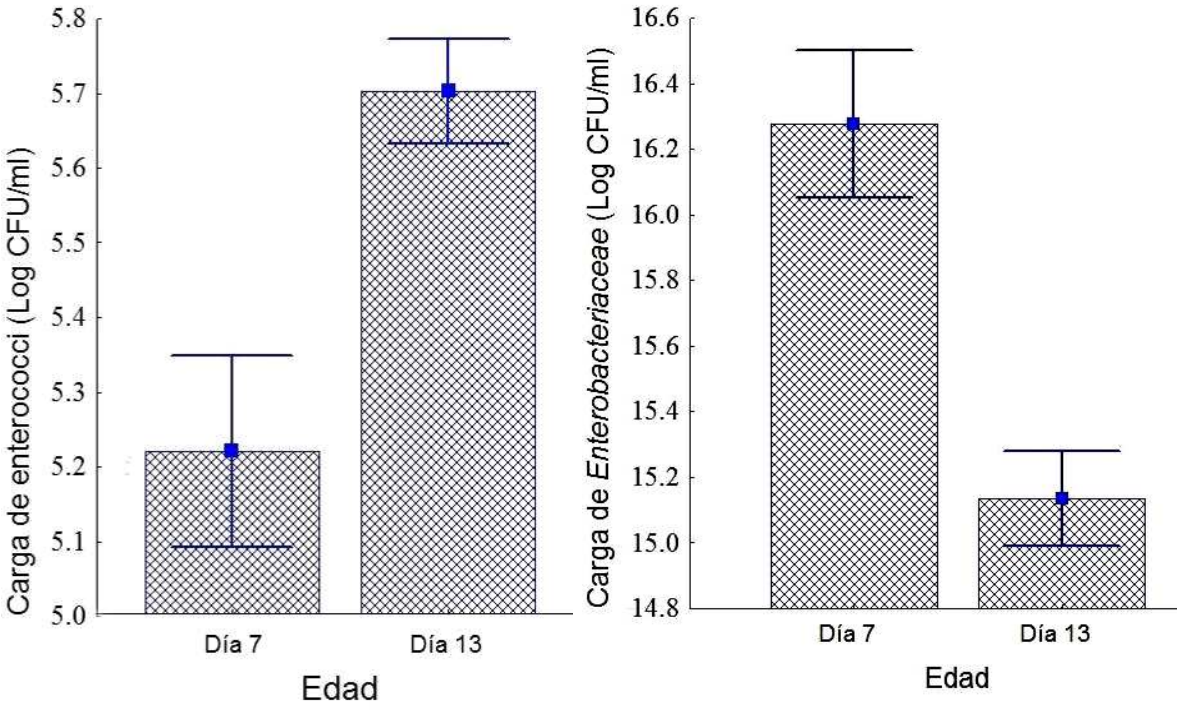

Figura 5. Cargas bacterianas a los 7 y 13 días de edad de los pollos (día de eclosión = día 1) de a) enterococos (log CFU/ ml) y b) enterobacterias (log CFU/ ml).

Con respecto a las variables biométricas de los pollos, se observó que las cargas de enterococos a edades tempranas estaban positivamente correlacionadas con el peso y

la longitud del ala a dicha edad. Por otro lado, las cargas de enterococos a día 13 no estaban asociadas a ninguna variable biométrica. Para enterobacterias no se observaron asociaciones con las medidas de los pollos a ninguna edad. En cuanto al crecimiento en sí, es decir la diferencia entre las medidas biométricas tomadas a día 13 y a día 7, sólo los conteos de enterococos a día 7 mostraron una correlación negativa con el crecimiento del tarso (Figura 6). Este resultado sugiere que las bacterias intestinales no son sólo simbiontes beneficiosos para el individuo sino que pueden sustraer los recursos nutritivos necesarios para el crecimiento de ciertas partes anatómicas. Asimismo estos resultados apoyan otros trabajos que encontraron una asociación negativa entre las bacterias intestinales y alguna variable biométrica, aunque hay que destacar que la mayoría sólo han muestreado dichas bacterias en una ocasión antes de que los pollos abandonaran el nido (Lombardo *et al*. 1996, Mills *et al*. 1999, Potti *et al*. 2002).

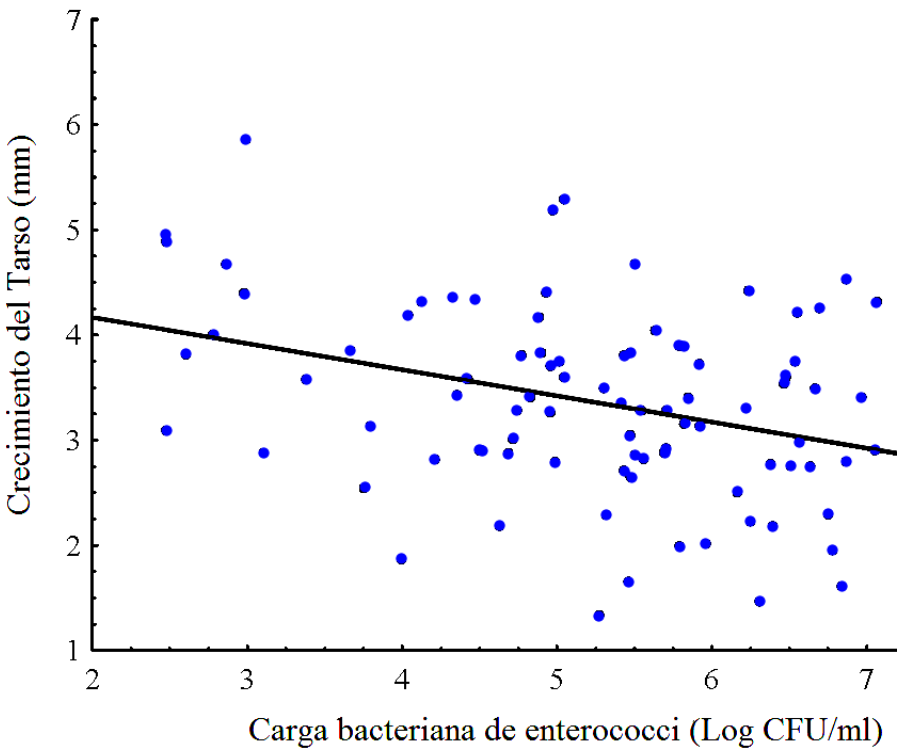

Figura 6. Correlación entre la abundancia de enterococci a día 7 (log CFU/ ml) y el crecimiento del tarso (mm).

CAPÍTULO II:

Una vez obtenidas en el capítulo anterior las estimas de enterococos y enterobacterias en el tracto intestinal de los pollos de papamoscas cerrojillo, intentamos desvelar sí, y en su caso cómo, las variables climatológicas a las que se encuentran sometidos los pollos en el nido pueden afectar a dichas bacterias. Las bacterias intestinales podrían verse afectadas por factores climatológicos como la lluvia o la temperatura ambiental, ya que estas variables afectarían a la temperatura corporal de los pollos al no tener éstos una capacidad termorreguladora eficiente a tempranas edades. Por consiguiente esta inestabilidad en la temperatura corporal podría repercutir en las poblaciones bacterianas.

Las variables climatológicas (temperatura media y precipitación) fueron obtenidas de la estación meteorológica "Casa de la Mata", situada a dos kilómetros de nuestra zona de estudio (40˚ 54' N, 4˚ 00' W, 1.150 m a.s.l.). Fueron calculadas dos temperaturas medias por cada nidada, una consistió en la temperatura media desde la fecha de eclosión hasta la edad más temprana (7 días) y la segunda la temperatura media comprendida entre las dos edades de muestreo. En cuanto a la lluvia se obtuvo la precipitación acumulada durante ambos períodos.

La abundancia de enterococos a tempranas edades está correlacionada positivamente con la temperatura ambiental. Como mencionábamos anteriormente, esto podría deberse a la escasa capacidad termorreguladora de los pollos a dichas edades (Starck & Ricklefs 1998), por lo que las fluctuaciones térmicas podrían afectar a la temperatura corporal a la que se desarrollan las bacterias. Ello a su vez podría modular el crecimiento bacteriano puesto que éstas tienen una temperatura óptima para su proliferación (Holt 1994, Foulquié-Moreno *et al*. 2005).

Con respecto al otro grupo bacteriano, su abundancia estuvo asociada negativamente con la fecha de eclosión a ambas edades. Que los pollos de nidos tardíos tengan una menor carga de enterobacterias probablemente se deba a que, como ya hemos mencionado en el **capítulo I**, los posibles cambios de alimentación a lo largo de la temporada afectan especialmente a este grupo de bacterias (Lombardo *et al*. 1996, Brittingham *et al*. 1988, Waldenström *et al*. 2002). Otra variable que afectó a la abundancia de enterobacterias en el día 7 fue la temperatura, que al igual que para enterococos, se asoció positivamente con ésta. Asimismo, la precipitación mostró una asociación negativa con la abundancia de enterobacterias a esta misma edad, lo cual también podía achacarse al efecto de enfriamiento del ambiente que produce la lluvia y a que los pollos a esta edad presentan una escasa capacidad de termorregulación (Starck & Ricklefs 1998). Por otro lado, la lluvia también tiene un efecto negativo sobre la capacidad de búsqueda de presas por parte de los adultos (Radford *et al*. 2001, Geiser *et al*. 2008, Arlettaz *et al*. 2010), lo que afectaría negativamente a la condición nutricional del pollo y podría por tanto conllevar una mayor competencia entre bacterias por los recursos nutritivos. La interacción significativa entre fecha de eclosión y precipitación (Figura 7) indica que la lluvia sólo tiene efectos negativos sobre la abundancia de enterobacterias en los nidos que eclosionaron en fechas tempranas que son aquellos que se enfrentaron a condiciones térmicas menos benignas.

Sin embargo a edades tardías no se han encontrado casi asociaciones entre las variables climatológicas y las abundancias bacterianas, siendo la única asociación hallada aquella entre abundancia de enterococos y temperatura que fue positiva. Ello probablemente se explique por el rango de temperatura de crecimiento de dicho género que oscila desde los 10 a 45ºC. El mayor rango térmico de estos microorganismos frente

al que presentan las enterobacterias podría explicar que una leve subida en la temperatura corporal del pollo afecte a la abundancia de enterococos.

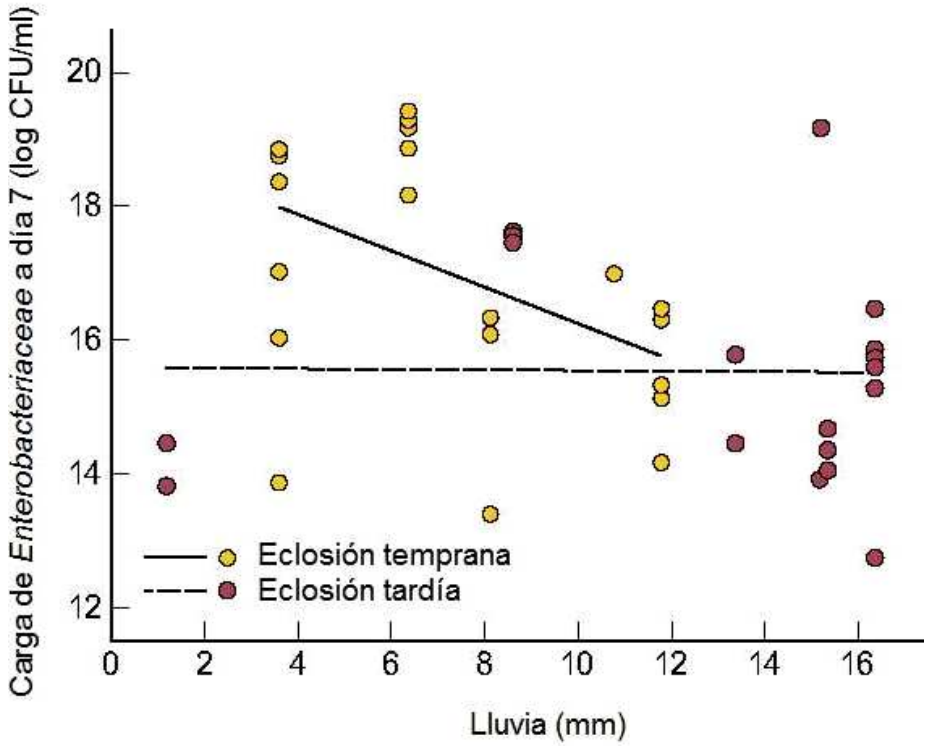

Figura 7. Asociación entre la abundancia de enterobacterias a día 7 con el día de eclosión y la lluvia.

CAPÍTULO III:

El intestino de las aves al eclosionar es estéril pero por poco tiempo puesto que inevitablemente las bacterias lo colonizan con rapidez por las diferentes vías de adquisición. Así mismo esta comunidad bacteriana se puede ver afectada por la edad pues a medida que el pollo crece se dan cambios morfológicos y fisiológicos en el tracto intestinal lo que posiblemente influirá en la estructura y composición de dicha comunidad.

En este **capítulo III** nos introducimos en las técnicas moleculares para obtener una aproximación a la comunidad bacteriana residente en el intestino de los pollos. La

técnica utilizada para estos fines fue el análisis automatizado del espaciador intergénico ribosomal (ARISA-PCR). Esta técnica amplifica la región intergénica del genoma comprendida entre los genes 16S y 23S del ADN ribosómico y se diferencia, con respecto a otras técnicas como RISA, en que uno de los primers utilizados en la PCR está marcado fluorescentemente y por lo tanto el paso electroforético se realiza con un sistema automatizado que proporciona la detección por láser de los fragmentos de DNA fluorescentes (Fisher & Triplett 1999, Ranjard 2001). Si bien esta metodología (ARISA) no nos permite identificar especies, si nos permite diferenciar entre unidades operativas taxonómicas (OTUs que son las siglas en inglés de *Operational Taxonomic Units*). Dichos OTUs equivalen en cierta medida a la tradicional categoría de especie bacteriana. Además la intensidad de la señal detectada ofrece una aproximación a la abundancia de cada OTU (Yannarell & Triplett 2005, Mennerat *et al.* 2009, White *et al.* 2010, 2011).

Se extrajo el ADN de un total de 100 muestras fecales de las obtenidas en el **capítulo I**, siguiendo el protocolo desarrollado por Martín-Platero *et al.* (2007) y posteriormente se amplificó mediante ARISA-PCR. Como primer resultado se obtuvo un número total de 91 OTUs o especies bacterianas entre las dos edades que conformaban el conjunto de las muestras. De estos 91 OTUs sólo cuatro se encuentran en el 20% de las muestras analizadas, siendo la media ± SE de OTUs encontrados en cada muestra 5.670 ± 0.383, resultado parecido a otros hallados en diversos estudios con aves adultas y pollos de otras especies (Ruíz-Rodríguez *et al.* 2009, Benskin *et al.* 2010, van Dongen *et al.* 2013). En cuanto al cambio con respecto a la edad, se encontró que el número de OTUs no cambió significativamente de una edad a otra. Ello no concuerda con otros resultados obtenidos con pollos de gaviota tridáctila *Rissa tridactyla*, en los que se observó que el número de especies bacterianas aumentaba con la edad (van

Dongen *et al.* 2013). Nuestro resultado sugiere que probablemente los pollos de papamoscas cerrojillo ya han adquirido su microbiota durante la primera semana tras la eclosión debido a que están confinados en el nido. Consecuentemente pueden adquirir en un corto período de tiempo las bacterias de su entorno. Ello no significa que dicha microbiota sea ya la definitiva en la etapa adulta ya que pueden adquirir nuevas especies una vez que abandonan el nido.

Otro resultado fue que la diversidad microbiana medida mediante el índice de Shannon ($H' = \Sigma\ p_i \log_2 (p_i)$, dónde p_i es la abundancia relativa observada de la especie i) fue mayor antes de abandonar el nido que a la semana de vida. Así pues, la comunidad microbiana en el intestino de los pollos aumenta su diversidad desde una etapa inicial que está constituida por unos pocos tipos bacterianos mayoritarios a una etapa previa al abandono del nido en que la diversidad es mayor e incluye un mayor número de bacterias comunes.

Bloque II:

Este bloque está formado por los **capítulos IV** y **V**, teniendo como objeto de estudio las bacterias heterótrofas existentes en el nido y en la piel de los pollos. En detalle, en el **capítulo IV** se estudió el efecto que puede tener la reutilización de los nidos sobre el crecimiento de los pollos como consecuencia de una posible mayor abundancia de bacterias en dichos nidos. Por otro lado, en el **capítulo V** se estudiaron las bacterias de la piel de los pollos, centrándonos en los factibles cambios en su abundancia durante el desarrollo de los pollos así como la posible asociación de esta abundancia con el crecimiento de los mismos.

CAPÍTULO IV:

En ocasiones las aves trogloditas se ven forzadas a reutilizar los nidos debido a la falta de disponibilidad de una nueva cavidad apropiada que no haya sido utilizada previamente. Esta tendencia puede estar correlacionada con la capacidad que tenga la especie para excavar nuevas oquedades, con los niveles de competencia inter o intraespecífica por las cavidades o con los años que han pasado desde su excavación (Mazgajski 2007, Bai & Mühlenberg 2008).

Por eso en 2011 realizamos un experimento para comprobar el efecto que podrían tener las bacterias presentes en nidos previamente utilizados (Singleton & Harper 1998) sobre el crecimiento de los pollos de papamoscas cerrojillo. Estimamos las cargas bacterianas de los nidos (viejos-nuevos) y sobre la piel de los pollos, concretamente en el vientre, relacionándolas con las cargas presentes sobre un objeto inerte control para comprobar que no se trataba de una mera colonización pasiva.

Si bien no existe ninguna diferencia significativa en cuanto a la carga bacteriana del nido dependiendo de si era reutilizado o no, sí se observó una relación con el tipo de nido para las bacterias de la piel, presentando una mayor carga bacteriana los pollos que crecieron en los nidos viejos (Figura 8).

Otro resultado destacable fue que la longitud alar de los pollos muestreados a los 13 días, debida fundamentalmente a la longitud de las primarias en crecimiento, se vio afectada por el tipo de nido así como por la abundancia de las bacterias propias del mismo. Los pollos en nidos reutilizados presentaron alas más cortas, lo que podría deberse a las bacterias degradadoras del plumaje (Burtt & Ichida 1999, Goldstein *et al.* 2004, Shawkey *et al.* 2009, Ruiz-de-Castañeda *et al.* 2012) ya que el género al que pertenecen han sido encontradas en el nido (Singleton & Harper 1998) y también sobre la piel de pollos (Berger *et al*. 2003).

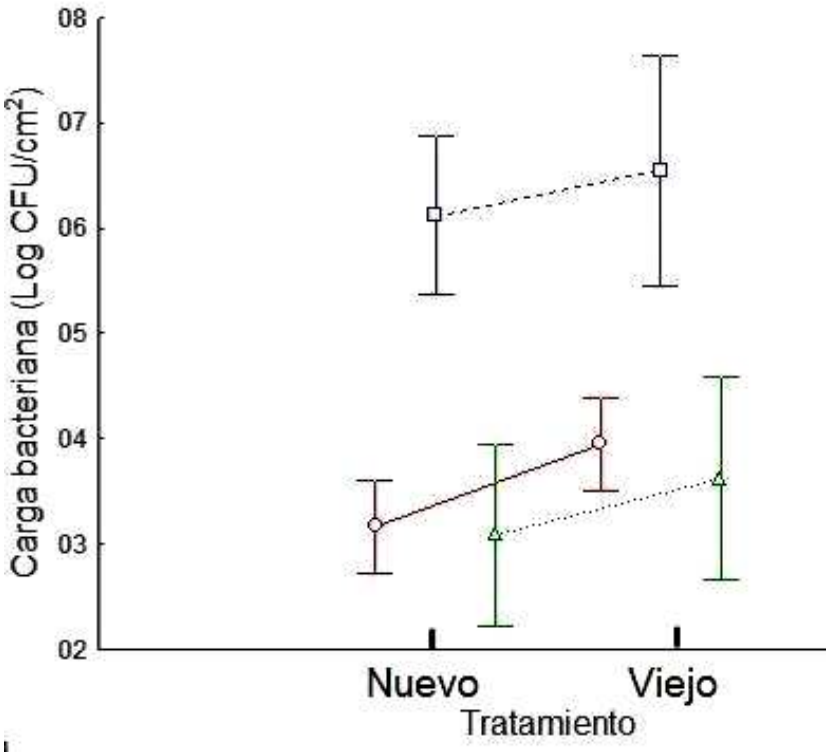

Figura 8. Abundancia bacteriana (media ± SE) sobre un objeto control (triángulos), sobre la piel de los pollos (círculos) y en el cuenco del nido (cuadrados) en relación al tipo de nido (objeto control: $F_{1,36}$ = 0.826, p = 0.369; piel: $F_{1,45}$ = 4.424, p = 0.041; nido: $F_{1,36}$ = 0.178, p = 0.675).

CAPÍTULO V:

Este capítulo se centra únicamente en las bacterias heterótrofas que colonizan la piel de los pollos, concretamente la piel de la parte ventral que está prácticamente desprovista de plumas a las edades de muestreo.

Observamos que las abundancias de bacterias no cambian con la edad de los pollos, probablemente porque los pollos ya han adquirido la microbiota de la piel durante los primeros días de vida por lo que los cambios podrían ser más cualitativos que cuantitativos, si existen. Además la abundancia de dichas bacterias a la edad más tardía de los pollos está asociada positivamente con el tamaño de la nidada. Ello se puede deber a que nidadas mayores ensucian más el nido y la cavidad debido a que los adultos sacrifican su capacidad para mantener unas buenas condiciones higiénicas en el nido en aras de poder proporcionar suficiente comida a los pollos. Por consiguiente se

daría una mayor acumulación de bolsas fecales en estos nidos que tendría como consecuencia un incremento de las poblaciones bacterianas de la piel. Asimismo se observó una asociación positiva de la abundancia bacteriana de la piel en el día 13 con la longitud alar a esta edad (Figura 9). Este posible efecto beneficioso de dichas bacterias sobre el crecimiento de las plumas puede deberse a la existencia de interacciones competitivas entre distintas bacterias, que llevaría a efectos nocivos de ciertos linajes bacterianos sobre la abundancia de agentes degradadores del plumaje. Así por ejemplo en las abubillas se ha encontrado que la bacteria *E. faecalis* produce sustancias antibacterianas efectivas frente a *B. licheniformis* (Soler *et al*. 2008, Ruiz-Rodríguez *et al*. 2009). Sin embargo existe otra explicación plausible basada en que los nidos con pollos que tengan una mayor longitud alar podrían contener mayores recursos nutritivos para el crecimiento bacteriano alrededor de las plumas lo cual beneficiaría una mayor población bacteriana que terminaría colonizando la piel del vientre, dando como resultado una mayor carga bacteriana en dicha zona.

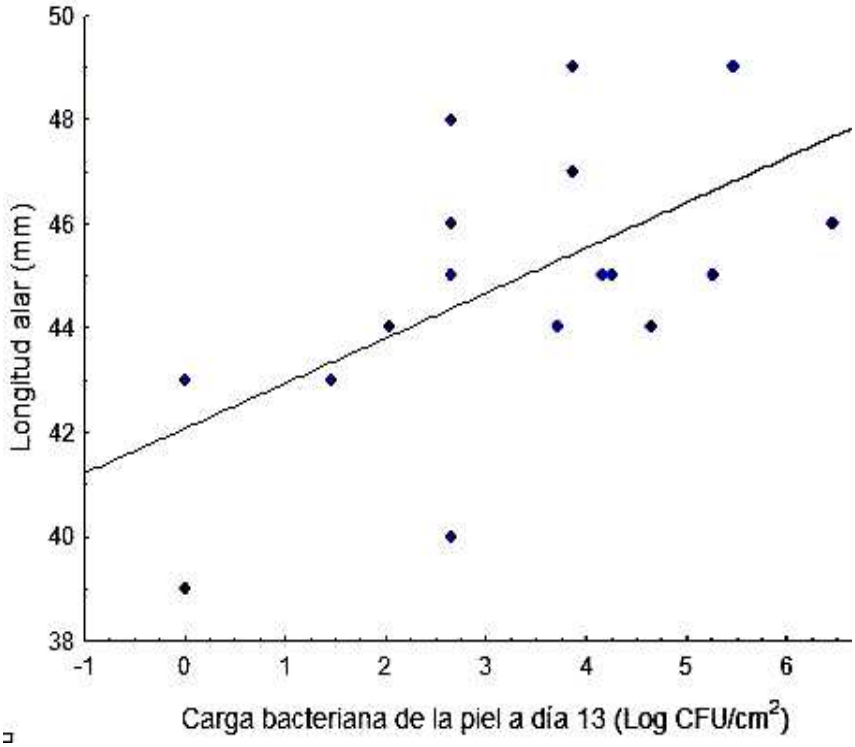

Figura 9. Asociación entre la abundancia bacteriana existente en la piel de los pollos a los 13 días y la longitud del ala medida a esta misma edad.

Bloque III:

Compuesto únicamente por el **capítulo VI**, se centra en diferentes medidas de la respuesta inmunitaria en los pollos, la posibilidad de que estén asociadas entre sí y el compromiso existente entre la inversión en dicha respuesta en fases tempranas del desarrollo y el crecimiento de los pollos nidícolas en el nido.

Capítulo VI:

Existen varios componentes independientes del sistema inmunitario en vertebrados, entre los cuales se han encontrado distintos tipos de relaciones desde positivas a negativas pasando por inexistentes (González *et al.* 1999, Møller *et al.* 2001, Møller & Petrie 2002, Buchanan *et al.* 2003, Morales *et al*. 2004, Matson *et al*. 2006, Mendes *et al*. 2006, Ardia 2007, Roulin *et al*. 2007, Arriero 2009). En este estudio se han medido tres componentes de la respuesta inmunitaria de pollos de papamoscas cerrojillo como son el nivel de inmunoglobulinas en suero (respuesta humoral), la reacción inflamatoria al antígeno fitohemaglutinina (PHA, respuesta mediada por células T; veáse Martín II *et al*. 2006), los niveles de anticuerpos naturales (NAbs) y la hemolisis (respuesta innata). Observamos que las distintas medidas de inmunidad no estaban correlacionadas entre sí, como ya han mostrado otros autores (Tabla 1; Matson *et al*. 2006, Mendes *et al*. 2006, Roulin *et al*. 2007). Por consiguiente, es difícil poder estimar la inmunocompetencia general de un ave en base a una sola medida de algún componente inmunitario (Adamo 2004, Matson *et al.* 2006). Por otro lado, el sistema inmunitario es costoso de desarrollar y de mantener (Klasing & Leshchinsky 1999, Adamo 2004). Esto se pone de relieve con el resultado obtenido en este capítulo, ya que existe una asociación negativa entre los niveles de anticuerpos naturales y la longitud del tarso a edades tempranas, evidenciando un posible compromiso entre crecimiento e

inmunidad innata (Soler *et al*. 2003, Brommer 2004). Es lógico que dicho compromiso se evidencie sólo a edades tempranas ya que el crecimiento esquelético en estos pollos es especialmente rápido durante la primera semana de vida (Lundberg & Alatalo 1992).

Tabla 1. Modelo lineal mixto con las diferentes medidas inmunológicas (nivel de IgY, respuesta a PHA y hemaglutinación) como variable dependiente y un modelo generalizado mixto para la hemolisis. En ambos modelos, incluímos el nido como factor aleatorio y el día de eclosión, tamaño de la nidada, longitud del tarso y ala y el peso medidos a día 7 como covariables usando la corrección de Satterthwaite para la estimación de los grados de libertad. Los modelos minímos fueron obtenidos a partir de los modelos completos mediante la eliminación de las variables no significativas ($\alpha = 0.05$).

	Coeficiente	*df*	*F*	*p*
Nível IgY				
Modelo completo				
Día de eclosión	-0.000	1,75.4	0.23	0.621
Tamaño nidada	-0.009	1,80.6	5.97	0.052
Longitud alar a día 7	0.002	1,124	1.93	0.313
Peso a día 7	-0.007	1,119	1.40	0.157
Longitud tarsal a día 7	0.006	1,121	0.02	0.364
Respuesta a PHA				
Modelo completo				
Día de eclosión	-0.001	1,66.8	1.00	0.320
Tamaño nidada	-0.000	1,68.1	0.01	0.939
Longitud alar a día 7	-0.006	1,119	1.50	0.222
Peso a día 7	0.015	1,112	2.13	0.146
Longitud tarsal a día 7	0.013	1,120	0.84	0.362
Hemaglutinación				
Modelo completo				
Día de eclosión	0.007	1,94	0.07	0.790
Tamaño nidada	0.153	1,94	1.40	0.238
Longitud alar a día 7	0.046	1,94	0.34	0.563
Peso a día 7	-0.100	1,94	0.43	0.512
Longitud tarsal a día 7	-0.408	1,94	3.14	0.079
Modelo mínimo				
Longitud tarsal a día 7	-0.367	1,98	5.40	**0.022**
Hemolisis				
Modelo completo				
Día de eclosión	-0.045	1,66	0.47	0.497
Tamaño nidada	-0.282	1,58.9	0.90	0.348
Longitud alar a día 7	0.143	1,95.2	0.69	0.409
Peso a día 7	-0.316	1,93.1	0.87	0.353
Longitud tarsal a día 7	0.068	1,97.9	0.02	0.884

DISCUSIÓN INTEGRADORA

La mayoría de los estudios que tratan las interacciones entre bacterias y aves se basan en la avicultura pues en esta industria se intenta minimizar las pérdidas económicas ocasionadas por bacterias patógenas como pueden ser *Campylobacter* spp. o *Salmonella* spp. (revisado en Benskin *et al*. 2009). En vivo contraste con la vasta bibliografía sobre el crecimiento de pollos nidícolas (O'Connor 1984, Starck & Ricklefs 1998) está el escaso interés mostrado hasta la fecha entre los ornitólogos por las asociaciones mutualistas o parasitarias entre bacterias y pollos nidícolas con posibles efectos sobre el crecimiento de éstos en el nido y por ende sobre su posibilidad de supervivencia a corto o largo plazo, una vez fuera del nido (ver Lombardo *et al.* 1996, Mills *et al.* 1999, Potti *et al.* 2002, Moreno *et al.* 2003). Por consiguiente la interacción entre aves y bacterias constituye un nuevo campo de estudio para los investigadores que trabajan con poblaciones silvestres.

El tracto gastrointestinal de los pollos de aves silvestres, al igual que en los pollos de corral, es colonizado por bacterias al poco tiempo de eclosionar (Malyszko *et al.* 1991, Mills *et al.* 1999, van der Wielen *et al.* 2002). A medida que el pollo crece, aumentan generalmente las abundancias de las bacterias que conforman esta microbiota (ver **capítulo I**, Mills *et al.* 1999). Si bien no debemos olvidarnos de las interacciones competitivas que pueden existir entre las bacterias que componen dichas comunidades, y que por lo tanto podrían variar las abundancias de algunos tipos bacterianos. Aparte de los cambios cuantitativos también se dan cambios cualitativos o de composición en la comunidad microbiana intestinal (van Dongen *et al.* 2013). Dichos cambios en general pueden estar influenciados por diferentes factores como pueden ser los cambios en la alimentación (Maul & Farris 2005, Waldenström *et al.* 2002).

Otro factor es la variación ambiental que también influirá en la composición de esta comunidad, siendo un ejemplo de los efectos ambientales directos, los derivados de dilatados períodos de lluvia durante los cuales los adultos reproductores no son tan eficientes en la búsqueda de alimento (Radford *et al*. 2001, Geiser *et al*. 2008, Arlettaz *et al*. 2010), lo que repercutiría negativamente sobre las poblaciones bacterianas debido a la falta de recursos nutritivos o a posibles cambios en la dieta. Por otro lado, las inclemencias climáticas tendrían un efecto sobre la temperatura corporal en los pollos a tempranas edades debido a su escasa capacidad de termorregulación (Starck & Ricklefs 1998). Así las fluctuaciones en la temperatura ambiental terminarían afectando a las poblaciones bacterianas del tracto intestinal que crecen mejor a temperaturas superiores ya que su temperatura óptima de crecimiento es alta (entorno a los 37ºC) frente a las que alcanzarían los pollos en estos períodos de bajas temperaturas producidos por la lluvia (Breed *et al*. 1957, Holt 1994, Madigan *et al*. 2006). En esta tesis hemos encontrado algunas de las primeras evidencias en aves silvestres de que las poblaciones bacterianas intestinales responden a factores ambientales posiblemente mediados por las condiciones en que se desarrollan los pollos (ver **capítulo II**). Ciertos tipos de bacterias, como son las enterobacterias en nuestro estudio, parecen mucho más sensibles a efectos ambientales directos que otras como los enterococos.

Además de los efectos debidos a posibles cambios en la composición del alimento suministrado por los padres y el efecto ambiental sobre la comunidad, también se dan cambios fisiológicos o morfológicos en el intestino asociados a la edad (Lumpkins *et al.* 2010, Uni *et al.* 1998). Existe también la posibilidad de que las propias bacterias establecidas puedan modificar dicho entorno favoreciendo o perjudicando el establecimiento de otras especies bacterianas (Barnes *et al.* 1972, Fuller 1977, Mead 2000). En un estudio en el cual se obtuvieron muestras cloacales de pollos de gaviota

tridáctila *Rissa tridactyla* durante su estancia en el nido, comprendiendo un período total de 25 días, se observó que el número de especies que constituía la comunidad microbiana intestinal aumentaba a medida que el pollo se hacía mayor (van Dongen *et al.* 2013). Sin embargo parece ser que la comunidad microbiana intestinal en los pollos de papamoscas cerrojillo se establece en la primera semana de vida a tenor de los resultados obtenidos en el **capítulo III** ya que observamos que no existen diferencias en el número de especies entre las dos edades muestreadas. Este resultado y que difiere de los obtenidos por otros autores (van Dongen *et al.* 2013), probablemente se deba, como ya hemos mencionado anteriormente, a que la comunidad microbiana esté establecida a edades más tempranas a las de nuestro muestreo como consecuencia de que los pollos se encuentran confinados en el nido durante su período de desarrollo. Por lo tanto debido a este confinamiento, los pollos se enfrentan a las mismas bacterias desde edades tempranas a diferencia de los pollos de gaviota tridáctila que se crían en colonias con nidos abiertos y por consiguiente con mayor probabilidad de adquirir especies bacterianas nuevas ya sean ambientales o por el íntimo contacto entre componentes de la colonia. Además a esto se sumarían las diferencias en cuanto a pautas comportamentales, ya que los adultos de papamoscas cerrojillo eliminan del nido en la medida de lo posible los excrementos de los pollos (Cantarero *et al.* 2013), evitando de esta manera una fuente de inoculación bacteriana.

En relación al crecimiento en aves silvestres, se ha encontrado que ciertas bacterias intestinales como es el caso de *E. faecium* o del género *Lactobacillus* están asociadas positivamente con el peso y tamaño de pollos antes de abandonar el nido (Lombardo *et al*. 1996, Moreno *et al*. 2003), aunque este último género también tiene efectos negativos sobre el crecimiento del tarso (Lombardo *et al.* 1996). De hecho, en avicultura se utilizan las bacterias mencionadas anteriormente como promotores del

crecimiento ya que pueden impedir la adherencia de bacterias patógenas además de ser beneficiosas para el desarrollo del pollo en términos de ganancia de peso (Fuller 2001, Mountzouris *et al*. 2007). Por otro lado, no todas las bacterias tienen efectos beneficiosos, sino que ciertas bacterias intestinales pueden tener efectos patogénicos sobre el hospedador, derivados de la utilización directa de los recursos nutritivos del ave, mientras que ciertas características bacterianas como su adhesión y colonización de la mucosa pueden causar enfermedades inflamatorias (Batt *et al.* 1996). Efectivamente se han observado efectos negativos de ciertos tipos bacterianos o géneros, ya que no se ha llegado a la especie bacteriana, con respecto a la calidad fenotípica de los pollos de especies silvestres antes de abandonar el nido (Lombardo *et al*. 1996, Mills *et al*. 1999). Además experimentalmente se ha demostrado que dichas bacterias conllevan costes para el hospedador puesto que al eliminarlas mediante la administración de un antibiótico, se observó un crecimiento más rápido en sujetos sometidos al tratamiento (Potti *et al.* 2002). Por consiguiente las bacterias intestinales no son sólo unos organismos simbiontes que no conllevan ningún coste para el hospedador sino que repercuten en el uso de los nutrientes que el hospedador puede utilizar para el desarrollo de estructuras esqueléticas o el aumento de masa corporal. Es por ello de vital importancia estudiar los efectos de las bacterias intestinales en el hospedador ya que el peso antes de volar así como las medidas de inmunocompetencia son buenos predictores en cuanto a la supervivencia futura de los individuos una vez han abandonado el nido (Cichón & Dubiec 2005, Moreno *et al*. 2005).

Los resultados obtenidos en el **capítulo I** muestran que ciertos tipos bacterianos tienen efectos negativos sobre el crecimiento esquelético (enterococos) mientras que otros parece ser que no tendrían ningún efecto (enterobacterias). Al haber muestreado la abundancia de estas bacterias a dos edades diferentes durante el desarrollo esquelético

de los pollos, en contraste con otros estudios con un solo muestreo, se han podido esclarecer los posibles efectos globalmente deletéreos de algunos tipos bacterianos sobre el crecimiento. Estos resultados requieren obviamente confirmación experimental pero sugieren cuales son las bacterias intestinales cuya abundancia puede retrasar y coartar el crecimiento de pollos nidícolas en aves insectívoras.

Los nidos de las aves proporcionan un ambiente idóneo para la proliferación bacteriana principalmente porque ofrecen unas condiciones térmicas relativamente constantes (Mehmke *et al*. 1992, Singleton & Harper 1998, Goodenough & Stallwood 2010). Asimismo muchas aves construyen dichos nidos en cavidades, reforzando aún más el carácter benigno de las condiciones microclimáticas para el crecimiento bacteriano (Goodenough & Stallwood 2011). Es por ello que el material del nido constituye una de las vías por las que el pollo puede adquirir la microflora bacteriana residente sobre la piel, puesto que los pollos altriciales se ven forzados a pasar la primera etapa de su vida confinados en éste hasta que su desarrollo se ha completado lo suficiente como para poder abandonarlo. La exposición a estas bacterias debe ser importante pues se ha observado que determinadas especies de aves introducen en el nido fragmentos de hierbas aromáticas, las cuales contienen compuestos volátiles capaces de reducir la riqueza y abundancia de la comunidad bacteriana de la piel de los pollos (Gwinner & Berger 2006, Mennerat *et al.* 2009) o del material del nido (Clark & Mason 1985).

En las aves trogloditas es frecuente la reutilización de los nidos viejos por ejemplo por motivos de baja disponibilidad de cavidades nuevas. Nuestra especie de estudio, el papamoscas cerrojillo, reutiliza nidos viejos pero construyendo un nuevo cuenco con material fresco encima del antiguo, aislando de esta forma a los huevos y pollos de contactar directamente con el material viejo. La presencia de nidos

previamente utilizados en las cavidades de nidificación facilita la permanencia de poblaciones bacterianas capaces de colonizar a los ocupantes de nuevos nidos construidos en la misma cavidad. Puesto que existen bacterias que son capaces de esporular y así permanecer en el material del nido durante el invierno cuando las condiciones no son las más óptimas para el crecimiento de sus poblaciones, los nidos construidos sobre nidos viejos podrían contener una mayor carga bacteriana que aquellos construidos en una cavidad vacía. Un caso de bacterias capaces de formar endosporas es *B. licheniformis* (Breed *et al*. 1957), una bacteria degradadora del plumaje de las aves (Burtt & Ichida 1999, Shawkey *et al*. 2003, Ruiz-de-Castañeda *et al*. 2012) cuya mayor abundancia podría conllevar costes para el desarrollo de las plumas de los pollos.

En el **capítulo IV**, mediante un estudio experimental, demostramos que los pollos que crecen en nidos construidos sobre nidos viejos presentan ciertamente una mayor carga bacteriana sobre la piel respecto a aquellos crecidos en nidos exclusivamente con material nuevo. Además una mayor abundancia de bacterias en el material del nido estuvo asociada a una menor longitud alar de los pollos. Ello podría deberse precisamente a la permanencia en el material viejo de bacterias ya sean degradadoras del plumaje u otros tipos bacterianos durante la temporada no reproductora, algo que merece un estudio más detallado en el futuro. Una menor longitud alar al abandonar el nido podría tener implicaciones sobre la capacidad de vuelo de los pollos, y por tanto sobre su supervivencia durante los primeros días como volantones (Nilsson & Gardmark 2001, Anim Behav 2001).

Al igual que ocurre con las bacterias del nido y su efecto sobre el crecimiento de los pollos, poco se sabe sobre las bacterias que colonizan la piel de éstos una vez que eclosionan. Las bacterias pueden desarrollar interacciones tanto mutualistas como

patógenas con sus hospedadores (Coles 1997, Messier *et al.* 1993, Clark *et al.* 2010). Si bien en uno de los pocos estudios que existen, no se encontró ningún efecto de dichas bacterias sobre el peso de los pollos antes de abandonar el nido (Berger *et al.* 2003), sin embargo no se puede descartar una posible asociación de la abundancia de bacterias sobre la piel con el crecimiento de estructuras dérmicas de los pollos como las plumas. En el **capítulo V** profundizamos más en este tema y esclarecemos la posible asociación que pueden tener estas bacterias con el crecimiento del ala. Curiosamente las bacterias cuya abundancia estimamos sobre la piel son aparentemente beneficiosas para el correcto desarrollo del ala. Ello podría deberse a la competencia de éstas con las bacterias degradadoras del plumaje. Un ejemplo de este tipo de relaciones competitivas entre bacterias se ha encontrado en la abubilla *Upupa epops*, existiendo una relación simbiótica del ave con poblaciones de *E. faecalis* residentes en la glándula uropigial durante la época de reproducción. Estas bacterias son capaces de producir bacteriocinas, las cuales son efectivas contra bacterias Gram-positivas, entre las cuáles se encuentra *B. licheniformis* (Martín-Platero *et al.* 2006, Ruiz-Rodríguez 2009, Soler *et al.* 2008). Queda por esclarecer experimentalmente si ciertas bacterias de la piel poseen asimismo la capacidad de contrarrestar los efectos negativos de las bacterias degradadoras del plumaje durante la permanencia de los pollos en el nido. Sin embargo a falta de demostración de dicha competencia entre bacterias en pollos nidícolas durante la fase de crecimiento, existe otra explicación plausible para dicho resultado consistente en que los pollos con una mayor longitud alar probablemente tengan una mayor cantidad de bacterias entre las que se pueden encontrar las bacterias degradadoras. Dichas bacterias por cuestiones competitivas podrán colonizar nuevas superficies como puede ser la piel ventral de los pollos. De esta manera existirá una asociación positiva entre las bacterias existentes en la piel y la longitud alar.

Por otro lado, existen evidencias de que el crecimiento de los pollos de las aves puede verse afectado por el desarrollo del sistema inmune, ya que éste puede requerir recursos nutritivos que son también necesarios para el crecimiento de otros tejidos y órganos (Klasing & Leshchinsky 1999, Mauck *et al*. 2005, Pihlaja *et al*. 2006, Szép & Møller, 1999). Este compromiso entre crecer y ser capaces de responder a los ataques de agentes patógenos ha sido demostrado experimentalmente a través de estudios realizados con pollos nidícolas a los que se les suministraba metionina, que actúa aumentando la respuesta inmunitaria mediada por linfocitos T, en comparación con otros pollos del mismo nido que no recibían este estimulante de la respuesta inmunitaria. Efectivamente se observó que dichos pollos mostraban una menor tasa de crecimiento durante el tiempo que duró el tratamiento pero una alta respuesta al antígeno PHA, el cuál se utiliza como medida indicadora de la respuesta inmune mediada por los linfocitos T (Brommer 2004, Soler *et al*. 2003). Este compromiso podría explicar nuestro resultado del **capítulo VI**, en el cuál se observó que la longitud del tarso en pollos de 7 días de edad estaba asociada negativamente con la medida de anticuerpos naturales, una medida de respuesta inmunitaria innata. Cabe destacar que en la especie estudiada, el esqueleto tiene un crecimiento rápido durante la primera semana de vida (Lundberg & Alatalo 1992), lo que puede explicar por qué las medidas posteriores no muestran ninguna asociación con la respuesta inmunitaria. Indudablemente, este resultado descriptivo debe ser confirmado mediante un experimento.

En dicho capítulo también abordamos las posibles asociaciones entre diferentes variables de respuesta, cuya existencia permitiría valorar cualquiera de ellas como indicador de la inmunocompetencia general del individuo (Adamo 2004). Posiblemente debido a las diferentes tasas de desarrollo de los distintos componentes del sistema

inmunitario en pollos nidícolas, no encontramos ninguna asociación entre los diferentes componentes del sistema inmune que hemos medido al igual que se ha encontrado en estudios de otros autores (Matson *et al*. 2006, Mendes *et al*. 2006). Existe sin embargo evidencia en la especie de estudio de que en pollos, tanto la respuesta mediada por células a la PHA (Moreno *et al.* 2005) como los niveles de inmunoglobulinas (Lobato *et al.* 2008) permiten evaluar las probabilidades de reclutamiento a la población de los individuos muestreados. Ello sugiere que aunque no exista ninguna asociación significativa entre distintas respuestas inmunitarias, varias de ellas están relacionadas con la capacidad de sobrevivir en un medio cargado de agentes patógenos. Es posible que la culminación del desarrollo del sistema inmunitario al completo permitiera visualizar mejor las asociaciones entre componentes en el individuo adulto.

CONCLUSIONES

- La abundancia de ciertas bacterias intestinales varía con la edad del pollo, los enterococos aumentan mientras que las enterobacterias disminuyen.

- La abundancia de enterococos a la edad de 7 días del pollo presenta una asociación negativa con el crecimiento de una estructura esquelética como es el tarso, mientras que la abundancia de enterobacterias no presenta ninguna asociación con el crecimiento en general de los pollos.

- Las variables climatológicas ambientales como la temperatura media y la precipitación conjuntamente con la fecha de eclosión, afectan a la abundancia de enterobacterias mientras que enterococos sólo se ve afectada por la temperatura.

- El número de especies bacterianas residentes en el intestino de los pollos no varía con la edad. Sin embargo esta comunidad bacteriana es más diversa a mayor edad del pollo.

- Los pollos que crecen en nidos construidos sobre material viejo presentan una mayor carga de bacterias heterótrofas sobre la piel comparado con los pollos residentes en nidos compuestos por material exclusivamente nuevo.

- El crecimiento de la longitud del ala es mayor en los pollos que crecen en nidos nuevos, y esta longitud es menor cuantas más bacterias están presentes en el material del nido.

- La abundancia de bacterias sobre la piel de los pollos no varía con la edad de los mismos. No obstante, esta carga bacteriana está asociada positivamente con el número de pollos de la nidada, probablemente debido a las condiciones higiénicas del nido.

- La carga bacteriana existente en la piel ventral de los pollos está positivamente asociada con la longitud alar que a su vez se debe al crecimiento de las plumas.

- Existe un compromiso entre la producción de anticuerpos naturales de la respuesta inmunitaria innata y el crecimiento del tarso a edades tempranas, en las cuales dicha estructura tiene una mayor tasa de crecimiento.

- No existe ninguna asociación estadística entre medidas de la respuesta inmunitaria innata, humoral y mediada por células en los pollos.

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I

Cambios relacionados con la edad en la abundancia de enterococos y enterobacterias en pollos de papamoscas cerrojillo (*Ficedula hypoleuca*) y su asociación con el crecimiento

Age-related changes in abundance of enterococci and enterobacteria in pied flycatcher (*Ficedula hypoleuca*) nestlings and their association with growth

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Resumen - Las bacterias han sido una importante fuerza de selección en la evolución de muchos aspectos de la biología aviar, incluyendo el crecimiento de los pollos. Estimamos la abundancia de dos tipos comunes de bacterias intestinales en aves (enterococci y *Enterobacteriaceae*) y su correlación con el crecimiento del tarso, peso y ala de 102 pollos (54 nidadas) de papamoscas cerrojillo (*Ficedula hypoleuca*), en una población que cría en España. Los pollos fueron pesados y medidos a los 7 y 13 días después de eclosionar, además a estas edades se obtuvieron muestras fecales con el fin de poder estimar la abundancia de las citadas bacterias. Las abundancias de ambos tipos bacterianos no estuvieron correlacionadas entre sí. Las cargas de enterobacterias disminuían desde el día 7 al 13, mientras que las de enterococos aumentaban durante el mismo periodo. En el día 7, las cargas de *Enterobacteriaceae* entre hermanos fueron similares pero no para enterococci. En el día 13, ninguna de las cargas de ambos tipos bacterianos fueron similares para hermanos del mismo nido. Las abundancias de enterococci estuvieron positivamente correlacionadas con el peso y la longitud alar del día 7, pero no a día 13. El crecimiento del tarso entre los días 7 y 13 estuvó negativamente correlacionado con la abundancia de enterococci de día 7.

Abstract - Bacteria have the potential to be important selective forces in the evolution of many aspects of avian biology, including nestling growth. We estimated abundances of two common gut bacterial types in birds (enterococci and enterobacteria) and their correlation with growth in tarsus length, mass and wing length of 102 nestlings (54 broods) of the pied flycatcher (*Ficedula hypoleuca*) in a population of central Spain. Chicks were weighed and measured on days 7 and 13 after hatching, at which ages faecal samples were obtained for detection and estimation of abundance of enterococci and enterobacteria. The loads of the two bacterial types were not correlated. Enterobacterial loads decreased from day 7 to day 13, while loads of enterococci increased during the same period. On day 7 loads of enterobacteria among nest mates were similar whereas loads of enterococci were not similar. On day 13 nest mates did not have similar loads of either bacterial type. Loads of enterococci were positively correlated with body-mass and wing length on day 7, but not on day 13. Tarsus growth between days 7 and 13 was negatively correlated with loads of enterococci on day 7.

Introduction

Bacteria may be important selective forces in avian evolution (Sheldon 1993, Nuttal 1997). Knowledge about the prevalence of bacteria in natural avian populations is limited, and their effects on the general health of wild birds are poorly understood. Recently, bird-bacteria interactions have received much attention, as shown by studies on the effect of bacteria on avian growth (Lombardo *et al.* 1996, Mills *et al.* 1999, Potti *et al.* 2002, Moreno *et al.* 2003, Lucas & Heeb 2005), their association with sexually transmitted diseases (Lombardo *et al.* 1999, Poiani & Wilks 2000, Kulkarni & Heeb 2007), their degradation of feathers (Burtt & Ichida 1999, 2004, Goldstein *et al.* 2004, Gunderson *et al.* 2009, Ruiz-Rodriguez *et al.* 2009c), the effect of nest sanitation on bacteria (Mennerat *et al.* 2009, Peralta-Sánchez *et al.* 2009) and uropygial gland secretions as antimicrobial defences (Shawkey *et al.* 2003, Soler *et al.* 2008, Møller *et al.* 2009, Martín-Vivaldi *et al.* 2010).

Early development is a crucial determinant of fitness in many animals. In birds, in addition to some environmental factors, the body-mass at fledging as well as body condition and measures of immunocompetence, are good predictors of individual post-fledging survival (Cichón & Dubiec 2005, Moreno *et al.* 2005). Nestling growth has traditionally been studied without consideration of microbial effects (Ricklefs 1979, O'Connor 1984, Starck & Ricklefs 1998). Inoculation of altricial nestlings with bacteria is inevitable, either through direct contact with their parents, through the food they receive from their parents (Kyle & Kyle 1993), from their siblings in the nest (Lucas & Heeb 2005), or through direct contact with the nest materials (Mills *et al.* 1999). Bacteria colonizing bird guts have been described mainly for poultry (Soerjadiliem *et al.* 1984), where continuous efforts are made to minimize losses due to diseases (Mead

1997). However, the information concerning gut bacterial communities in natural avian populations is scant and information concerning the development of these communities during the period of nestling growth extremely limited. Most previous studies have sampled bacterial communities in chicks only once, just before fledging (Lombardo *et al.* 1996, Potti *et al.* 2002, Berger *et al.* 2003, Moreno *et al.* 2003, Lucas & Heeb 2005). However, Mills *et al.* (1999) detected cloacal microbes shortly after nestlings hatched and found that the nestlings were colonized by more types of microbes as they got older, indicating that development of gut microbial communities occurs gradually after hatching. This study estimates bacterial loads in nestlings half-way through their development in the nest and to compare these bacterial communities with those present in nestling guts shortly before fledging. Changes in gut bacterial loads during this crucial period may be correlated with nestling growth and possibly with survival in the nest and thereby affect avian life histories. We have here analyzed the most prevalent bacterial types in avian guts, namely Enterococci and Enterobacteria (Barrow 1994, González *et al.* 2000, Janiga *et al.* 2007). The gut of chicks is colonized by bacteria from hatching so it is expected that gut bacterial loads increase with age until the establishment of an intestinal balance (Malyszko *et al.* 1991, Mills *et al.* 1999). However, this balance may be attained at different growth stages for different bacterial groups. Thus, loads of enterococci and enterobacteria loads may change throughout the nestling period in different ways (Mills *et al.* 1999).

A related issue is whether chicks in the same nest develop similar intestinal bacterial communities due to similar inherited genetic propensities or to environmental effects induced by similar parentally-determined diets (Glunder 2002), or alternatively, if nest-mates are different in this respect due to individual differences induced by sex, competitive hierarchy in the nest, provisioning by different parents, or slight genetic

differences (Tannock 1997, Moreno *et al.* 2009, Ruiz-Rodríguez *et al.* 2009a). We would expect high intra-nest similarities in gut microbial loads and community composition in the first case, but not in the second. Moreover, similarities could be high initially due to strong effects of common genes for immune response or to strong common-environment effects (Brinkhof *et al.* 1999), and decrease during development as individual differences in immunity and diet begin to show effects on acquisition and retention of bacterial strains. There is scarcely any information available on these aspects of bird-bacteria symbioses, despite their potential implications for avian fitness.

There is some evidence of negative correlations of intestinal bacteria with nestling growth rate in the wild (Lombardo *et al.* 1996, Mills *et al.* 1999, Potti *et al.* 2002). In the only experimental study the effects of bacteria on growth in wild birds, Potti *et al.* (2002) observed that Magellanic penguin *Spheniscus magellanicus* chicks that were treated with a wide-spectrum antibiotic grew faster than control chicks which were not treated. In another study, Moreno *et al.* (2003) found a significantly positive correlation between nestling mass shortly before fledging and the presence of *Enterococcus faecium*. These enterococci could be acting as growth promoters through their competitive interactions with facultative pathogenic bacteria like *Enterococcus faecalis*. There is thus scope for both positive and negative correlations of nestling growth with bacterial loads and community composition. Our expectation is that some bacterial types may negatively affect nestling growth and condition, while others may have positive effects. Moreover, bacterial loads of different strains could operate at different stages of nestling development. Understanding the timing of effects requires repeated sampling of the same chicks with respect to gut bacterial loads throughout development.

In this study we explore the effects of intestinal bacteria on the growth of altricial nestlings, focussing to that end on a model-species in avian ecology, the pied flycatcher *Ficedula hypoleuca* (Lundberg & Alatalo 1992). We have measured mass, tarsus length and wing length as expressions of muscle and organ growth, skeletal growth and integumentary growth respectively. Given different patterns of flexibility in the growth of these traits (Schew & Ricklefs 1998), we have analyzed them separately. We have hypothesized that: (1) enterococci and Enterobacteria may show significant intra-nest similarities with respect to nestling age due to common genetic, maternal or environmental effects, (2) bacterial loads will increase with age of the chicks, (3) bacterial loads will be related to measures of development, (4) these relationships will depend on nestling age, and (5) bacterial loads will be correlated with growth in mass and linear measurements between sampling periods. To test these hypotheses we have analyzed the most prevalent bacterial types (enterococci and enterobacteria) in avian guts, sampling nestlings at 7 days (during peak growth) and at 13 days of age (shortly before fledging).

Methods

The study was conducted during the 2009 breeding season in a deciduous forest of Pyrennean oak (*Quercus pyrenaica)* at an elevation of 1.200 m a. s. l. in Valsaín, Segovia province (40˚ 54’ N, 4˚ 01’ W). A study of a population of pied flycatchers breeding in nest-boxes in that area has been conducted since 1991 (Sanz *et al.* 2003). Nest-boxes are cleaned every year after the breeding season (for nest-box design and manipulation see Lambrechts *et al.* 2010). Nest-boxes were checked daily for nest-

building activity by pied flycatchers, and the dates of clutch initiation, clutch sizes, and numbers of fledged young were ascertained.

The pied flycatcher is a small (12-13 g) passerine bird, which breeds in many forested areas of the Palaearctic region (Lundberg & Alatalo 1992). It only stays in European woodlands for the spring and summer, spending the rest of the year on migration or in the wintering areas in tropical West Africa. It breeds naturally in tree cavities, but if nest-boxes are provided, they are preferred over natural cavities. Egg laying in the population under study typically begins in late May, and clutch sizes range from 4 to 7 eggs with a mode of 6 eggs (mean 5.5 ± 0.6). The female incubates alone and receives part of her food from her mate (Lundberg & Alatalo 1992). Young are brooded by the female only up to an age of 7 days (Sanz & Moreno 1995). Both sexes feed the young. Young fledge within 14–16 days of hatching. This occurs in the second half of June in our study area (Moreno *et al.* 2001).

A sample of 54 broods of four to six chicks was used for this study. Of these nests, we obtained samples from two randomly selected chicks in 43 nests and one randomly chick in the remaining nests. Nestlings were measured and weighed at the ages of 7 and 13 days (hatching day = day 1). On each nestling the length of the tarsus was measured with a digital calliper to the nearest 0.01 mm, mass was obtained with a Pesola® spring balance (precision of 0.25 g) and wing length was measured with a stopped ruler to the nearest mm. Chicks were banded on day 7 with numbered aluminium bands.

Bacterial sampling

As cloacal samples cannot safely be obtained due to the size of nestling cloacae, we have sampled freshly produced faecal sacs as a substitute. We assume that most bacteria sampled will be derived from gut cloacal communities. Faecal sacs were

collected at 7 and 13 days in sterile eppendorf tubes. These tubes were sterilized prior to use by being autoclavated at 121˚C during 15 min to obtain sterile conditions. Samples were transported in a portable cooler until their processing in the laboratory (3-6 h after collection). Once in the laboratory, we introduced one sterile cotton swab in the sterile eppendorf tube containing the faecal sac during 1-3 seconds trying to uniformly impregnate the cotton with faecal matter. Then, we transferred the swabs to transport media Amies *(Sterile R, Meus s.r.l.,* Piove di Sacco, Italy*)* and chilled the samples (4˚C) until processed. All samples were conserved during 20 days.

Swabs were transferred from Transport Media Amies into 1 ml of phosphate buffered saline (pH = 7.2, Química Clínica Aplicada, Tarragona, Spain). Later, 100 µl of the original sample were sequentially diluted in 900 µl of sterile physiological saline (0.85% NaCl) in order to obtain the optimal bacterial concentration for the quantification (Herbert 1990). For 20 initially collected samples, we cultured 100 µl of all serial dilutions from 10^{-1} to 10^{-5} looking for the optimal range of 30-300 CFU (Herbert 1990). For the following samples, we cultured only in the previously established optimal dilutions, 10^{-2}, 10^{-3} and 10^{-4}. We cultured on the Mac Conkey agar (bioMérieux, Madrid, Spain) and D-Coccosel agar (bioMérieux, Madrid, Spain). Mac Conkey agar is a special bacterial growth medium that is selective for gram-negative bacteria and can differentiate those bacteria that are able to hydrolyze lactose (enterobacteria), while D-Coccosel is used for isolation of enterococci. Owing to problems with D-Coccosel medium delivery, some samples (4 for 7-day samples and 16 for 13-day samples) were cultured on Enterococcosel (Difco, Detroit, Michigan, USA), that is selective for enterococci. There were no significant differences in bacterial counts obtained with these two media (both $p > 0.1$), so data were pooled. We incubated plates for 48 ± 2 h at 37 ± 1˚C. All the samples were analyzed exactly 20 days after

collection. The quantifications were made by counting all the CFU (Colony Forming Units) present in the plate after the incubation with a colony counter "sensor" (Suntex Instruments Co., Ltd., Taipei County, Taiwan) by the same observer (SG-B). CFU counts represent the number of colonies on those plates presenting between 30 and 300 colonies (to assure their legibility) multiplied by the dilution factor used to obtain the viable count of the original sample (Herbert 1990). A total of 102 chicks were sampled at the two different ages.

Statistical analyses

Three zero values for enterococci on day 7, six for Enterobacteria on day 7 and four for enterococci on day 13, were eliminated as they were extreme outlying values (3 corresponded to the same chicks for both bacterial types). All CFU counts were normalized through logarithmic transformation. Analyses were conducted with *Statistica 7.0* (*Statsoft 2001*). We tested for a correlation between individual bacterial loads of the two bacterial types on day 7 and day 13. To test for repeatability among chicks of the same nest and two different ages for enterococci and Enterobacteria, we used repeated-measures ANOVAs with Enterobacteria load or enterococci load as dependent variable and age, nest ID, and the interaction age*nest ID as factors to test whether bacterial loads changed with age and whether bacterial loads of nestling varied more among than within broods. As loads of enterobacteria were similar within nests on day 7 (see below), we selected one chick per nest randomly for analyses of these loads to avoid pseudoreplication.

To test for the correlations between bacterial loads and nestling measurements at two ages, we used the Variance Components module. Non-significant factors were removed from the final models. In the model, we included a variable of growth (tarsus length, wing length or mass) as dependent variable, and brood size at that age (day 7 or

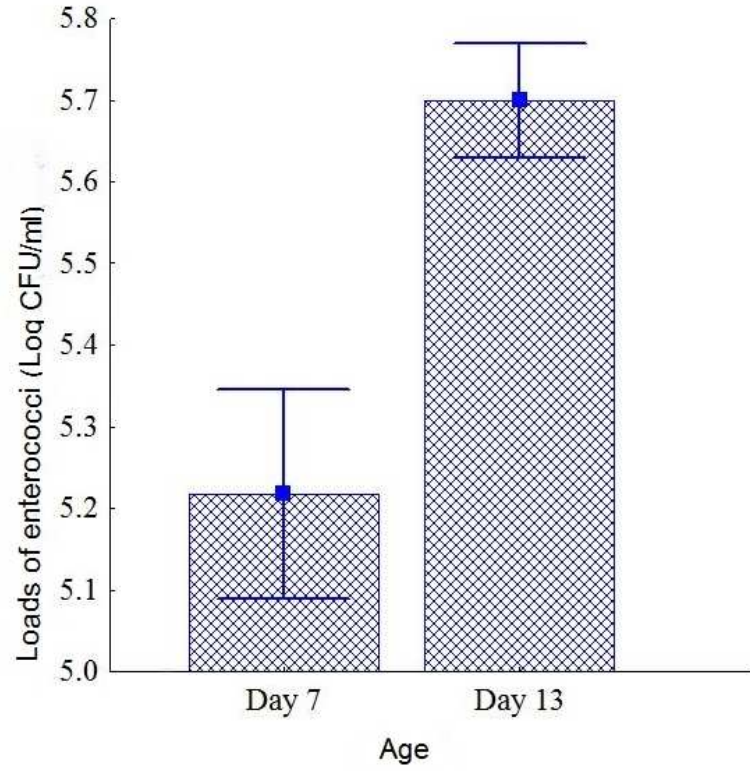
day 13), hatching date and bacterial loads (enterococci and enterobacteria) as covariates, and nest as random factor. The two bacterial loads were included in the same analysis because there is no correlation between them (see below). This module uses the Satterthwaite correction for estimating degrees of freedom. For estimating the correlations of bacterial loads with growth between days 7 and 13 (i.e. the difference between 7 and 13) we also used the Variance components module, introducing bacterial loads on day 7 as an independent term. This model was based on the hypothesis that only bacterial loads at the inception of the observed growth period were explanatory.

Results

The loads of the two bacterial types were not correlated either on day 7 (r = 0.116; p = 0.319; n = 75) or on day 13 (r = 0.082; p = 0.463; n = 83). The loads on day 7 of enterobacteria but not of enterococci were significantly similar within broods (Table 1). No similarity was found for loads on day 13 (Table 1). Enterobacterial loads decreased from day 7 to day 13, while loads of enterococci increased during this period (Table 1; Fig. 1).

Table 1. Results of repeated-measures ANOVAs with enterococci load or enterobacteria load as dependent variable and nest, age and their interaction as factors.

	Enterococci			**Enterobacteria**		
	df	*F*	*p*	*df*	*F*	*p*
Nest	1,26	1,407	0,188	1,23	2,85	**0,007**
Age	1,1	7,374	**0,011**	1,1	11,01	**0,002**
Age*Nest	1,26	1,245	0,284	1,23	2,16	**0,035**



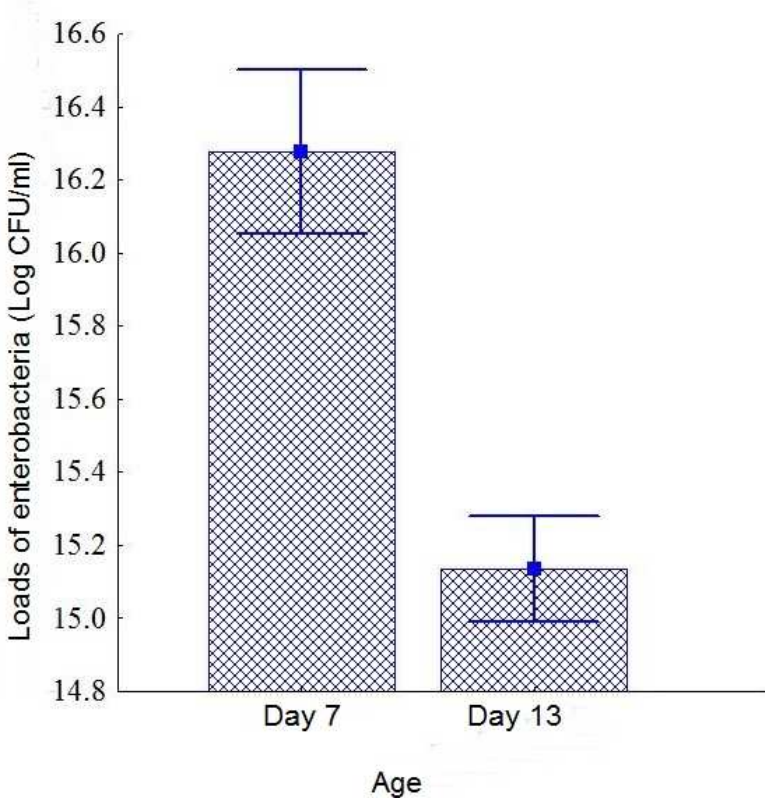


Figure 1. Bacterial loads at 7 and 13 days of (a) enterobacteria (log CFU /ml), and (b) enterococci (log CFU /ml).

Enterococci loads showed positive correlations with body-mass and wing length on day 7 but not on day 13 (Table 2). Hatching date showed negative associations with tarsus length in these analyses, while brood size was not related to any measurement at any age (Table 2). Enterobacterial loads were not related to nestling measurements at any stage (Table 2). Enterococci loads on day 7 showed a negative correlation with tarsus growth (Fig. 2). Hatching date in these analyses was also negatively correlated with tarsus growth (Table 2). Enterobacterial loads were not related to growth in any trait (Table 2).

Table 2. Variance components analyses of effects of loads of Enterococci and Enterobacteria, hatching date and brood size, with nest as random factor, on nestling linear measurements, mass and growth using the Satterthwaite correction for estimating degrees of freedom.

	df	*F*	*p*	*r*
Tarsus length on day 7				
Nest	1,52	2.952	**<0.001**	-0.38
Hatching date	1,52	5.500	**0.022**	0.44
Body-mass on day 7				
Nest	1,50	3.185	**<0.001**	-0.23
Enterococci on day 7	1,50	6.410	**0.013**	0.32
Wing length on day 7				
Enterococci on day 7	1,50	10.825	**0.001**	0.32
Nest	1,50	2.285	**0.004**	-0.29
Growth of tarsus length				
Nest	1,49	3.152	**<0.001**	0.33
Enterococci on day 7	1,49	6.656	**0.012**	-0.38
Hatching date	1,49	5.747	**0.020**	-0.47
Growth of body-mass				
Nest	1,48	5.193	**<0.001**	0.12
Growth of wing length				
Nest	1,52	6.042	**<0.001**	0.15
Tarsus length on day 13				
Nest	1,51	6.285	**<0.001**	-0.02
Body- mass on day 13				
Nest	1,52	5.269	**<0.001**	-0.11
Wing length on day 13				
Nest	1,52	2.882	**<0.001**	-0.19

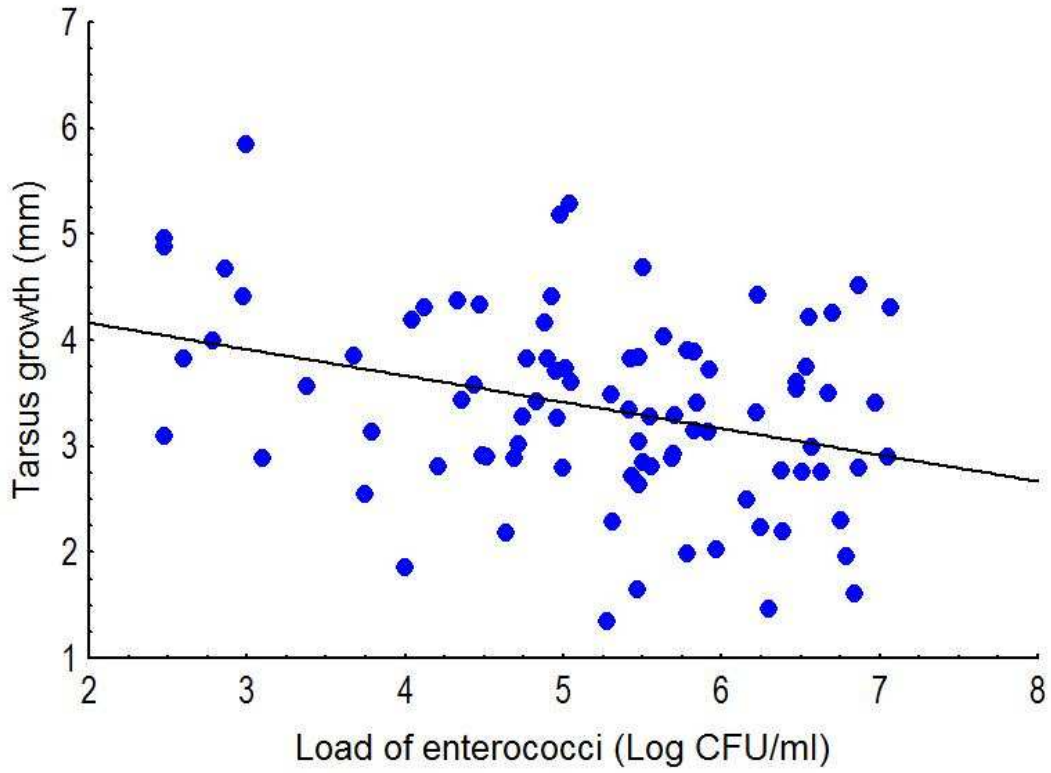


Figure 2 . Correlation between loads of Enterococci and growth in tarsus length.

Discussion

There is no correlation between loads of the two main gut bacteria. The load of enterobacteria is similar among nest mates, but only at young ages. These bacterial loads change throughout the nestling period, enterobacterial loads decrease while enterococci loads increase during the same period. Moreover, enterococci loads are positively correlated with mass and wing length of nestlings on day 7 but not on day 13, while no correlation is found for enterobacterial loads. Finally, skeletal growth between days 7 and 13 is negatively related to prior enterococci loads. We emphasize that the last result would not have been detected if we had only sampled nestlings shortly before fledging as has been done in other studies (Lombardo *et al.* 1996, Potti *et al.* 2002, Moreno *et al.* 2003, Lucas & Heeb 2005).

Within-nest consistency in Enterobacterial loads among brood-mates was found on day 7, but no significant effect detected on day 13. This result indicates that common genetic, environmental and food-mediated effects are important during the first week of nestling life but less so shortly before fledging. This change may be attributed to within-nest individual differences in immunity, sex-related differences or possibly differences in diet correlated with dominance hierarchies among siblings.

Loads of enterobacteria and enterococci change significantly during the nestling period. These different tendencies may reveal competition between them, with enterococci apparently gaining the upper hand. Competition for resources and space among intestinal microbes is a common phenomenon expressed as chemical conflicts through bacteriocin production (Klaenhammer 1988, Riley & Wertz 2002, Soler *et al.* 2009). Thus, competition among bacteria was used to explain the contrary effects on nestling growth of different enterococci in the pied flycatcher (Moreno *et al.* 2003). Another explanation for these changes may be due to variation in the diet of chicks or in parental saliva when feeding them (Kyle & Kyle 1993). Food is an important source of bacterial inoculation to young hosts and that consumption of particular food items may result in the establishment of specific microbial communities (Maul & Farris 2005). Waldeström *et al.* (2002) found that the prevalence of *Campylobacter* spp. was highly influenced by feeding habits in birds. In insectivorous and granivorous species, these bacteria were rarely or never isolated, while prevalence was found to be high in raptors and opportunistic feeders. A study on the raptor *Milvus milvus* found differences in composition, richness and prevalence of faecal microflora associated with diet (Blanco *et al.* 2006).

Studies on the correlations of intestinal bacterial communities with nestling growth are scarce. Most of them have only sampled gut bacteria at the end of the

nestling period (Lombardo *et al.* 1996, Potti *et al.* 2002, Moreno *et al.* 2003, Lucas & Heeb 2005). In the present study, we have sampled gut microbial communities at two stages of the nestling period encompassing the peak growth in passerine nestlings. This has allowed us to relate bacterial loads not only to measurements of chicks at two ages, but to correlate bacterial loads directly with growth. Larger and heavier nestlings at 7 days of age had higher loads of enterococci, and although we did not measure microbial richness but loads of two different groups of bacteria, this result was in agreement with the positive correlation between cloacal microbial richness and body condition found in female spotted towhees, *Pipilo maculatus* (Klomp *et al.* 2008). Another study found significant relationships between microbial diversity and nestling phenotypic traits related to probability of recruitment in magpies and great spotted cuckoo (Ruiz-Rodríguez *et al.* 2009b). Taken together, this may indicate that initially more efficiently provisioned broods were also those with a more intense development of enterococci. However, there was no correlation on day 13, indicating that contrary to previous studies (Lombardo *et al.* 1996, Potti *et al.* 2002, Moreno *et al.* 2003, Lucas & Heeb 2005), nestling size is more strongly correlated with initial than with final bacterial loads.

We have been able to confirm a negative correlation between nestling growth and bacterial load of enterococci but no relationship with enterobacterial loads. Lombardo *et al.* (1996) found that enterobacteria showed a negative correlation with prefledging mass and a positive one with wing length. We may expect that bacterial loads are higher at early ages in heavier chicks, but these higher bacterial loads might do that chicks grow more poorly, so no correlation would be found at age 13. In any case, it was tarsus length that correlated with bacterial loads, a trait which has been shown to affect post-fledging survival in pied flycatchers (Alatalo & Lundberg 1986). Potti *et al.*

(2002) showed experimentally that Magellanic penguin chicks grew better after antibiotic treatment. Moreno *et al.* (2003) found a significant positive effect of *E. faecium* on prefledging (at age 13) tarsus length and mass of chicks if *E. faecalis* was not present in nestling pied flycatchers, whereas our data show a significant positive correlation between body-mass and wing length on day 7 but not on day 13. We cannot unravel in our data the implications of different enterococci, but our results agree with the notion that high gut bacterial loads compete for substance in the digestive track with nestling growth. This result suggests that microbial symbionts are not only beneficial for growing altricial birds, but may also slow down growth, with negative implications for postfledging fitness (Alatalo & Lundberg 1986). These effects may be due to direct food detraction by gut bacteria (Stevens & Hume 1998, Ruiz- Rodríguez *et al.* 2009b) or through the negative implications of high levels of immunity necessary to control intestinal microbes for growth (Ruiz-Rodríguez *et al.* 2009b). The fact that bacterial loads on day 7 and not on day 13 were correlated with growth rate suggests that sampling bacteria only at the end of the growth period as normally done in field studies would not have revealed the link between growth and bacterial loads. We recommend that future studies of bacteria-growth correlations sample bacteria at the beginning of the peak growth period as well as the end of peak growth.

To conclude, growing altricial birds develop bacterial communities in their guts throughout the nestling period which are only intra-nest consistent for some bacteria. Some bacterial types increase while others decrease during this period, possibly as an expression of competitive interactions or due to changes in nestling diet with age. Finally, nestling growth may suffer from high bacterial loads of some bacterial types like enterococci during the period of peak growth. More studies are needed to clarify the patterns revealed in the present study.

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II

Fuentes de variación en las abundancias de enterococos y *Enterobacteriaceae* en pollos de un paseriforme troglodita

Sources of variation in enterococci and *Enterobacteriaceae* loads in nestlings of a hole-nesting passerine

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Ardea 100: 71-77

Resumen.- Las cargas bacterianas intestinales en pollos pueden verse afectadas por factores como el clima, la estacionalidad y el tamaño de la nidada. Sin embargo no existe nada publicado sobre este tema en aves silvestres, a pesar de que es importante para la salud y crecimiento del pollo. Por ello, estudiamos la asociación de dichos factores con las abundancias de dos bacterias intestinales, enterococci y enterobacterias en pollos de papamoscas cerrojillo (*Ficedula hypoleuca*) en una población que cría en España. Para este propósito, obtuvimos muestras fecales de 54 nidadas (102 pollos) a los 7 y 13 días desde su eclosión, estimando las abundancias de dos tipos bacterianos (enterococos y enterobacterias). Obtuvimos que la abundancia de enterobacterias a edades tempranas estuvó positivamente correlacionada con la temperatura media, y negativamente correlacionada con la precipitación y el día en el cuál eclosionaban los pollos. Si bien, esta asocicación negativa de enterobacterias con la precipitación sólo fue encontrada para las nidadas tempranas y a bajas temperaturas. Al igual que el otro tipo bacteriano, la carga de enterococos estuvó positivamente correlacionada con la temperatura media aunque a diferencia de enterobacterias, dicha correlación se obtuvó a ambas edades. Por otro lado, la abundancia de enterobacteria estuvó negativamente correlacionada con el día de eclosión. Todo esto sugiere que enterobacteria podría ser más sensitiva a los cambios estacionales y variaciones en cuanto al clima, posiblemente debido a cambios en la dieta suministrada a los pollos. Por el contrario, enterococos sólo se ve afectada por cambios termales. La capacidad termoreguladora que va adquiriendo el pollo reduce los efectos climatológicos que afectan a la abundancia de enterobacterias.

Abstract.- Gut bacterial loads in avian nestlings may be affected by factors such as climate, seasonality and brood size. There is no published information on this subject for wild birds despite its potential importance for nestling welfare and growth. We studied the associations of these factors with abundances of two common gut bacterial types, enterococci and *Enterobacteriaceae*, in nestling pied flycatchers *Ficedula hypoleuca* in central Spain. To that end, we obtained faecal samples from 54 broods (102 nestlings) on day 7 and 13 after hatching for detection and estimation of bacterial abundance. *Enterobacteriaceae* loads on day 7 were positively correlated with mean temperature during the preceding seven days and negatively with rainfall and hatching date. The negative associations of *Enterobacteriaceae* loads with rainfall were only found for early broods and at low temperatures. Enterococci loads on day 7 were positively associated with mean temperature. On day 13, *Enterobacteriaceae* loads were negatively correlated with hatching date while enterococci loads were positively correlated with mean temperature. *Enterobacteriaceae* are apparently more sensitive to seasonal changes and climatic variation than enterococci, possibly in relation with variation in diet and nutrition. By contrast, enterococci are only sensitive to thermal variation. The attainment of full thermoregulatory capacity by nestlings reduces climatic effects on *Enterobacteriaceae* loads.

Introduction

The study of host-parasite interactions has been of primary interest for avian ecologists. However, only recently have bacteria received attention in this respect (Maul & Farris 2005, Benskin *et al*. 2009). Associations between birds and bacteria may involve pathogenic interactions, but also positive symbiotic interactions (Martín-Platero *et al*. 2006, Ruiz-Rodríguez *et al*. 2009). These interactions begin in the nest and may affect growth and survival of altricial and semialtricial nestlings (Potti *et al*. 2002, Moreno *et al*. 2003, González-Braojos *et al.* 2012). The early stage of the posthatch period is critical for establishment of the gut microbial community. This process starts from a sterile gastrointestinal environment at the moment of hatching and continues toward establishing a relatively stable status as the nestling ages. Thus, Mills *et al.* (1999) reported that microorganisms colonize nestling cloacae shortly after hatching, suggesting the source of microbes to be adults, local food items, or their local environment. Understanding the factors modulating bacterial abundances in nestling digestive tracts could improve our understanding of bird-bacteria interactions in the wild.

Several factors including climate, food, age and health state affect the composition of the gut microbiota of individual birds (Brittingham *et al*. 1988, Lombardo *et al*. 1996). Nutrient richness in the environment, humidity and temperature have been identified as important factors affecting growth in bacterial cultures (Ratkowsky *et al*. 1982, Madigan *et al.* 2006). Nutrient availability for digestive bacteria may change with seasonal variation in diet of both adults and nestling birds (Blanco *et al*. 2006, Novotny *et al*. 2007). Nutritional quality may also affect bacterial growth, especially for nestlings competing for parental food deliveries. Late-breeding

pairs tend to offer less or poorer quality food to their nestlings (Naef-Daenzer *et al*. 2000, Rossmanith *et al*. 2007, Wilkin *et al*. 2009). Nestlings in larger broods may also suffer nutritionally from stronger competition with brood mates (Naguib *et al*. 2004, Pichorim & Monteiro 2008). Thus, breeding phenology and brood size could affect the growth of bacteria in digestive tracts through nestling nutrition in terms of quantity and quality. It has been shown that gut mass declines in conditions of poor nutrition (Brzek & Konarzewski 2001), possibly driving higher competition among bacteria for space. To our knowledge, there is no published information about associations of environmental factors with growth of bacteria in nestling digestive tracts. The only study, to our knowledge, relating gut bacterial growth to seasonal climate changes did not include nestlings (Janiga *et al*. 2007). Ambient temperature may affect bacterial growth through the thermoregulatory capacity of altricial nestlings which improves with age until thermal independence from adult brooding behaviour (O'Connor 1978, Starck & Ricklefs 1998). If body temperature of non-thermally independent offspring fluctuate more when ambient temperature is low (Starck & Ricklefs 1998, Bize *et al*. 2007), bacterial growth might suffer accordingly. Thermally independent nestlings may offer more stable thermal regimes for gut bacteria. Rainfall may affect the foraging capacity of adults and thereby nestling nutritional condition (Rosa & Murphy 1994, Elliott *et al*. 2005, Spencer 2005, Geiser *et al*. 2008, Morrison *et al*. 2009, Arlettaz *et al*. 2010).

In this study we assessed whether environmental factors modulate the abundance of two types of gut bacteria (enterococci and *Enterobacteriaceae*) at two nestling ages in the pied flycatcher *Ficedula hypoleuca*. Enterococci are widely distributed in animal gastrointestinal tracts (Foulquié-Moreno *et al*. 2005) and may exist as commensal organisms of the alimentary tract of chickens (Klein 2003) and wild birds (e.g. Moreno *et al*. 2003). They have probiotic properties and are able to limit the colonization of the

digestive tract by pathogenic bacteria (Mazur-Gonkowska *et al*. 2006). Moreno *et al*. (2003) found a significantly positive association between nestling mass shortly before fledging and the presence of *Enterococcus faecium*. *Enterobacteriaceae* are also common in the intestinal microflora of wild birds. Thus, Winsor *et al*. (1981) showed that the most prevalent intestinal bacteria of this group in Turkey vultures *Cathartes aura* were *Escherichia coli* and *Proteus mirabilis*. Moreover, *Enterobacteriaceae* contribute to the digestion of food and play an important role in controlling other gut bacteria (Hudault *et al*. 2001, Reid *et al*. 2001). It has been shown that both enterococci and *Enterobacteriaceae* grow best at temperatures between 22 and 45 ºC (Ron 1975, Martínez *et al*. 2003, Foulquié-Moreno *et al*. 2005), so thermal fluctuations in thermally dependent nestlings during parental absences may affect bacterial growth conditions.

Accordingly, we hypothesized that: (1) later broods will have lower bacterial counts due to poor nestling nutrition, (2) larger broods will have lower bacterial counts due to poor nestling nutrition, (3) lower ambient temperatures will result in lower bacterial growth in non-thermally independent offspring (7 days) while having smaller effects in nestlings about to fledge (13 days), (4) higher rainfall will induce poorer bacterial growth due to restricted parental food deliveries to nestlings. Finally, (5) we looked for synergistic effects of rainfall and temperature in driving bacterial growth in guts of nestlings, as low nutrition may have especially strong effects when the costs of thermoregulation are high.

Methods

The study was conducted during the 2009 breeding season in a deciduous forest of Pyrennean Oak (*Quercus pyrenaica)* at an elevation of 1.200 m a.s.l. in Valsaín, Segovia province (40˚ 54' N, 4˚ 01' W), Spain. The local population of pied flycatchers breeds in nest-boxes and has been under study since 1991 (Sanz *et al*. 2003). Nest-boxes are cleaned every year after the breeding season. For the current study, nest-boxes were checked daily for nest-building activity, and the hatching dates and brood sizes were recorded.

The pied flycatcher is a small (12-13 g) passerine bird, which breeds in many forested areas of the Palaearctic region (Lundberg & Alatalo 1992). It only stays in European woodlands for the spring and summer, spending the rest of the year on migration or in the wintering areas in tropical West Africa. It breeds naturally in tree cavities, but if nest-boxes are provided, these are preferred over natural cavities. Egg laying in the population under study typically begins in late May, and clutch sizes range from 4 to 7 eggs with a mode of 6 eggs (mean 5.5 ± 0.6). The female incubates alone and receives part of her food from her mate (Lundberg & Alatalo 1992). Young are brooded by the female only up to day 8 (hatching day = day 1) (Sanz & Moreno 1995). Both sexes feed the young. Young fledge within 14–16 days of hatching. This occurs in the second half of June in our study area (Moreno *et al*. 2001).

A sample of 54 broods of four to six chicks was used for this study. Of these nests, we obtained samples from two randomly selected chicks in 43 nests and one randomly selected chick in the remaining nests. Nestlings were measured and weighed at two ages (7 and 13 days).

Bacterial sampling

Bacterial samples were obtained as described in González-Braojos *et al.* (2012). Briefly, we sampled freshly produced faecal sacs and assumed that most bacteria contained in those were derived from gut cloacal communities. Faecal sacs were collected at 7 and 13 days in sterile eppendorf tubes and were processed in the laboratory 3-6 h after collection. Here, we impregnated one sterile cotton swab per faecal sac with faecal matter, transferred this to transport media Amies *(Sterile R, Meus s.r.l.*, Piove di Sacco, Italy*)* and conserved the samples at 4˚C until processed. All samples were analysed after exactly 20 days to avoid effects of differences in time elapsed between sampling and laboratory processing.

Swabs were transferred into 1 ml of phosphate buffered saline (pH = 7.2, Química Clínica Aplicada, Tarragona, Spain). Optimal bacterial concentration for the quantification (Herbert 1990, Maier *et al*. 2000) was determined by serial dilution in sterile physiological saline (0.85% NaCl). The samples were cultured by plating out 100 µl of the following dilutions: 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. Samples were cultured on the following solid selective and differential bacterial media: Mac Conkey agar (bioMérieux, Madrid, Spain; *Enterobacteriaceae*) and D-Coccosel agar (bioMérieux, Madrid, Spain; enterococci) or Enterococcosel for 20 samples (Difco, Detroit, Michigan, USA; enterococci). There were no significant differences in bacterial counts obtained with the latter two media (both $p > 0.1$), so data were pooled. Plates were incubated for 48 ± 2 h at 37 ± 1˚C, after which colonies were counted using a colony counter "sensor" (Suntex Instruments Co., Ltd.,Taipei County, Taiwan) by the same observer (SG-B).

Environmental data

The data of daily environmental mean temperature and rainfall were obtained from the meteorological station "Casa de la Mata", located 2 km from the study area (40˚ 54' N, 4˚ 00' W, 1.150 m a.s.l.). Two temperature averages were obtained for each brood: (1) the average of mean temperatures between hatching date and day 7, and (2) the average of mean temperatures between days 7 and 13. For rainfall, we used the rainfall accumulated (1) between hatching date and day 7, and (2) between days 7 and 13.

Statistical analyses

Bacterial loads were successfully normalized through logarithmic transformation prior to analyses. We first tested for intra-brood repeatability of bacterial loads for the two bacterial types and two nestling ages separately (Statistica 6.0).

Loads of the two types of bacteria and for the two nestling ages were included separately in four different linear mixed models using Satterthwaite's correction for estimating degrees of freedom. Each model included hatching date, mean temperature, rainfall, brood size and their interactions as fixed effects and nest as random effect. Model selection was based on the Corrected Akaike Information Criterion (AICc), which is more suitable than AIC at moderate sample sizes. We present only models with a ΔAIC_c smaller than 2 with respect to the best model for each bacterial type and nestling age. We also present the sign and strength of the significant effects included in the selected models. Linear mixed models were performed in SAS 9.2. For graphical representation of significant interactions between variables, we split one of the variables according to the median and plotted these separately.

Results

The loads on day 7 of *Enterobacteriaceae* (r = 0.433; p = 0.011), but not of enterococci (r = 0.229; p = 0.126) were significantly correlated within broods. No significant within-brood similarity was found for loads on day 13 either for *Enterobacteriaceae* (r = 0.159; p = 0.192) or enterococci (r = 0.014; p = 0.468). Thus, we did not calculate intra-brood average bacterial counts.

Table 1. Linear mixed models of bacterial loads (two bacterial types at two nestling ages) in relation to mean temperature, rainfall, hatching date, brood size and their interactions. Nest was included as random effect. Only the best models for each analysis are presented as no alternative models showed ΔAIC_c lower than 2. K refers to the number of parameters in each model and weight estimate the probability that the model is the best model in the model set.

Models	K	AIC_c	Weight
***Enterobacteriaceae* on day 7**			
H. Date + Rainfall + M. Temperature + H. Date*Rainfall + M. Temp*Rainfall	6	294.6	0.8
***Enterobacteriaceae* on day 13**			
H Date	2	254.2	0.7
Enterococci on day 7			
M. Temperature	2	286.7	0.7
Enterococci on day 13			
M. Temperature	2	196.3	0.9

For each bacterial type and nestling age, we present only the best model as alternative models showed ΔAICc values higher than 2. The best model for *Enterobacteriaceae* on day 7 included hatching date, mean temperature, rainfall and the interactions between hatching date and rainfall and mean temperature and rainfall during the preceding period (Table 1). All variables and interactions included in the model with lowest AICc were significant (Table 2). There a negative association of *Enterobacteriaceae* loads on day 7 with rainfall, but only for early broods (Fig. 1).

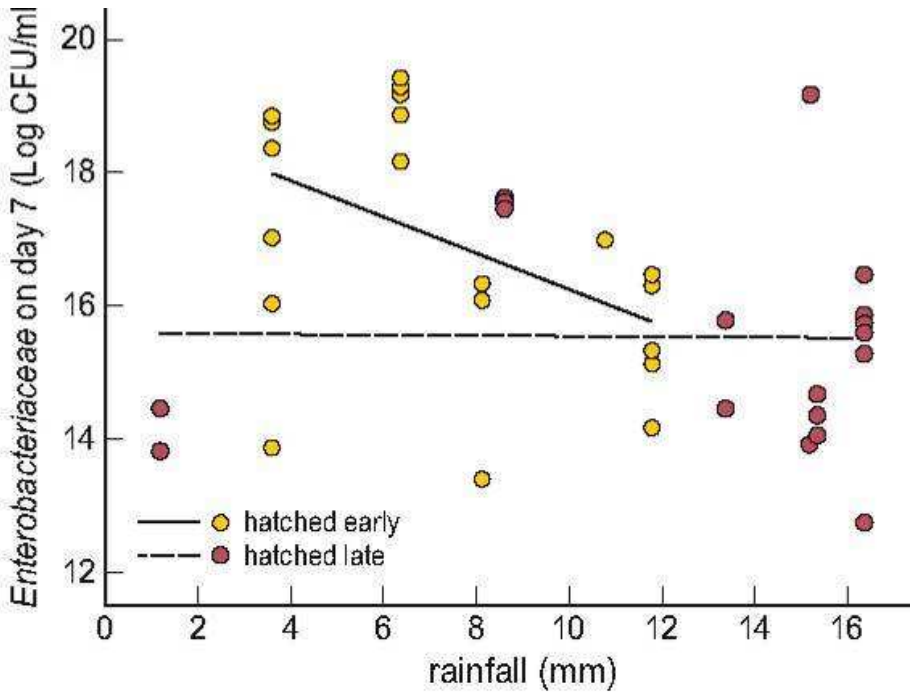

Figure 1. Association of *Enterobacteriaceae* loads on day 7 with rainfall for broods hatched before or after the median hatching date in the population.

Likewise, rainfall showed a negative association with *Enterobacteriaceae* loads on day 7, but only at low ambient temperatures. The best model for *Enterobacteriaceae* on day 13 included only hatching date (Table 1), which showed a negative association with bacterial loads (Table 2, Fig. 2).

Table 2. Parameter estimates of effects included in the best models (lowest AICc) of *Enterobacteriaceae* and enterococci loads at two nestling ages.

	Estimate coefficient ± SE	*df*	*F*	*p*
***Enterobacteriaceae* on day 7**				
H. Date	-1.345 ± 0.202	1,74	44.12	**<0.001**
M. Temperature	2.852 ± 0.497	1,74	32.92	**<0.001**
Rainfall	-2.571 ± 0.792	1,74	10.54	**0.001**
H. Date * Rainfall day 7	0.085 ± 0.017	1,74	23.67	**<0.001**
M. Temp. * Rainfall day 7	-0.194 ± 0.040	1,74	22.98	**<0.001**
Enterococci on day 7				
M. Temperature	0.127 ± 0.046	1,54.6	7.37	**0.008**
***Enterobacteriaceae* on day 13**				
H. Date	-0.127 ± 0.038	1,44.1	10.76	**0.002**
Enterococci on day 13				
M. Temperature	0.162 ± 0.071	1,91	5.15	**0.025**

The best model for enterococci on day 7 included only mean temperature (Table 1), which showed a positive association with bacterial loads (Table 2). For enterococci on day 13 included only mean temperature (Table 1), which showed a positive association with bacterial loads (Table 2).

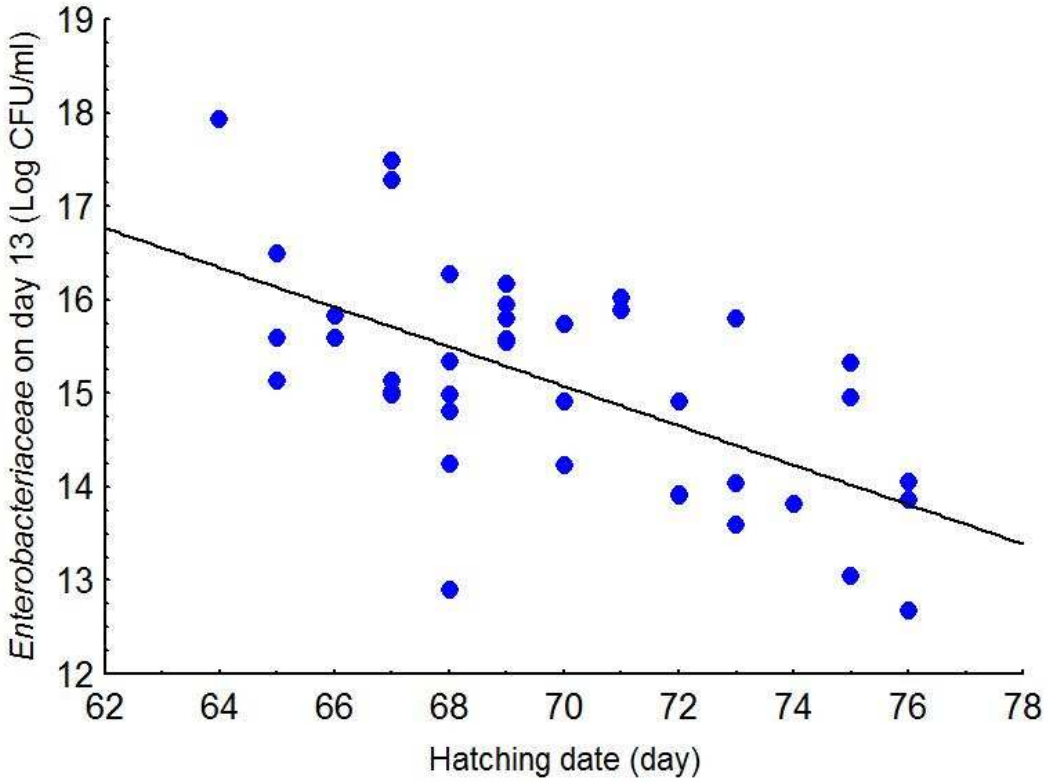

Figure 2. Association of hatching date (day 1= 1 of April) with loads of *Enterobacteriaceae* on day 13.

Discussion

Our results showed that *Enterobacteriaceae* loads were lower in later-hatching nestlings. We found no association between brood size and loads of either bacterial type. Ambient temperature was positively correlated to *Enterobacteriaceae* loads, but only on day 7. While the temperature, was positively correlated with enterococci loads

at two nestling ages. Higher rainfall resulted in lower *Enterobacteriaceae* loads on day 7.

Nestlings in late-hatched broods have fewer *Enterobacteriaceae* in their guts at both ages than early-hatched nestlings. This effect may be due to seasonal changes in diet. For example, Waldeström *et al*. (2002) found that the prevalence of *Campylobacter* spp. in migrating birds was highly influenced by feeding habits. Also, Lombardo *et al*. (1996) suggested that different feeding habits might explain the greater prevalence of bacteria in insectivorous than in omnivorous birds. Blanco *et al*. (2006) found differences in composition, richness and prevalence of faecal microbiota associated with the diet of adult red kite *Milvus milvus*. Furthermore, they found that *Klebsiella* sp. showed a higher prevalence in January than in February whereas Novotny *et al*. (2007) found that the occurrence of *Yersinia enterocolitica* in adult alpine accentors *Prunella collaris* was high in summer, especially during the nestling period. It is possible that late-breeding parents are less efficient at collecting prey, and that late nestlings may therefore be undernourished. Malnourished nestlings may be able to sustain lower bacterial growth in their guts due to intestinal shrinkage (Brzek & Konarzewski 2001) or to lower nutrient input. However, the non-significant effect of brood size suggests that malnourishment induced by competition among siblings is less important than seasonal changes in diet. In contrast to *Enterobacteriaceae*, enterococci were not responsive to differences in hatching date which suggests that they are relatively insensitive to nutritional effects.

As expected, loads of *Enterobacteriaceae* and enterococci of chicks at early nestling ages are positively associated with mean temperature while this association for grown nestlings is only found for enterococci. At low ambient temperatures, poorly thermoregulating chicks (younger than 7 days) may lose relatively more heat, drop their

body temperature and reduce their metabolism (Starck & Ricklefs 1998), thereby negatively affecting bacterial growth in the gut. Nestlings about to fledge maintain body temperature within a much smaller range than nestlings of 7 days (O' Connor 1984). The responsiveness of enterococci to temperature at late nestling ages indicates the extreme thermal sensitivity of these bacteria when compared with other gut bacteria.

Enterobacteriaceae loads showed a negative association with rainfall, but only of young nestlings. The negative effects of rainfall on day 7 on loads of *Enterobacteriaceae* could also be explained by poor thermoregulation as temperatures in the nest drop during rain showers. Rainfall also has a negative effect on the foraging capacity of adults (Radford *et al*. 2001, Geiser *et al*. 2008, Arlettaz *et al.* 2010). This could adversely affect nestling nutritional condition, so we would expect a negative effect of rainfall on the capacity to sustain large bacterial populations. By contrast, enterococci were not affected by rainfall at any nestling age. This suggests that enterococci are less responsive to rainfall-mediated nestling nutritional condition than *Enterobacteriaceae*.

Only early hatched broods experienced nesting environments conductive to strong predicted effectsof high rainfall on bacterial loads in nestling guts. In fact, loads of *Enterobacteriaceae* on day 7 in early-hatched broods, but not in late-hatched broods, showed a negative association with rainfall. This may be related to poor nestling nutrition due to low foraging capacity of adults during periods of high humidity. Finally, low temperatures and high rainfall may interact synergistically as predicted to induce thermoregulatory problems for small nestlings, thereby inducing reduced bacterial growth. In contrast, enterococci showed no response to rainfall at any ambient temperature.

To conclude, growth of important intestinal bacteria appears sensitive to seasonal and climatic factors, presumably mediated by nestling diet, thermoregulatory capacity and nutritional state. Different bacterial types vary in their responsiveness to environmental and seasonal variation.

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III

La diversidad bacteriana del tracto gastrointestinal se incrementa con la edad en los pollos de un paseriforme silvestre, *Ficedula hypoleuca*

Gut bacterial diversity increases with age in nestlings of a wild passerine, *Ficedula hypoleuca*

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Resumen.- Las bacterias del intestino juegan un importante papel en la salud de los animales puesto que éstas tienen efectos en la morfología del intestino, la nutrición o incluso pueden prevenir la colonización de dicho tracto por parte de bacterias patógenas. Sin embargo poco se sabe sobre los cambios en cuanto a diversidad bacteriana debidos a la edad. Por lo tanto, mediante la técnica ARISA hemos obtenido las diferentes Unidades Taxonómicas Operativas (OTUs) los cuales nos permiten caracterizar la comunidad bacteriana existente en el intestino de los pollos de un ave paseriforme silvestre como es el papamoscas cerrojillo en una población de España. Un total de 100 muestras fueron analizadas, en concreto 58 muestras pertenecen a una edad temprana (7 días desde su eclosión) y 42 muestras de la segunda edad (13 días). Obtuvimos 91 OTUs, de los cuales sólo cuatro fueron encontrados en un 20% de todas las muestras. Finalmente, analizamos la diversidad bacteriana por medio del índice de Shannon, dando como resultado que la diversidad bacteriana aumenta a medida que el pollo va creciendo si bien el número de OTUs no cambió con la edad. Nuestros resultados muestran que la comunidad bacteriana estaba casi establecida a edades tempranas en el pollo de papamoscas cerrojillo o bien que una nueva adquisición bacteriana fue mínima además la abundancia de los diferentes tipos bacterianos llegan a un equilibrio antes de que el pollo abandone el nido.

Abstract.- Gut bacterial play an important role in the health of animals given that they have effects in gut morphology, nutrition or can prevent that other pathogenic bacteria colonize intestinal tract. Little is known about how it changes the gut bacterial diversity due to age. Therefore, we have obtained by technique ARISA the different Operational Taxonomic Units (OTUs) that characterize the bacterial community in the gut of nestlings of a wild passerine bird, pied flycatcher, in a population of Spain. A total of 100 samples were analyzed, 58 samples at early age (7 days from hatching date) and 42 for the second age (13 days). 91 OTUs were found, which only four were found in over 20% of all samples. Finally, we analyzed the diversity by means of Shannon index resulting in bacterial diversity increases as nestling grows while the number of OTUs didn't change with the age. Our results show that the gut microbial community was almost established at early ages in the pied flycatcher or that a new input was minimum and the abundance of the different bacterial types reach equilibrium before the nestling leaves the nest.

Introduction

Bacteria are very ubiquitous and can be found in most environments with sufficient nutrients necessary for growth (Horner-Devine *et al.* 2004, Madigan *et al.* 2004). Animals offer such conditions and are colonized by many bacterial strains. Animal-bacteria interactions can have important implications for individual host fitness for example in relation to growth of young birds (Lombardo *et al.* 1996, Mills *et al.* 1999, Potti *et al.* 2002, Moreno *et al.* 2003, González-Braojos *et al.* 2012a, 2012b) or to reproduction (Lombardo & Thorpe 2000, Westneat & Rambo 2000, White *et al.* 2010). Animal guts constitute an ideal environment for bacterial growth (Barnes 1972, Madigan *et al.* 2004). Gut bacteria play an important role in animal health through their effects on gut morphology (Barnes 1972, Shapiro & Sarles 1974), metabolic functions such as fermentation of non-digestible dietary residues (Stanley *et al.* 2013) protection against colonization by pathogens such as *Salmonella* spp. (Rantala & Nurmi 1973, Gleeson *et al.* 1989, Fuller 2001) and stimulation of the immune response (Cebra 1999, Brisbin *et al.* 2008, Ruiz-Rodríguez *et al.* 2009a). When birds hatch their gut is sterile, but quickly acquires a numerous and highly diverse microbial community (Barnes 1972, Mills *et al.* 1999) that remains stable throughout the adult stage as described by Benskin *et al.* (2010) in a study with zebra finches *Taeniopygia guttata* under controlled conditions. The acquisition of gut microbiota can occur either by infection from the environmental surroundings (Lucas & Heeb 2005, Klomp *et al.* 2008), by parents through saliva when young are being fed (Kyle & Kile 1993), through contact with siblings or through the food consumed (Glunder 2002, Waldenström *et al.* 2002, Wienenmann *et al.* 2011). Acquisition of gut bacteria through food can affect the composition of this community. Thus, Waldenström *et al.* (2002) observed that the

prevalence of *Campylobacter* spp. was influenced by diet, being low in granivorous or insectivorous birds. Moreover, certain members of the community may affect its composition through competitive exclusion through for example the use of bacteriocines to prevent the establishment of competing bacteria (Soerjadi-Liem *et al.* 1984, Riley & Wertz 2002, Martín-Platero *et al.* 2006). Finally, age may affect bacterial community structure and composition due to changes throughout nestling development in the morphology and physiology of the intestinal tract. For example, Mills *et al.* (1999) have shown that relative abundance of bacteria increased with nestling age with culture-dependent methods while van Dongen *et al.* (2013) have obtained the same result with molecular techniques.

The aim of the study is to study the age-dependent variation in the number of operational taxonomic units (OTUs) and community diversity as measured with the Shannon index. We expect that the number of OTUs increases as chicks grows (see van Dongen *et al.* 2013) and that diversity attains a state of equilibrium during nestling development. To that end we have focussed on nestlings of pied flycatchers *Ficedula hypoleuca*, a species for which there is now some information on changes in nestling gut bacteria (Moreno *et al.* 2003, Gonzalez-Braojos *et al.* 2012a). However, unlike van Dongen *et al.* (2013), we have measured OTUs and diversity in the same nestlings of pied flycatchers at two different ages. To quantify OTUs and diversity we have used a set of samples used previously for culture-dependent analyses (González-Braojos *et al.* 2012a). We have used the automated ribosomal intergenic spacer analysis (henceforth ARISA) technique, which describes bacterial communities by amplifying a sequence of the rRNA intergenic spacer 16S-23S that varies in length and sequence according to taxonomic groups (Ranjard *et al.* 2001). Using this technique, we obtained the OTUs, also called phylotype, which are assumed to denote bacterial lineages or species (Atlas

& Bartha 1997). The ARISA technique has been used mainly to characterize environmental bacterial communities (e. g. Fisher & Triplett 1999; Drakare & Liess 2010; Smith *et al.* 2010), but is progressively being used to describe bacterial communities associated with wild and domestic birds (Scupham 2007; 2009; White *et al.* 2010; Corrigan *et al.* 2011; 2012; van Dongen *et al.* 2013).

Methods

The study was conducted in the breeding season of 2009 on a population of pied flycatchers nesting in a deciduous forest of Pyrennean Oak (*Quercus pyrenaica)* at an elevation of 1.200 m a.s.l. in Valsaín, Segovia province (40˚ 54' N, 4˚ 01' W), Spain. This population breeds in nest-boxes (for detail of this nest-boxes see appendix in Lambrechts *et al.* 2010), which are cleaned every year after the breeding season. Nest-boxes were checked daily for nest-building activity, and the hatching dates and brood sizes were recorded. For details on the study species and population see González-Braojos *et al.* (2012a, b).

Bacterial sampling

We have sampled freshly produced faecal sacs as a substitute for cloacal samples, and therefore we assume that most bacteria sampled will be derived from gut communities. A total of 100 faecal sacs between days 7 and 13 were collected in sterile Eppendorf tubes. Samples were transported in a portable cooler until their processing in the laboratory (3–6 h after collection). In the laboratory, one sterile cotton swab was introduced in the sterile Eppendorf tube containing the faecal sac for 1–3 seconds trying to uniformly impregnate the cotton with faecal matter. Then, we transferred the swabs to transport media Amies (Sterile R; Meus, Piove di Sacco, Italy) and chilled the

samples (4ºC) until processed. All samples were preserved for 20 days from collection. Swabs were transferred from transport media Amies into 1 ml of phosphate buffered saline (PBS, pH = 7.2; Química Clínica Aplicada, Tarragona, Spain). Later, 500 µl were transferred to screw cap tubes of 1.5 ml that contains 1 ml of freezing medium (20 g skim milk (Difco, Laboratories, Detroit, MI, USA), 30g Tryptone (Pronadisa, CondaLab, Torrejón de Ardoz, Madrid, Spain), 8 ml Glycerol (Panreac Química, s. l., Castellar del Vallés, Barcelona, Spain) and 1000 ml of distilled water). These tubes were frozen at -80ºC until further analysis.

DNA extraction and amplification

To characterize the structure and the diversity of bacterial communities existing in each gut sample of nestling, we performed automated ribosomal intergenic spacer analyses (ARISA). This DNA-fingerprinting method is based on the amplification of the 16S-23S intergenic spacer region (ISR) from the bacterial rRNA operon. The ISR region is extremely variable in both sequence and length for different prokaryotic species, thus the DNA amplification profile obtained with ARISA for a given bacterial community sample allows straightforward estimation of bacterial diversity.

We extracted bacterial DNA using the protocol developed by Martín-Platero *et al.* (2007). Before beginning DNA extraction, 500 µl of our sample were washed two times with 500 µl of PBS and centrifuged at 20.000 g for 60 sec to clean the sample of faecal debris. Once this step was made, the pellet was resuspended in 100 µl of TES-lysozime buffer and incubated for 30 min at 37ºC. Afterwards the cells were lysed by adding 600 µl of lysis buffer with the tube being gently inverted for mixing and incubated for 15 min at room temperature. The lysate was treated with 10 µl of proteinase K (10 mg/ml) and incubated for 15 min at 37ºC.This step serves to increase the purity of the DNA. Then the tube was incubated at 80ºC for 5 min followed by

cooling to room temperature for 10 min. Later, 200 µl of sodium acetate (3M, pH 5.2) was added and the solution was vortex-mixed for 15 sec, chilled on ice for 15 min and again centrifuged at 20.000g for 10 min. At last, the supernatants from the previous step, which contains the nucleic acids, were precipitated with 600 µl of isopropanol. This tube was gently inverted several times and centrifuged at 20.000 g for 10 min, obtaining the DNA pellet, which was washed with 1 ml of ethanol at 70 % and dried at room temperature. Finally, the DNA was resuspended in 200 µl of 0.5 x TE buffer. Subsequently, these samples (9 µl) were analyzed by 0.7 % agarose gel electrophoreses for 1 h at 100 V.

Once verified that DNA extraction was successful, we performed the polymerase chain reaction (PCR) to amplify such DNA. The primers used were 72 F (5'-TGCGGCTGGATCTCCTT-3') and the HEX (6-carboxyhexafluorescein)-labeled primer 38 R (5'-[6HEX] CCGGGTTTCCCCATTCGG- 3') (Ranjard *et al.* 2001). The PCR was performed in 50 µl , with 3 µl of DNA template (50 ng), 1 x PCR buffer (QUIAGEN), 6 µl $MgCl_2$ (25 mM), 5 µl of each primer (10 pmol/ µl), 1 µl of dNTPs (10 mM), 0.3 µl of Taq polymerase (QUIAGEN) and milliQ water to make 50 µl. The amplification reaction was performed using an initial denaturing at 94ºC for 3 min, followed at 94ºC for 1 min, 55ºC for 30 sec, 72ºC for 1 min, a total of 30 cycles from initial denaturing to this step, and a final extension at 72ºC for 5 min. Afterwards, we carried out 1% agarose gel electrophoreses for 1 h at 100 V with the PCR products in order to determine that the amplification was successful.

Once, we obtained the products of PCR and verified the amplification, 5 µl of our product of PCR diluted 15 times in milliQ water were analyzed with a sequencer 3130 Genetic Analyzer (Applied Biosystems) at the Centro de Instrumentación Científica (CIC) of the University of Granada.

Analyses of bacterial communities

The sequencer detected fragments ranging from 100 to 1000 bp in size. These fragments were analyzed by using Peak Scanner Software version 1.0 (Applied Biosystems). This software separates DNA fragments according to their size. Once we obtained the peaks and their respective sizes and area, we followed Ramette (2009) to fix the bin size and get the OTUs. Then, we considered the intensity of the signal detected as a proxy for the abundance of individual OTUs.

We calculated for each gut sample the Shannon diversity index, [$H' = \Sigma\ p_i \log_2 (p_i)$], where p_i is the observed relative abundance of OTU i, using the R-package "vegan" (Oksanen *et al.* 2011).

Statistical analysis

All variables had a normal distribution. The statistical procedures were implemented in the R-software v. 2.12.2 (R Development Core Team, 2011). We performed a mixed model with the R-package "nlme" (Pinheiro *et al.* 2011). For this we included the Shannon index as dependent variable, age as repeated factor and nest ID as random factor. The same test was performed for the number of OTUs including this variable as dependent.

Results

We identified 91 OTUs from a total sample size of 100 nestlings. No OTU was present in all samples; only four OTUs were present in over 20% of all individuals and 35% of OTUs were found in a single sample. The number of OTUs obtained per faecal sample averaged 5.670 ± 0.383 (SE, n=100).

We observed that Shannon diversity index was affected by age ($F_{1, 57}$ = 5.239; p = 0.025), diversity being higher in faecal samples on day 13. There was a slight albeit non-significant increase ($F_{1, 57}$ = 0.910; p = 0.343) in number of OTUs with age (7 days (mean ± SE): 5.396 ± 0.509; 13 days (mean ± SE): 6.047 ± 0.584).

Discussion

We obtained 91 OTUs in the faecal samples of nestlings of pied flycatcher, with only four being found in over 20% of the samples whereas 35% of OTUs were found in a single sample. The number of OTUs or bacterial lineages (average 5.670 ± 0.383) did not change with respect to age but community diversity as measured by the Shannon index, increased with nestling age. The average number of OTUs obtained in faecal samples of nestlingpied flycatcher was similar to those reported for other species such as zebra finch adults (6 OTUs, Benskin *et al.* 2010), nestlings of magpie *Pica pica* and of great spotted cuckoo *Clamator glandarius* (5 and 9 OTUs respectively, Ruiz-Rodriguez *et al.* 2009b) and nestlings and adults of kittiwake *Rissa tridactyla* (6 and 9 OTUs, van Dongen *et al.* 2013). The result that 35 % of OTUs were found in a single individual contrasts with the results of van Dongen *et al.* (2013) where 63% of OTUs found in nestlings belonged to a single individual.

Our results with respect to the effect of age on the number of OTUs differ from those obtained by van Dongen *et al.* (2013) since they obtained that as nestling grew, the number of OTUs contained in the gastrointestinal tract increased. The absence of age-dependent effects suggests that nestlings had already acquired at a very early age the environmental bacteria from their surroundings and those provisioned by their parents (Kyle & Kyle 1993, Mills *et al.* 1999, Lucas & Heeb 2005). Nestling pied

flycatchers unlike kittiwake nestlings are not raisedin large colonies with little space between open nests and thus may experience a lower probability of contacting with new environmental bacterial strains throughout their stay in the nest. Furthermore the diet of pied flycatcher nestlings is mainly composed of caterpillars (15-65% in deciduous forests; Lundberg & Alatalo 1992, Sanz 1998). In our population the food given to nestlings of 5-7 days was composed of Lepidoptera (mostly caterpillars, about 50%) followed by Hemiptera and Coleoptera (about 20% both) (Moreno *et al.* 1995). The specialized diet may explain why nestlings do not continue to incorporate OTUs after the first week in the nest. Pied flycatchers also remove faecal sacs from nest cavities (Cantarero *et al.* 2013), while kittiwakes are not able to maintain nest hygiene in crowded colonies which may imply a source of bacterial inoculation. Finally, we have not covered changes during the first week of life unlike of article of van Dongen *et al.* 2013. Thus, Mills *et al.* (1999) found that the percentage of positives in different selective media increased during the first days of life (approximately 30% at the age of 2-3 days to about 55% on day 7) while the increase was not as marked after the first week However, the results obtained with culture-depemdent methods are not really comparable to our results. While molecular techniques, van Dongen *et al.* (2013) have obtained almost the same results as ours in a similar period because the number of OTUs found in nestlings at 5 days were 4 whereas at age 10, the total average was around 5-6 OTUs. Therefore, the increase in number of species is in the early days of life since having sterile gastrointestinal tract, it rapidly colonized by bacteria in the surrounding environment and the last days spent in the nest when diet may be more similar to the adult while in intermediate period changes are weak.

The increase in community diversity can be explained if at early ages one or a few bacterial strains are more dominant than at later ages. Dominance may gradually

dilute because bacteria increasingly compete with each other for space and nutrients. Effects of this competition was observed by Moreno *et al.* (2003) in nestlings of pied flycatchers, which showed a negative association between scores of *E. faecalis* and of the rest of enterococci. In a study on the bacterial communities found in the ceca of broilers according to age, it was found that at 4 days of life this community was composed mainly of *Salmonella* spp. (40% of relative abundance) followed by *Lactobacillus* spp. (approximately 20 %). Subsequently this community changed until the age of 14 days by becoming more homogeneous with no species exceeding 30% in relative abundance (Amit-Romach *et al.* 2004). These changes in relation to abundances of bacteria have also been found with the same samples with culture-dependent techniques in relation two types of gut bacteria (González-Braojos *et al.* 2012a).

More studies on this subject are needed to know how gut microbiota are acquired and how they change with age during development by incorporating also the first days of life of nestlings (2-3 days). Furthermore, it is recommended that repeated samples come from the same individual at different periods of development because there may be individual variation in gut microbiota (Benskin *et al.* 2010) which is not efficiently controlled through cross-sectional age studies. .

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IV

Nidos reutilizados y las bacterias residentes en la piel en relación al crecimiento en pollos de papamocas cerrojillo

Nest reuse and skin bacteria in relation to nestling growth in pied flycatchers

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Resumen. Las bacterias pueden colonizar los nidos de las aves sin que se sepa las repercusiones que éstas puedan tener en el crecimiento y la salud de los pollos, aunque dichas bacterias del material del nido pueden colonizar la piel y las plumas en crecimiento de los pollos. Las aves trogloditas en ocasiones construyen sus nidos sobre un antiguo nido, debido a la escasez de cavidades. La reutilización de los nidos podría favorecer la colonización por parte de las bacterias del nuevo material así como de la piel del pollo y consecuentemente afectar el crecimiento de las plumas de éste. Para testar estas posibilidades, llevamos a cabo un estudio con el papamoscas cerrojillo *Ficedula hypoleuca*, en una población que cría en cajas-nido en España. Para ello dejamos una muestra de cajas-nidos sin limpiar de la temporada anterior, por lo tanto contenían el nido viejo de 2010 y se comparó la carga bacteriana del material del nido, de un objeto control y de la piel de la parte ventral del pollo en los nidos viejos con las existentes en los nidos nuevos de 2011. Los pollos que crecieron en los nidos viejos tuvieron mayores cargas bacterianas en su piel comparado con los de los nidos nuevos, mientras que no se encontró ninguna diferencia para las otras medidas tomadas. Se hayó una tendencia marginalmente significativa con la longitud alar del pollo, siendo dicha longitud menor en los nidos viejos aunque ninguna tendencia se observó para las otras medidas biométricas. La carga bacteriana del nido mostró una asociación negativa con la longitud alar únicamente. Este resultado indica una asociación entre el nido reutilizado y las bacterias que crecen sobre la piel no detectada. También sugiere un posible deterioro de la capacidad de vuelo del volanton mediada por la comunidad bacteriana del nido que está en contacto continuo con la piel y plumas en crecimiento.

Abstract. Bacteria may colonize avian nests with unknown repercussions on nestling growth and health, although bacteria on nest materials may easily colonize nestling skin and growing feathers. Cavity nesters may have to build their nests on top of used nest materials, given restrictions on cavity availability. Nest reuse may favour bacterial colonization of nest materials and nestling skin and thereby affect nestling feather growth. To test these possibilities, we conducted a study of pied flycatchers *Ficedula hypoleuca* breeding in nest-boxes in central Spain. We left a sample of nest-boxes without removing old nest materials in 2010 and compared bacterial loads of nest materials, control inert objects and nestling belly skin in reused nests with those in new nests in 2011. Nestlings raised in reused nests had higher bacterial loads on their belly skin than those in new nests, while no difference between nest types for nest materials and control inert objects were found. There was a marginally significant tendency for wing length before fledging to be lower in reused nests, but no trend for mass or tarsus length. The bacterial loads of nests showed a negative association with feather growth of nestlings as expressed through wing length but not with tarsus length or mass growth. These results indicate an association between nest reuse and bacterial growth on nestling skin not hitherto detected. They also suggest a possible impairment of flight capacity at fledging mediated by nest bacterial communities which are in direct contact with nestling skin and growing feathers.

Introduction

Altricial birds use nests for incubating eggs and raising young (Hansell 2000). Avian nests are micro-environments very likely to be colonized by bacteria due to the presence of debris, faeces and discarded food. The factors affecting bacterial colonization of nest environments are still poorly understood. Nests in cavities may offer more constant and suitable environmental conditions for bacterial growth, so we may expect them to harbour richer bacterial communities. Only a few studies to date have been conducted on nest bacterial communities in cavity-nesting species (Mehmke *et al.* 1992, Singleton & Harper 1998, Goodenough & Stallwood 2010). In one of them, Singleton & Harper (1998) detected the presence of three potentially pathogenic bacterial genera (*Pseudomonas*, *Bacillus* and *Staphylococcus*) in house wren *Troglodytes aedon* nests. Nest composition may affect bacterial colonization given that different nest materials may offer different microclimatic conditions. Goodenough & Stallwood (2010) have studied bacterial assemblages in nest materials used by two common and related hole-nesting passerines, the great tit *Parus major* and the blue tit *Cyanistes caeruleus*, and found that the dominant bacteria were again lineages of *Pseudomonas*, *Bacillus* and *Staphylococcus*. The same genera of bacteria were found on European starling *Sturnus vulgaris* nestling skin (Berger *et al.* 2003) except *Pseudomonas*. However, *Pseudomonas* is present in both nest materials and on vertebrate skin (D'Aloia 1996, Goodenough & Stallwood 2010, Remold *et al.* 2011). In this regard, some studies have also shown that specific materials added to nests may have antibacterial properties, suggesting a potential deleterious role of nest bacteria for nestling development (Clark & Mason 1985, Petit *et al.* 2002, Gwinner & Berger 2005, Mennerat *et al.* 2009a, 2009b).

Nest reuse in birds is rare as most birds construct new nests for each breeding attempt (Hansell 2000). One reason for not reusing nests may be avoiding contact of adults, eggs and nestlings with rich bacterial communities. However, for cavity-nesting passerines, nest site availability is one of the main factors constraining reproduction (Newton 1994). Given that appropriate cavities are scarce, hole-nesting birds may often reuse those that were occupied in the previous season, incurring costs caused by an increase in the risk of predation (see Mazgajski 2007a) or the presence of ectoparasites (e.g. Loye & Carroll 1998, Mazgajski 2007a, 2007b, Tomás *et al.* 2007). Experimental studies have explored the importance of nest reuse in cavity nests for arthropod colonization of nests and for nestling health and growth (Rendell & Verbeek 1996b, Allander 1998). Moreover, bacteria can remain dormant in nests and faeces for several months (Maier *et al.* 2000), so there may exist an increased risk of bacterial infection for breeding birds. Indeed, Singleton & Harper (1998) found *Salmonella* and *Yersinia*, two potentially pathogenic bacteria, in old wren nests. Moreover, the behaviour of removing old nest material that has been observed in some nests of several cavity-nesting species (e.g. Merino & Potti 1995, Pacejka *et al.* 1996, Mazgajski *et al.* 2004) could function to reduce bacterial loads.

Bacteria are ubiquitous organisms that can interact with growing nestlings in many different ways, going from positively (Moreno *et al.* 2003) to negatively (Potti *et al.* 2002, González-Braojos *et al.* 2012). Avian skin is an adequate environment for bacterial colonization (Rajchard 2010), although very little is known about the role of tegumental bacteria during avian development. Potentially pathogenic bacteria may colonize the bare skin of growing altricial nestlings (Berger *et al.* 2003) and affect feather growth. Effects on plumage development in nestlings may impact survival after fledging if flight or insulation is somehow impaired. Nestling skin is in direct contact

with nest cup materials and is therefore exposed to bacterial colonization during the whole nestling period if nest materials harbour significant bacterial communities. It thus appears plausible that bacteria in nest materials may colonize nestling skin and plumage in direct contact with the nest-cup. Berger *et al.* (2003) found no clear association of skin bacterial loads with nestling biometry and mortality although they suggested deleterious impacts for late nests. This is the only study to date relating skin bacterial loads to nestling fitness.

Given the need for more information concerning the association of nest composition and nest reuse with bacterial colonization of nest materials, we have conducted a study on pied flycatchers *Ficedula hypoleuca*, in which old nests were left in some nest-boxes and others cleaned according to a standardised procedure commonly used in studies of nest reuse (Pacejka & Thompson 1996, Blem *et al.* 1999, Cavitt *et al.* 1999, Stanback & Dervan 2001, Mazgajski 2007b, Tomás *et al.* 2007). Pied flycatchers in cavities with old nests usually add some nest material to construct the nest cup on top of old nest material, the largest part of reused nests thus being composed of old material (Moreno *et al.* 2010). We assume that new nest cups covering old nest material are rapidly colonized by bacteria resident in reused material. We have estimated heterotrophic bacterial loads on nestling skin, an inert control object to estimate passive colonization of objects in nests and on nesting material to test the following predictions:

1) Reused nests should harbour larger bacterial populations than new nests.
2) Nestlings in reused nests should show higher bacterial loads on the skin and consequently should grow more poorly given potential effects of nest reuse on ectoparasites and bacteria.
3) Nestling mass and size before fledging should be correlated with bacterial loads on nest or skin.

4) Feather growth should be more impaired than skeletal or mass growth given the close contact of skin with nest materials.

Methods

We conducted the study during the spring of 2011 on a population of pied flycatchers breeding in artificial nest-boxes in a montane forest of Pyrenean oak, *Quercus pyrenaica*, at 1500 m.a.s.l. close to the village of Lozoya, central Spain (40º 58' N, 3º 48' W). The pied flycatcher is a small hole-nesting passerine of European woodlands which is frequently used as a model species in behavioural ecology (Lundberg & Alatalo 1992). Females are the main nest builders (Martínez-de la Puente *et al.* 2009). Egg laying in the population under study typically begins in late May, and modal clutch size is six. The female incubates alone and receives part of her food from her mate (Moreno *et al.* 2011). Young are brooded by the female only up to day 8 (hatching day = day 1) (Sanz & Moreno 1995). Both sexes feed the young. Young fledge within 14–16 days after hatching (Lundberg & Alatalo 1992). This occurs in the second half of June in our study area. Nests were followed and hatching dates estimated through daily visits.

In the study area, there are 100 wooden nest-boxes which are occupied by pied flycatchers, great tits and blue tits (nest-boxes are opened by removing the front; see Lambrechts *et al.* 2010 for dimensions, structure and placement). After the breeding season of 2010, a randomly selected half of these nest-boxes containing nests of pied flycatchers, great tits and blue tits were not cleaned as is usually done after the breeding season. Of these nest-boxes with old nests, 21 were occupied by pied flycatchers in 2011, while 34 pairs nested in nest-boxes cleaned in 2010. Of 21 reused nests, 12

belonged to pied flycatchers in 2010 and 9 to great or blue tits. Reused nests refer in this case to old nest material covered by some fresh material (Moreno *et al.* 2010). As an inert control available for bacterial colonization of objects in nests we have used a plastic sheet of the same surface as that sampled on nestlings. The plastic was chosen as the most suitable material because it was easy to wash with alcohol, dried quickly and is an inorganic material which may function as a standard surface for bacterial colonization. On the day when nestlings hatched, we introduced into the nest material near the nest-cup a square of 1.5 cm^2 (total area of both sides = 3 cm^2) cut out of plastic sleeves with a rough surface, which was previously washed with alcohol (Fig. 1 a).

On day 13 (hatching day = day 1), we ringed nestlings and measured their tarsus length with a digital calliper to the nearest 0.01 mm and their wing length with a stopped ruler to the nearest mm. Nestlings were also weighed with a Pesola spring balance to the nearest 0.25 g.

Bacterial sampling

On day 13 of nestlings' life, we sampled two randomly selected nestlings per nest, the nest cup and the inert control object. Wearing sterile rubber gloves, one of us (SG-B) covered the unfeathered ventral side of nestlings (henceforth called belly) with a rigid plastic sheet where a rectangle of 3 cm^2 had been cut out, so the naked skin of the belly could only be swabbed through this surface (Fig. 1b). Feather patches on the belly were avoided when covering the belly with the sheet. We passed the delimited belly area during 30 s with a sterile cotton swab previously moistened with sterile phosphate buffered saline (pH = 7.2, Química Clínica Aplicada, Tarragona, Spain). The belly swabs were then transferred into transport media Amies (*Sterile R, Meus s.r.l.,* Piove di Sacco, Italy). We sampled the nest cup in the same way by applying the sheet to the nest-cup wall on a randomly selected spot and swabbing through the 3 cm^2 square

during 30 s carefully avoiding contact with faeces or discarded prey (Fig. 1c). The plastic sheets were washed with alcohol between samplings, avoiding contamination between nests. The control object was collected from the nest material with a sterile forceps. The control object was swabbed on both sides during 30 s in the same manner as for nestling bellies. Seven control objects were not found (five for new nests and two corresponding to old nest). All samples were transported in a portable cooler until their processing in the laboratory (3-6 h. after collection).

Figure 1. (a) inert control object inserted into the nest material near the nest-cup, (b) sampling of unfeathered belly of nestling through a section not covered by a rigid plastic sheet (feathers were separated before swabbing the skin) and, (c) sampling of the nest-cup in the same way as nestling's belly.

Once in the laboratory, we transferred the swabs to tubes containing 1 ml of freezing medium composed of 20 g skim milk (Difco, Laboratories, Detroit, MI, USA), 30g Tryptone (Pronadisa, CondaLab, Torrejón de Ardoz , Madrid, Spain), 8 ml Glycerol (Panreac Química, s. l., Catellar del Vallés, Barcelona, Spain) and 1000 ml distilled

water. These tubes were frozen at -80 º C until processed less than one month later. The samples were cultured by plating out 100 µl of the dilutions 10^0 and 10^{-1} on Tryptic Soy Agar (TSA, Scharlau, Barcelona, Spain). Plates were incubated for 48 ± 2 h at 25 ± 1˚C, and colonies were then counted using a colony counter "sensor" (Suntex Instruments Co. Ltd., Taipei County, Taiwan) by the same observer (SG-B). Bacterial loads are expressed as colony forming units (CFU)/cm^2. TSA is a general medium to estimate abundances of aerobic culturable bacteria (Mills *et al.* 1999, Cook *et al.* 2003, Cook *et al.* 2005, Soler *et al.* 2008, Møller *et al.* 2009, Shawkey *et al.* 2009, Goodenough *et al.* 2011).

Statistical analyses

All variables were normally distributed or successfully normalized through logarithmic transformation prior to analyses. Analyses were conducted with Statistica 6.0 (Statsoft) and SAS (Institute 2001). First, we tested if the type of old nest (moss in tit nests or leaves and grass in flycatcher nests, Moreno *et al.* 2009) affected bacterial loads on nest material, inert control object or skin. As there were no significant effects (Nest: $F_{1, 12}$ = 0.569, p = 0.464, R^2 *adjusted* = -0.034; Object: $F_{1,15}$ = 0.329, p = 0.574, R^2 *adjusted* = -0.043; Skin: $F_{1,17}$ = 0.143, p = 0.709, R^2 *adjusted* = -0.049), both types of reused nests have been pooled into a single category of reused nests. Then we tested if nest type (old, new) affected bacterial loads on nest material and control object while controlling for hatching date in mixed model analyses. Subsequently we tested for effects of nest type on skin bacterial load as dependent variable controlling for hatching date and brood size at fledging. Then, we tested for effects of nest type on mean nestling biometrical variables (tarsus length and wing length) and mass per nest as dependent variables with nest type as factor and hatching date and brood size as covariables. Finally, we tested for associations of biometrical variables of swabbed nestlings with skin and nest

bacterial loads while controlling for hatching date and brood size besides nest type. All analyses where two samples per nest were introduced as dependent variable (skin bacterial loads, biometrical variables) were run into a mixed model while introducing nest as random factor. Non-significant variables were sequentially removed until obtaining a final model including only significant effects.

Results

Nest reuse showed no significant effect on nest bacterial loads when controlling for hatching date (Table 1, Fig. 2). The bacterial loads on inert control objects were not affected by nest reuse, nest bacterial load or hatching date (Table 1, Fig. 2). The bacterial loads of nestling skin were associated with nest type when controlling for hatching date, brood size and nest bacterial load (Table 2). Loads of bacteria were higher on nestling skin in reused nests compared with new nests (Fig. 2).

Table 1. General lineal model analyses of effects of nest type and hatching on nest bacterial load, and effects of nest type, hatching date and nest bacterial load on inert control object bacterial load.

	df	*F*	*p*	R^2 *adjusted*
Nest bacterial load				
Nest type	1,36	0.178	0.675	
Hatching date	1,35	0.668	0.410	
Model	2,35	0.106	0.898	-0.052
Inert control object				
Nest type	1,36	0.826	0.369	
Hatching date	1,36	2.497	0.122	
Nest bacterial load	1,25	0.142	0.708	
Model	3,25	1.475	0.184	0.076

Table 2. Mixed model analyses of effects of nest type, nest bacterial load, hatching date and brood size, with nest as random factor, on belly skin bacterial loads of swabbed nestlings. Minimal models are obtained from full models by successive backward deletion of variables when the variance explained does not significantly improve the model (α = 0.05).

	Coefficient	*df*	*F*	*p*
Full model				
Hatching date	0.070	1,32	1.53	0.224
Brood size	0.349	1,32	3.31	0.077
Nest bacterial load	0.135	1,32	2.08	0.158
Nest type	1.122	1,32	8.79	0.005
Minimal model				
Nest type	1.258	1,48	8.19	0.006

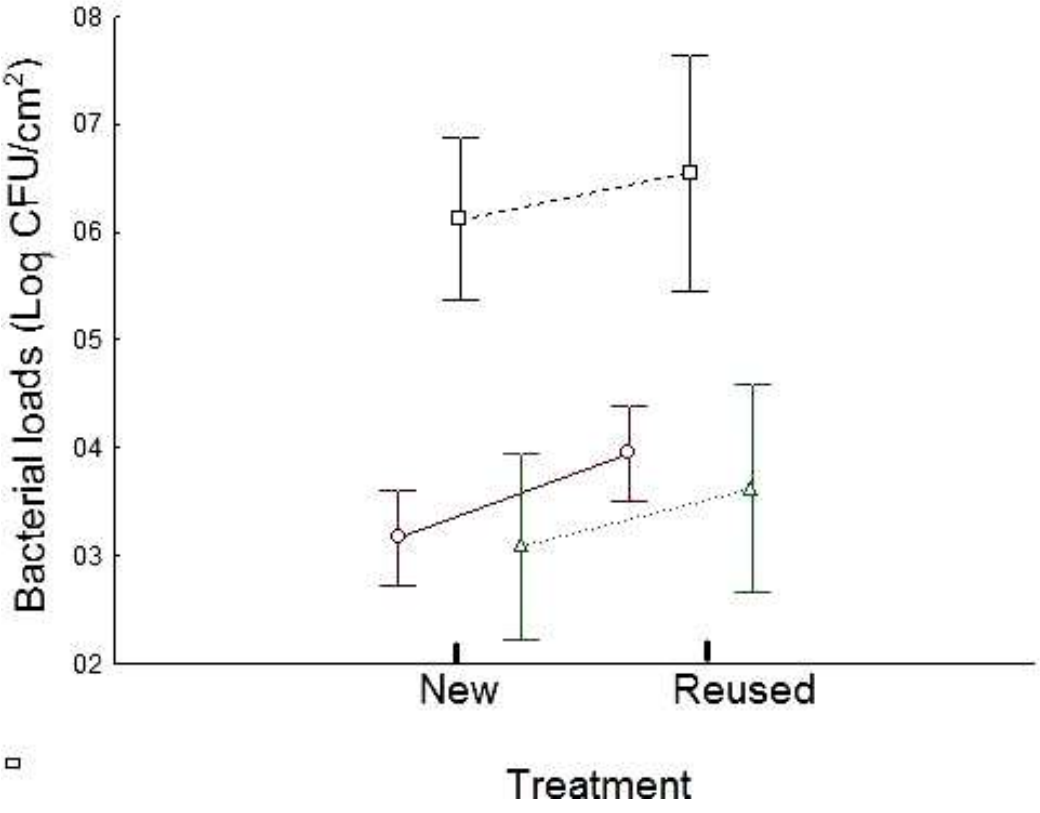

Figure 2. Relationships between loads of bacteria (inert control object = empty triangles, nestling belly skin = empty circles, nest-cup = empty squares) in relation to nest type (inert control object: $F_{1, 36} = 0.826$, $P = 0.369$; skin: $F_{1, 45} = 4.424$, $P = 0.041$; nest: $F_{1, 36} = 0.178$, $P = 0.675$).

Nest reuse showed a marginally significant effect on wing length when controlling for hatching date and brood size (Tables 3, 4). There was no effect for mass and tarsus length (Tables 3, 4).

Table 3. Mean values (± SE) of biometrical variables of nestling of pied flycatcher.

	New Nest	Old Nest
Tarsus length	17.304 ± 0.053 (67)	17.255 ± 0.086 (42)
Body-mass	14.213 ± 0.090 (68)	14.006 ± 0.143 (40)
Wing length	45.352 ± 0.318 (68)	43.809 ± 0.445 (42)

Table 4. Mixed model analyses of effects of nest type, brood size and hatching date on nestling linear measurements and mass. Minimal models are obtained from full models by successive backward deletion of variables when the variance explained does not significantly improve the model ($\alpha = 0.05$).

	Coefficient	*df*	*F*	*p*
Tarsus length				
Full model				
Hatching date	-0.014	1,48	0.13	0.710
Brood size	0.092	1,48	0.46	0.496
Nest type	0.022	1,48	0.23	0.627
Body mass				
Full model				
Hatching date	-0.134	1,48	3.49	0.067
Brood size	0.127	1,48	1.30	0.259
Nest type	-0.149	1,48	0.78	0.380
Minimal model				
Hatching date	-0.287	1,52	4.61	0.036
Wing length				
Full model				
Hatching date	0.046	1,48	1.42	0.239
Brood size	0.432	1,48	1.32	0.255
Nest type	0.213	1,48	1.70	0.198
Minimal model				
Nest type	0.264	1,53	3.97	0.051*

* Marginally significant effect

Wing length was negatively associated with nest bacterial loads but not with skin bacterial loads (Table 5, Fig. 3). No associations with nest or skin bacterial loads were found for nestling tarsus length or body mass (Table 5).

Table 5. Mixed model analyses of effects of belly skin and nest bacterial loads, brood size, hatching date and nest type, with nest as random factor, on nestling linear measurements and mass of swabbed nestlings. Minimal models are obtained from full models by successive backward deletion of variables when the variance explained does not significantly improve the model ($\alpha = 0.05$).

	Coefficient	df	F	P
Tarsus length				
Full model				
Hatching date	0.009	1,34	0.13	0.724
Brood size	0.139	1,34	2.84	0.101
Skin bacterial load	-0.076	1,34	4.70	0.037
Nest bacterial load	-0.038	1,34	0.49	0.488
Nest type	-0.064	1,34	0.19	0.665
Body-mass				
Full model				
Hatching date	-0.060	1,35	2.01	0.164
Brood size	0.123	1,35	0.90	0.348
Skin bacterial load	-0.095	1,35	2.82	0.102
Nest bacterial load	0.036	1,35	0.18	0.672
Nest type	0.132	1,35	0.32	0.573
Wing length				
Full model				
Hatching date	0.005	1,35	0.00	0.971
Brood size	0.686	1,35	2.40	0.130
Skin bacterial load	-0.188	1,35	0.59	0.448
Nest bacterial load	-0.483	1,35	2.82	0.102
Nest type	-0.164	1,35	0.04	0.838
Minimal model				
Nest bacterial load	-0.578	1,42	5.70	0.021

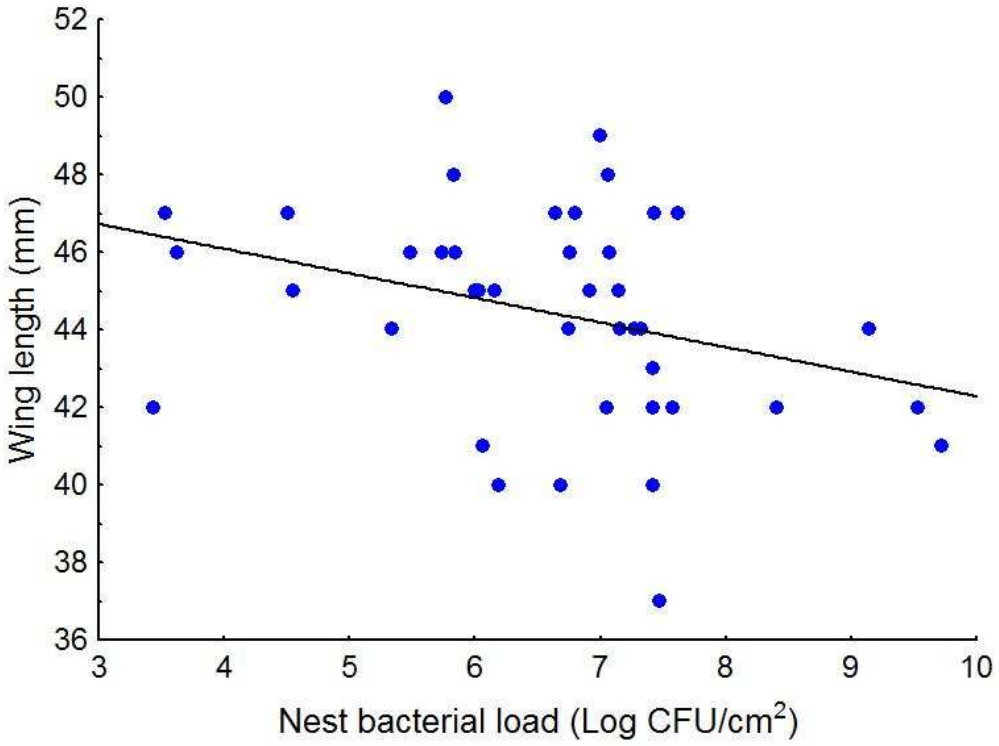

Figure 3. Correlation between nest bacterial load and wing length of nestlings at 13 days of age. A randomly selected single nestling per nest has been presented (r = -0.321, P = 0.037, n = 40).

Discussion

We have found that nestlings raised in reused nests had higher bacterial loads on their belly skin, while this was not the case for sampled nest-cups and for inert control objects. The growth of nestlings with respect to tarsus length and mass was not affected by being raised in reused nests. However, there was a marginally significant negative effect of nest reuse on wing length before fledging. Moreover and as predicted, the bacterial loads of nests but not of skin were negatively associated with feather growth of nestlings as expressed through wing length but not with skeletal or mass growth. These results indicate an association between nest reuse and bacterial load on nestling skin not hitherto detected. It also points out a possible impairment of flight capacity at fledging mediated by nest bacterial communities which are in direct contact with nestling skin

and growing feathers. It should be emphasized that bacteria were not determined to genus level.

Avian nests are colonized by bacterial communities as judged from the scant information available (Mehmke *et al.* 1992, Singleton & Harper 1998, Goodenough & Stallwood 2010). Cavity nests offer suitable conditions for growing bacterial communities given their stable microclimatic conditions. One consequence of a restricted availability of nesting cavities is that hole-nesting birds may be forced to reuse previously used nests. However, the tendency to reuse cavities could be influenced by the ability to excavate, the ability to compete for nest-sites or even how many years have passed since the hole was excavated (Mazgasjki 2007c, Bai & Mühlenberg 2008). The common practice of cleaning nest-boxes after uses every year removes the possible implications of nest reuse for arthropod communities (Møller 1989, Pacejka & Thompson 1996, Mazgajski 2007b). Thus, old nests may harbour larger populations of some ectoparasites (Rendell & Verbeek 1996a, Rytkonen *et al.* 1998, Mazgajski 2007b). There is conflicting evidence concerning the effects of nest reuse on nestling growth, health and mortality. While some studies have found negative effects of nest reuse on nestling fitness and reproductive success (Oppigler *et al.* 1994, Tomás *et al.* 2007, García-Navas *et al.* 2008), others have found no effects or even positive effects (Rendell & Verbeek 1996b, Allander 1998, Blem *et al.* 1999, Mazgajski 2007a, 2007b). The focus of these studies of cavity nesters has been on ectoparasites and their higher or lower attraction to old nest material. Some studies report preferences of pied flycatchers for nest-boxes with old nests (Orell *et al.* 1993, Mappes *et al.* 1994, Olsson & Allander 1995) while such a preference has not been detected in Spanish populations (Merino & Potti 1995). This difference could be related to climatically-induced differences in bacterial loads between populations. We have detected a weak

marginally significant negative effect of nest reuse on nestling wing length. Nestling wing length is the only biometrical variable measured in our study that is related to nest bacterial loads, which suggests that this result may be related to bacterial effects on feather growth. In the nests here studied, the numbers of different ectoparasites (blowflies, fleas and mites) were not associated with biometric variables of nestlings (López-Arrabé *et al*. 2012), so the detected effects of nest type on wing length should not be due to ectoparasite loads.

Despite the interest in effects of nest reuse on reproductive success in cavity nesting birds (Tomás *et al.* 2007; Wiebe *et al.* 2007; Garcia-Navas *et al.* 2008), their implications for nest bacterial communities have not been hitherto explored. We have been unable to show any effect of nest reuse on bacterial loads of samples obtained from nest material or a plastic control objects for unknown reasons. In the case of nest material, bacterial loads in new nests have had more time to build up than on control objects or nestling skin thereby potentially diffusing any initial differences with old nest material. Thus, the density of attached bacteria on feathers of female great tits increased between nest initiation and nest completion suggesting a regular build-up of nest bacterial communities during nest construction (Kilgas *et al.* 2012). However, there was an effect of nest reuse on bacterial loads of nestling belly skin at the end of the nestling period. This effect has not been reported earlier and may constitute a further cost of nest reuse in cavity nesters. Inert control objects showed a strikingly parallel trend in bacterial loads to nestling skin, although levels were lower and the effect of nest reuse was not significant. This indicates that bacteria colonize nestling skin in preference to inert objects and that a larger sample of nests could have increased the significance of trends. We may conclude that reused nests do in fact have higher loads of bacteria

capable of colonizing nestling skin which our measures of nest and control object bacterial loads have been unable to detect.

Bacteria present on nest materials may colonize the skin of growing nestlings soon after hatching as they apparently colonize the plumage of attending adults (Saag *et al.* 2011). Bacteria on nestling skin may affect especially the growth of tegumentary structures like feathers, while they may not be able to interact with the growth of internal organs and tissues. It is well known that feathers are consumed by keratinolytic bacteria like *Bacillus* (Burtt & Ichida 1999, Goldstein *et al.* 2004, Shawkey *et al.* 2009, Ruiz-de-Castañeda *et al.* 2012), one of the bacterial genera found in nests (Singleton & Harper 1998) and on nestling skin (Berger *et al.* 2003). Our results support a role for impairment of feather growth by nest bacteria, although not by bacteria on belly skin. *Bacillus* and other feather consuming bacteria may constitute only one component of skin bacterial communities not detected in our skin samples. Moreover, there may be differences between bacterial communities on belly skin and on wing skin and feather sheaths. It is also possible that bacteria on nest materials may be in direct contact with wing skin and growing feathers without having to colonize belly skin. How bacteria on nest materials affect feather growth remains to be elucidated. In any case, impairment of wing feather growth may have implications for flight ability at fledging.

To conclude, our results are the first to show that nest reuse may stimulate bacterial growth on nestling skin. It may also depress feather growth on nestlings. Furthermore, bacteria on nest materials may impair feather growth through unknown mechanisms. Future studies should address other determinants and implications of bacterial colonization of nestling skin and feathers.

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V

Bacterias sobre la piel de los pollos en relación con el crecimiento en el papamoscas cerrojillo

Bacteria on nestling skin in relation to growth in pied flycatchers

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Resumen. La piel de las aves puede albergar una compleja comunidad de bacterias desde edades tempranas, como la etapa de estancia en el nido. La existencia de asociaciones patogénicas o mutualistas entre las comunidades de bacterias de la piel y pollos nidícolas ha recibido hasta ahora escasa atención. En este estudio estimamos la abundancia de bacterias heterótrofas en la piel de pollos y su asociación con el crecimiento en peso, longitud de tarso y ala en el papamoscas cerrojillo *Ficedula hypoleuca*. Con este fin, muestreamos 40 pollos de 20 nidadas en una población reproductora en el centro de España. Los pollos fueron pesados y medidos a los 7 y 13 días después de la eclosión, edades a las cuales también muestreamos mediante hisopo un área delimitada de la piel desnuda ventral de los pollos además del mismo modo muestreamos un objeto inerte control de la misma superficie que el área de piel muestreada. Las cargas bacterianas de los pollos en el día 7 no estuvieron correlacionadas con ninguna de sus medidas, mientras a los 13 días estuvieron positivamente asociadas con el tamaño de nidada y con la longitud alar. Nidadas más grandes se desenvuelven en condiciones menos higiénicas en el nido, especialmente durante sus últimos días de estancia en el mismo, lo que podría explicar la asociación positiva entre tamaño de nidada y carga bacteriana. Las bacterias de la piel pueden favorecer el crecimiento de las plumas del ala al competir con bacterias degradadoras del plumaje, o un rápido crecimiento de las plumas podría facilitar el crecimiento bacteriano por la acumulación de restos orgánicos sobre la piel que rodea a las plumas en crecimiento. Más estudios son necesarios para comprobar dichas posibilidades.

Abstract. Avian skin may harbour a complex community of bacteria since early ages, such as the nestling stage. The existence of pathogenic or mutualistic associations between skin bacterial communities and nestlings has hitherto received scant attention. We estimated the abundance of heterotrophic bacteria on skin of nestlings and their association with growth in body mass, tarsus and wing length in the pied flycatcher *Ficedula hypoleuca*. To that end we sampled 40 nestlings of 20 broods of in a population breeding in central Spain. Nestlings were weighed and measured 7 and 13 days after hatching, at which ages we also swabbed a delimited area of the naked skin of the belly and in the same manner an inert control object of the same area inserted in the nest material. Bacterial loads of nestlings at 7 days were not correlated with any nestling measurement, while on day 13 they were positively associated with brood size and with nestling wing length. Larger broods develop in less hygienic conditions especially shortly before fledging, which could explain the positive association of brood size with bacterial loads at this age. Skin bacteria may favour wing feather growth through competition with harmful bacteria, or faster feather growth may facilitate bacterial growth through accumulation of remains on skin surrounding growing feathers. Further studies are needed to test these possibilities.

Introduction

Bacteria are well-known causes of disease and mortality, while pathogenic bacteria have also been isolated from birds in the wild (Lombardo *et al.* 1996, Nuttal 1997, Brittingham *et al.* 1998, Mills *et al.* 1999, Lombardo & Thorpe 2000, Westneat & Rambo 2000). Thus, bacterial infections have the potential to be important selective forces in the evolution of many aspects of avian biology (Benskin *et al.* 2009, Rachjard 2010, Soler *et al.* 2010). Research on bird-bacteria interactions have focussed mainly on eggs and feathers. Thus, it has been shown that bacteria have the potential to affect egg hatchability (Cook *et al.* 2005a, Wang *et al.* 2011, Soler *et al.* 2012). It is also well known that avian plumage harbours a complex community of bacteria, several of which are capable of degrading feather keratin (Burtt & Ichida 1999, Lucas *et al.* 2003, Shawkey *et al.* 2003, Ruiz-de-Castañeda *et al.* 2012). Much less is known about the role of bacterial assemblages on bare skin. Bacteria on vertebrate skin constitute a case of close coexistence that is expressed by preferential colonization of certain microbial taxa (Bandyopadhyay & Bhattacharyya 1996, D'Aloia *et al.* 1996, Berger *et al.* 2003). The effects of these symbiotic bacteria can go from mutualistic to pathogenic (Messier *et al.* 1993, Coles 1997, Clark *et al.* 2010).

Nestling development has frequently been treated without consideration of microbial effects (O'Connor 1984, Starck & Ricklefs 1998). However, some studies have found associations of certain gut bacteria with nestling growth (Potti *et al.* 2002, Moreno *et al.* 2003, Lucas & Heeb 2005, González-Braojos *et al.* 2012a). The effects of skin bacteria have not been considered although nest materials in close contact with the bare skin of nestlings could favour microbial colonization. Nest materials may be an important source of bacterial colonization of nestling skin given their rich microbial

communities (Goodenough & Stallwood 2010). Thus, potentially pathogenic bacteria may colonize the bare skin of growing altricial nestlings and could thereby affect their growth and development by affecting thermoregulation, invading other tissues or degrading growing feathers (Burtt & Ichida 1999, Clayton 1999, Muza *et al.* 2000). Berger *et al.* (2003) sampled bacteria of the naked belly skin of nestlings and found that bacterial abundance did not affect growth. Their conclusion was that a high skin bacterial load may not necessarily be harmful. However, Berger *et al.* (2003) did not show an increase in bacterial colonization of nestling skin above the level expected from passive colonization of inert objects. We should expect that symbiotic skin bacteria should show higher loads than those colonizing inert objects placed in the nest. Moreover, bacterial loads on nestling skin should not correlate with loads on inert objects placed in the nest if microbial associations with nestlings depend on elements other than passive colonization like interactions with the nestling immune system, associations with nestling condition or competitive interactions between bacterial strains on nestling skin.

It is also unknown if bacterial loads on skin increase throughout the nestling period or remain constant after initial colonization. In temperate areas, ambient temperature tends to increase in the course of the breeding season and temperature has positive effects on bacterial growth on eggs (Ruiz-de-Castañeda *et al.* 2011a, 2011b) and in nestling guts (González-Braojos *et al.* 2012b). Thus, we could expect positive effects of hatching date on skin bacterial loads if bacterial colonization of skin is strictly ambient-dependent. Alternatively, late-breeding parents are usually of lower parental quality which could affect bacterial colonization through nest hygiene (see below).

González-Braojos *et al.* (2012c) in a study of the reuse of old nests by cavity-nesting birds, found that nestlings raised in old nests had higher bacterial loads on their

belly skin than those raised in freshly built nests. The results concerning old nests raise the possibility that nest hygiene could have effects on skin bacterial loads. Nest hygiene may be affected by the ability of parents to remove detritus and faecal materials, which in turn may be related to brood food demand and brood size. Moreover, they found that bacterial loads of nests showed a negative association with nestling feather growth. The association with feather growth suggests that feather-degrading bacteria (FDB) (Burtt & Ichida 1999) may be able to colonize growing feathers already during the nestling stage. We could thus expect a deleterious effect of bacterial abundance on feather growth in the nest. On the other hand, bacteria that are not involved in feather degradation could compete with FDB and, if they themselves are not pathogenic, have a positive effect on nestling feather growth. Such competitive effects among bacteria have been detected in studies of egg hatchability (Ruiz-Rodríguez *et al.* 2009, Peralta-Sánchez *et al.* 2010, Soler *et al.* 2010) and nestling growth (Moreno *et al.* 2003). On the other hand, FDB affect feather degradation rather than feather growth, while feather degradation may not affect wing length, unless it implies feather breakage. Nests with nestlings with more developed wing feathers would also contain greater amounts of keratin, fat and other tissues derived from encircling developing feathers. These materials would favour the growth of keratinolitic and other bacteria that may access nestling belly skin. According to this possibility, nestlings with more developed feathers would also contain more bacteria on their skin.

The purpose of this study was to explore the possible changes in abundances of heterotrophic bacteria on skin and the potential associations of skin bacteria with nestling growth in altricial birds. To that end, we have conducted a study on pied flycatchers *Ficedula hypoleuca*, in which we have estimated heterotrophic bacterial loads on nestling skin and on inert control objects at the ages of 7 and 13 days. We have

measured mass, tarsus and wing length as expressions of muscle and organ growth, skeletal growth and feather growth, respectively. We have hypothesized that:

(1) Colonization of nestling skin may occur during the first days after hatching or gradually throughout the nestling period which would result in either no changes in skin bacterial loads from day 7 to day 13 or an increase between these ages.

(2) Given that microbial colonization may be temperature-dependent and/or that late breeders are of lower quality, bacterial loads on skin should increase with hatching date if there is a seasonal increase in temperature.

(3) Bacterial loads should be positively related to brood size if parents of large broods have reduced capacity to clean nests by removing faecal sacs or because these nests are warmer when chicks maintain high body temperatures.

(4) If bacteria on skin affect thermoregulation or are able to invade other tissues, skin bacterial loads should be negatively associated with general nestling growth.

(5) Feather development could be related to skin bacterial loads if bacteria on skin affect feather growth.

Methods

We conducted the study during the spring of 2011 on a population of pied flycatchers breeding in artificial nest-boxes in a montane forest of Pyrenean oak, *Quercus pyrenaica*, at 1200 m.a.s.l. in Valsaín, central Spain (40˚ 54’ N, 4˚ 01’ W). The pied flycatcher is a small hole-nesting passerine of European woodlands (Lundberg & Alatalo 1992). Egg laying in the population under study typically begins in late May, and modal clutch size is six. The female incubates alone and receives part of her food from her mate (Moreno *et al.* 2011). Young are brooded by the female only up to day 8

(hatching day = day 1). Both sexes feed the young. Nests were followed and hatching dates estimated through daily visits.

In the study area, there are 252 wooden nest-boxes which are occupied by pied flycatchers, great tits and blue tits (see appendix in Lambrechts *et al.* 2010 for dimensions, structure and placement). As an inert control available for bacterial colonization of objects in nests we have used a plastic sheet of the same area as that sampled on nestlings. On the day when nestlings hatched, we introduced into the nest material near the nest-cup two squares of 1.5 cm^2 (total area of both sides = 3 cm^2) cut out of plastic sleeves with a rough surface, which was previously washed with alcohol. One control object was sampled at each sampling of nestlings (7 and 13 days of age).

On day 7 (hatching day = day 1), we ringed nestlings and measured their tarsus length with a digital calliper to the nearest 0.01 mm and their wing length with a stopped ruler to the nearest mm. Nestling were also weighed with a Pesola spring balance to the nearest 0.25 g. On day 13, nestlings were again measured in the same way.

Bacterial sampling

At age 7 days, we sampled two randomly selected nestlings per nest and one inert control object in 20 broods. Nestlings were not introduced in a bag before sampling to avoid contamination. Wearing sterile rubber gloves, one of us (SG-B) covered the unfeathered ventral side of nestlings (henceforth called belly) with a sterile rigid plastic sheet where a rectangle of 3 cm^2 had been cut out, so the naked skin of the belly could only be swabbed through this surface. Feather patches on the belly were avoided when covering the belly with the sheet (Fig 1a). We passed the delimited belly area during 30 s with a sterile cotton swab previously moistened with sterile phosphate buffered saline (pH = 7.2, Química Clínica Aplicada, Tarragona, Spain). The belly swabs were then

transferred into transport media Amies (*Sterile R, Meus s.r.l.,* Piove di Sacco, Italy). One of the control objects was collected from material with a sterile forceps (Fig. 1b). The control object was swabbed on both sides during 30 s in the same manner as for nestling bellies. All samples were transported in a portable cooler until their processing in the laboratory (3-6 h. after collection). On day 13, we repeated bacterial sampling on the same nestlings and we collected the other control object with a sterile forceps. A total of 8 control objects were lost (3 on day 7 and 5 on day 13). One nestling was found dead in each of two nests.

Figure 1. Pictures shows: (a) sampling of unfeathered belly of nestling through a section not covered by a rigid plastic sheet (feathers were separated before swabbing the skin) and, (b) inert control object inserted into the nest material near the nest-cup.

Laboratory work

Once in the laboratory, we transferred the swabs to tubes containing 1 ml of freezing medium (20 g skim milk (Difco, Laboratories, Detroit, MI, USA), 30g Tryptone (Pronadisa, CondaLab, Torrejón de Ardoz (Madrid), Spain), 8 ml Glycerol (Panreac Química, s. l., Castellar del Vallés, Barcelona, Spain) and 1000 ml distilled water. These tubes were frozen at -80ºC until processed less than one month later. The samples were cultured by plating out 100 µl of the dilutions 10^0 and 10^{-1} on Tryptic Soy Agar

(TSA, Scharlau, Barcelona, Spain). Plates were incubated for 48 ± 2 h at 25 ± 1˚C, and colonies were then counted using a colony counter "sensor" (Suntex Instruments Co. Ltd., Taipei County, Taiwan) by the same observer (SG-B). Bacterial loads are expressed as density of colony forming units (CFU/ cm^2). TSA is a general medium to estimate abundances of aerobic cultivable bacteria (Mills *et al.* 1999, Cook *et al.* 2003, Cook *et al.* 2005b, Soler *et al.* 2008, Møller *et al.* 2009, Shawkey *et al.* 2009, Goodenough & Stallwood 2012).

Statistical analyses

All variables were normally distributed or successfully normalized through natural logarithms prior to analyses. Analyses were conducted either with IBM SPSS Statistics 21 (2012) (Table 1-3) or Statistica (Statsoft) (Table 4). For testing hypotheses 1-3 for which bacterial loads are the dependent variable, we have used a mixed linear model with repeated-measures by age with bacterial skin loads and bacteria of control object as dependent variable, nest ID as random factor, surface (nestling-plastic) as fixed factor and brood size and hatching date as covariables to test whether bacterial abundance changed with age, the effect of different surface (nestling vs plastic) and the effect of breeding parameters. For testing hypotheses 4-5 for which nestling biometry variables are the dependent variable, we have used mixed linear models to test for associations of biometrical variables of swabbed nestlings with skin bacterial loads at different ages while controlling for hatching date and brood size, including nest ID as random factor. For growth, we have used the Variance components module in Statistica. Non-significant variables were sequentially removed until obtaining a final model including only significant effects.

Results

Bacterial loads on skin were significantly higher than those on control objects (Day 7: skin bacterial loads (mean ± SE): 3.652 ± 0.258 and control object (mean ± SE): 2.747 ± 0.567; Day 13: skin bacterial loads (mean ± SE): 3.425 ± 0.272 and control object (mean ± SE): 1.449 ± 0.327; Table 1). Bacterial loads did not change with nestling age either on skin or on control objects (Table 1). Bacterial loads were positively correlated with brood size (Table 1, figure 2), but this significance was due only to differences at age 13 (R^2 = 0.006 for nestling and R^2 = 0.008 for object control on day 7, R^2= 0.300 for nestling on day 13 and R^2= 0.133 for object control on day 13). Bacterial loads were not correlated with hatching date (Table 1).

	df	*F*	*P*
Bacterial loads			
Age	1,81	1.173	0.282
Surface (nestling-plastic)	1,66	12.000	**0.001**
Brood size	1,15	7.000	**0.013**
Hatching date	1,15	3.000	0.067

Table 1. Mixed linear model with repeated measures (age) of associations of surface, hatching date, brood size and nest as random factor with bacterial loads of skin and control object.

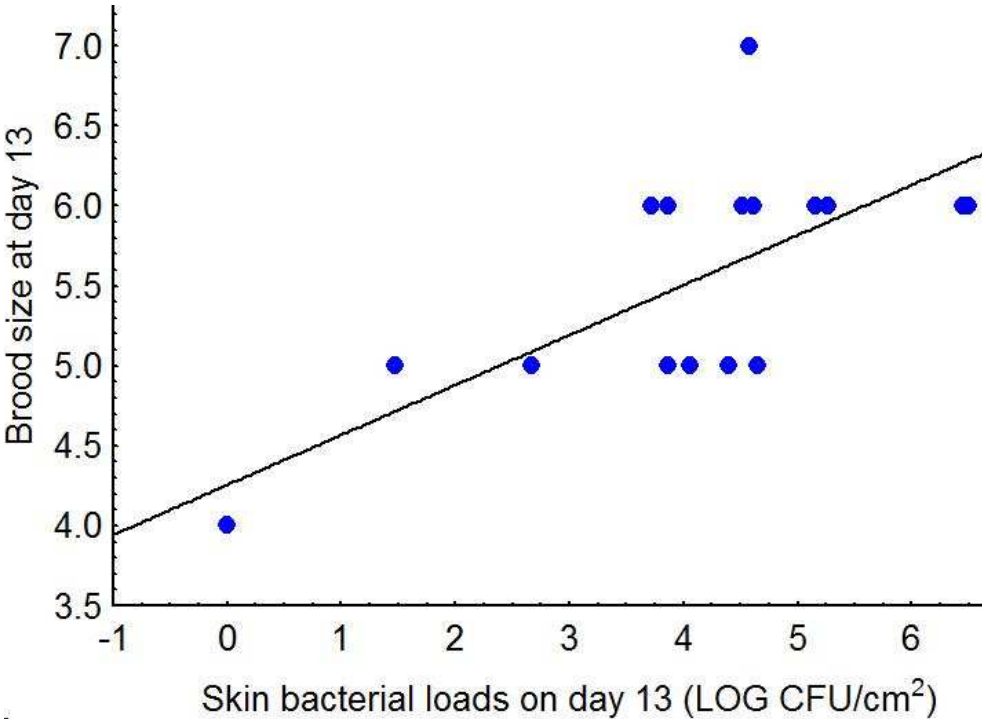

Figure 2. Association between skin bacterial loads on day 13 with brood size at the same age.

Table 2. Mixed linear models of associations of bacterial skin loads, hatching date, brood size and nest as random factor with nestling linear measurements and mass on day 7.

	Coefficient	*Df*	*F*	*p*
Tarsus length				
Full model				
Hatching date	0.042	1,12.478	0.691	0.421
Brood size	0.450	1,12.664	3.853	0.072
Skin bacterial loads	0.018	1,27.355	0.049	0.827
Body-mass				
Full model				
Hatching date	0.064	1,15.907	1.014	0.329
Brood size	0.299	1,15.442	1.421	0.251
Skin bacterial loads	-0.015	1,32.606	0.023	0.881
Wing length				
Full model				
Hatching date	0.095	1,15.528	0.284	0.602
Brood size	0.896	1,15.056	1.622	0.222
Skin bacterial loads	0.114	1,33.988	0.190	0.665

No biometrical variable on day 7 was significantly associated with hatching date, brood size or skin bacterial loads (Table 2). However, wing length on day 13 showed a significant positive association with skin bacterial loads at that age, while mass and tarsus length showed no association with skin bacterial loads (Table 3, Fig. 3). For growth, no variable included in the analyses was significant (Table 4).

Table 3. Mixed linear models of effects of bacterial skin loads, hatching date and brood size with nest as random factor on nestling linear measurements and mass on day 13. Minimal models are obtained by sequentially removing the most non-significant effects until a model with only significant effects is obtained.

	Coefficient	*df*	*F*	*p*
Tarsus length				
Full model				
Hatching date	-0.064	1,16.530	1.040	0.323
Brood size	0.336	1,17.125	1.759	0.202
Skin bacterial loads on day 13	-0.059	1,17.644	1.681	0.211
Body-mass				
Full model				
Hatching date	0.090	1,17.822	1.314	0.267
Brood size	0.457	1,19.235	1.996	0.174
Skin bacterial loads on day 13	-0.026	1,25.780	0.067	0.797
Wing length				
Full model				
Hatching date	0.012	1,15.119	0.003	0.954
Brood size	0.666	1,16.673	0.552	0.468
Skin bacterial loads on day 13	0.209	1,26.501	0.457	0.505
Minimal model				
Skin bacterial loads on day 13	0.554	1, 34.496	4.394	**0.043**

Table 4. Variance components analyses of associations of skin bacterial loads, hatching date, brood size and nest as random factor with increases in nestling linear measurements and mass between days 7 and day 13 using the Satterthwaite correction for estimating degrees of freedom. Minimal models are obtained by sequentially removing the most non-significant effects until a model with only significant effects is obtained.

	Df	*F*	*p*
Tarsus length growth			
Full model			
Hatching date	1,12.73	1.084	0.317
Brood size	1,12.83	0.933	0.351
Skin bacterial loads on day 7	1,16.67	1.964	0.179
Skin bacterial loads on day 13	1,15.70	0.610	0.446
Nest	14,12	3.984	**0.010**
Minimal model			
Nest	17,18	6.290	**<0.001**
Body-mass growth			
Full model			
Hatching date	1,13.43	0.688	0.421
Brood size	1,13.68	0.343	0.567
Skin bacterial loads on day 7	1,20.86	3.001	0.097
Skin bacterial loads on day 13	1,19.28	0.665	0.424
Nest	16,14	2.006	0.098
Wing length growth			
Full model			
Hatching date	1,13.85	0.320	0.580
Brood size	1,14.06	0.024	0.877
Skin bacterial loads on day 7	1,20.11	0.477	0.497
Skin bacterial loads on day 13	1,18.75	0.998	0.330
Nest	16,14	2.398	0.053
Minimal model			
Nest	19,20	2.713	**0.015**

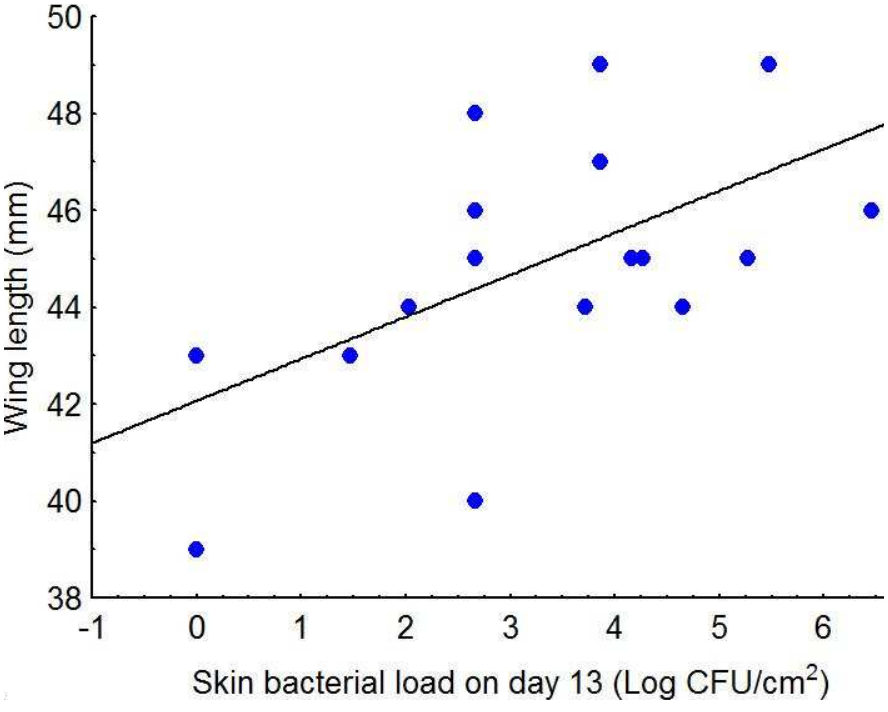

Figure 3. Association between skin bacterial loads on day 13 with wing length at the same age. We have included only a randomly selected nestling per nest to avoid a possible pseudoreplication.

Discussion

We have shown that skin bacterial loads are due to symbiotic relationships between bacteria and nestlings and not due to mere passive colonization. We have established that colonization of nestling skin occurs already during the first week of nestling life. The absence of an association of bacterial loads with hatching date suggests that seasonal thermal effects are not important for bacterial growth on skin. We have supported the possibility that larger broods imply improved bacterial growth possibly through restricted parentally mediated nest hygiene at late nestling ages. We have not confirmed any effect of skin bacteria on general nestling growth. Finally, we have found a positive association of skin bacteria with primary feather length. We will discuss these results in turn.

Bacterial loads on nestling skin were higher than on control objects. This may be due to bacteria finding skin more favourable than inert plastic objects due to more stable thermal conditions on skin. Brooding and thermoregulation may maintain optimal conditions for bacterial growth on skin (Zwietering *et al.*1991). Moreover, bacteria will obtain more nutrients on skin compared to an inert surface. Our data thus support that bacteria colonize nestling skin over and above the level expected from mere occupation of a bare surface inserted in the nest.

We did not find any significant change on skin bacterial loads between different ages probably because on day 7 the bacterial community was already established. Bacteria from the nest material, the environment or even parents may colonize the skin of nestlings rapidly in their first few days of life, and subsequent changes may be more qualitative than quantitative. González-Braojos *et al.* (2012a) found that loads of certain types of gut bacteria changed in their abundances between 7 and 13 days. However, gut

bacteria are influenced by age-dependent changes in nestling diet and in the amount of food processed. On the other hand, colonization of skin may occur rapidly after hatching given the continuous contact of nestlings with each other, the nest material and brooding adults. Unfortunately we have not been able to study changes in terms of bacterial taxon diversity and relative abundance. It would be good for future studies to sample skin bacteria at earlier ages to observe the gradual acquisition of the microbiota and molecularly analyze microbial diversity.

We didn't find any association between bacterial loads of skin and hatching date, although there is a positive correlation between hatching date and temperature for this year ($F_{1,17}$ = 24.983, p < 0.001, R^2 adjusted = 0.595). Berger *et al.* (2003) found that numbers of bacteria on belly skin at 14 days increased as the breeding season of Starlings progressed. Our sampling period may have been too short to detect such changes as only 8 days elapsed between collecting of the first and last samples at each age as compared to one month for the other study. However, brooding by females and subsequent development of thermoregulation in nestlings may preclude the operation of changes induced by ambient temperature. Moreover, the lower parental quality of late breeders may no affect bacterial colonization of nestling skin.

The result that large broods showed higher bacterial loads on day 13 may be explained by the higher parental provisioning intensity at the end of the nestling period, which may preclude efficient nest sanitation. Therefore faecal sacs containing intestinal bacteria would accumulate in nests with larger broods allowing these bacteria to colonize the skin of nestlings. In 2009 we measured the number of faecal sacs in nest-boxes (nest-cup and nest-box walls) in the same population of pied flycatchers when we measured nestlings at 13 days, obtaining that nest-boxes with larger broods contained more faecal sacs (R^2 *adjusted* = 0.093; $F_{1,43}$ = 5.552, p = 0.023) providing some support

to our explanation. Deficient nest hygiene will probably contribute to bacterial colonization of nestling skin also in other populations. In addition to this explanation, this increase in terms of number of bacteria with brood size may also be affected by temperature in the nest since the greater number of nestlings could lead to thermal increases given the high body temperatures of thermoregulating grown nestlings. This could also benefit the growth of bacteria on the skin of nestlings.

Only wing length on day 13 was associated with bacterial loads of belly skin, i.e nestlings with longer wings had more bacteria when feathers are growing fast. The lack of associations of skin bacterial loads with mass or skeletal growth indicates that skin bacteria are not removing important resources for growth in these birds, a result also found by Berger *et al.* (2003). It should be noted that these authors did not look for a relationship of skin bacteria with wing length.

A possible explanation for our result concerning wing length may be related to competition between bacterial strains for space and nutritive resources offered by the skin. Some bacteria can modify the chemical environment and create a physicochemical barrier that impedes the establishment of other bacteria, using bacteriocines (Riley & Wertz 2002, Rajchard 2010). This may contribute to exclude bacteria harmful for the host in some host-bacteria associations (e.g. Martín-Platero *et al.* 2006, Soler *et al.* 2008, Ruiz-Rodríguez *et al.* 2009). As we have not studied the bacterial diversity on skin, we cannot support this possibility. On the other hand, FDB affect feather degradation rather than feather growth, while feather degradation may not affect wing length, unless it implies feather breakage. Nests with nestlings with more developed wing feathers would also contain greater amounts of keratin, fat and other tissues derived from encircling developing feathers. These materials would favour the growth of keratinolitic and other bacteria that may access nestling belly skin. According to this

possibility, nestlings with more developed feathers would also contain more bacteria on their skin. We cannot at present test any of these explanations for the positive association of feather development with skin bacterial loads.

Summarising, this is the first study sampling naked belly skin of altricial nestlings at two different ages (7 and 13 days) and showing the potential effects of nest hygiene and the positive association of skin bacteria with nestling wing growth. More studies are needed to clarify the patterns revealed in the present study.

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VI

No existen asociaciones entre medidas de inmunidad en pollos de papamoscas cerrojillo (*Ficedula hypoleuca*)

No association between measures of immunity in nestling pied flycatchers (*Ficedula hypoleuca*)

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*Resumen***.-** Los costes inmunológicos se cree que juegan un papel importante en la evolución de la historia de vida, pero muchos estudios ecológicos de la inmunología han considerado sólo los aspectos individuales de la función inmune. Los pollos altriciales dependen principalmente de la inmunidad innata y anticuerpos maternales para la defensa inmune. Hemos llevado a cabo un estudio en pollos de papamoscas cerrojillo *Ficedula hypoleuca* en el que se midió los niveles de anticuerpos naturales (NAb) y hemólisis, se estimó los niveles de inmunoglobulinas en suero y finalmente se obtuvó una medida específica de la inflamación después de la inoculación de un antígeno, fitohemaglutinina (PHA). Por lo tanto se obtuvieron estimaciones independientes para explorar las relaciones entre los diferentes brazos del sistema inmune. No se encontró asociaciones entre las variables medidas tanto a nivel individual como a nivel de nidada. Esto indica que los diferentes aspectos de la inmunidad son independientes y difíciles de integrar en una medida general de la capacidad de respuesta inmune en los pollos altriciales. Se encontró que sólo NAbs se asoció negativamente con la longitud del tarso a la edad de 7 días. Por lo tanto, la evidencia de los recursos basados en los compromisos entre el crecimiento de los pollos y la inmunidad es débil en nuestra población de estudio.

Abstract.- Costs of immunity are widely believed to play an important role in life history evolution, but many studies of ecological immunology have considered only single aspects of immune function. Young of altricial birds are dependent primarily on innate immunity and maternally derived antibodies for immune defense. We conducted a study on nestling pied flycatchers *Ficedula hypoleuca* in which we measured levels of natural antibodies (NAbs) and hemolysis, estimated serum immunoglobulin levels and obtained a specific measure of inflammation after inoculation of an antigen, Phytohaemagglutinin (PHA). Thus we obtained independent estimates to explore the relationships among different arms of the immune system. We found that no immune variable measured was associated with any other variable at the individual and brood levels. This indicates that different aspects of immunity are independent and difficult to integrate in a general measure of immune response capacity in altricial nestling birds. We found that only NAbs was negatively associated with tarsus length at the age of 7 days. Thus, the evidence for resource-based trade-offs between nestling growth and immunity is weak in our study population.

Introduction

The immune system of an organism comprises its main defence mechanism against pathogens (Zuk & Stoehr 2002, Davison *et al*. 2008). Besides the obvious benefits in terms of animal health and survival (Hõrak *et al*. 1999, Christe *et al*. 2001, Ardia *et al*. 2003, Hanssen *et al*. 2004), immune responses also convey costs, including a higher risk of autoimmune diseases (Råberg *et al*. 1998) and the depletion of energy or other resources that could be used for other organismic functions (Sheldon & Verhulst 1996, Martin II *et al*. 2003). Individual immune responses have also been shown to vary with environmental conditions during development or reproduction (Morales *et al*. 2004).

The immune system is highly complex and is generally classified into two main components: innate and acquired immunity (Janeway & Travers 1996). Innate immunity is particularly important during the initial stages and is the primary means of controlling bacterial infections (Roitt *et al*. 1998), which includes factors present in the blood before antigenic exposure as well as natural antibodies produced by B cells of the adaptive branch (Forsman *et al*. 2010). Acquired immunity is usually classified into humoral and cell-mediated responses (Roitt *et al*. 1998). The humoral response, which includes B cells, acts against extracellular pathogens while the cell-mediated response mainly attacks intracellular pathogens (Roitt *et al*. 1998).

Immunocompetence is sometimes defined as "the magnitude and effectiveness of an animal's immune response" (Adamo 2004), assuming that a greater magnitude of an immune response is better for anti-parasite defence, although a maximum immune response is not necessarily optimal (Viney *et al*. 2005). Vinkler and Albrecht (2011) propose to use a more rigorous terminology, defining it as the ability to produce anti-parasite or anti-antigen immune responses. There is thus some controversy concerning

the term “immunocompetence”, although it is meant to summarize the effectiveness of one or more components of the immune system against potential parasite infections. However, studies in natural populations frequently evaluate it with a single immune assay, while the reliability of one component of immunity to measure the overall strength and efficacy of the immune system has been questioned (Norris & Evans 2000, Adamo 2004, Matson *et al*. 2006). In the literature, the correlations between the diverse indices of immunity measured have shown different results at the individual, family or species level (Matson *et al*. 2006, Mendes *et al*. 2006, Forsman *et al*. 2008). Thus some authors have found negative correlations among components of immunity (González *et al*. 1999, Møller & Petrie 2002, Buchanan *et al*. 2003), which have been interpreted as based on energetic trade-offs within the immune system. However, other studies have found positive associations between components of immunity, which have been interpreted as indicating a general ability to fight disease and pathogens (Møller *et al*. 2001, Morales *et al*. 2004, Ardia 2007, Arriero 2009). Furthermore, studies reporting no significant association between components of immunity (Matson *et al*. 2006, Mendes *et al*. 2006, Roulin *et al*. 2007) claim that different immunological responses are triggered by different types of challenges, and may therefore be regulated independently or be under divergent selection and show no association. Many of these studies concern altricial nestlings (Ilmonen *et al*. 2003, Morales *et al*. 2004, Forsman *et al*. 2008, Arriero 2009), for which differences in the rate of development of different arms may preclude any association between measures of immunity and thereby prevent the establishment of a single measure of immunocompetence.

Newly hatched avian nestlings are dependent on innate immunity and maternal immunoglobulins (Apanius 1998, Klasing & Leshchinsky 1999, Pihlaja *et al*. 2006). Adaptive defenses, on the other hand, are poorly developed in nestlings and take more

time to become fully functional. Differential rates of development of these main arms of the immune system are likely explained by the different costs and processes involved in the ontogeny of each component (Klasing & Leshchinsky 1999, Palacios *et al*. 2009). A trade-off between different components of the immune system during nestling growth would be predicted if the development and maintenance of different aspects of immunity implies different costs and compete for resources with other physiological activities (Deerenberg *et al*. 1997, Norris & Evans 2000). Innate immune function is especially important to altricial nestlings as their relatively short incubation periods may result in poorly developed immune systems at hatching and as their stay in the nest may result in greater exposure to parasites (Ricklefs 1992, Ardia & Schat 2008). Altricial nestlings also experience strong selective pressures to grow rapidly to fledge (O'Connor 1984). This suggests that the rate of maturation of immune defenses reflects an evolutionary trade-off with growth rate and tissue maturity required to fledge (Soler *et al*. 2003, Tschirren & Richner 2006).

In this study, we explored associations between different arms of the immune system at the nestling stage in altricial birds as exemplified by the pied flycatcher (*Ficedula hypoleuca*), a model organism for eco-immunological studies (Ilmonen *et al*. 2003, Kilpimaa *et al*. 2004, Morales *et al*. 2004, Grindstaff *et al*. 2006, Morales *et al*. 2006, Moreno *et al*. 2008). We also aimed at detecting associations between nestling growth and immune activity, although in a non-experimental setting. To these ends, we measured the activity of natural antibodies (NAbs) and of the complement cascade as components of the innate immune system. NAbs serve as recognition molecules capable of opsonizing invading microorganisms and initiating the complement enzyme cascade, which ends in cell lysis (Ochsenbein & Zinkernagel 2000). The acquired immune system was measured through the injection of the mitogen Phytohemagglutinin (PHA)

and by quantifying total Immunoglobulin (Ig) levels. These maternally derived antibodies may have blocking activity by binding to antigenic targets and thereby preventing the stimulation of the neonatal immune mechanisms (Apanius 1998, Starck & Ricklefs 1998, Klasing & Leshchinsky 1999). PHA has been used in several studies in wild birds and has been used to measure the T-cell mediated inflammation, thereby providing a good measure of immune response (Moreno *et al*. 1998, Martin *et al*. 2001, Moreno *et al*. 2001, Tella *et al*. 2002). Martin II *et al*. (2006) have confirmed that the PHA swelling response involves both innate and adaptive components of the immune system. Moreover, although some mild systemic stress could be produced, it has been suggested that PHA does not provoke potential confounding effects associated with physiological stress (Merino *et al*. 1999).

Methods

The study was conducted during the 2009 breeding season in a deciduous forest of Pyrennean oak (*Quercus pyrenaica)* at an elevation of 1.200 m. a. s. l. in Valsaín, Segovia province (40˚ 54' N, 4˚ 01' W), Spain. A study of a population of pied flycatchers breeding in nest-boxes in that area has been conducted since 1991 (Sanz *et al*. 2003). Nest-boxes are cleaned every year after the breeding season. Nest-boxes were checked daily for nest-building activity by pied flycatchers, and the dates of clutch initiation, clutch sizes, and numbers of fledged young were recorded.

The pied flycatcher is a small passerine bird, which breeds in many forested areas of the Palaearctic region (Lundberg & Alatalo 1992). It breeds naturally in tree cavities, but if nest-boxes are provided, they are preferred over natural cavities. Egg laying in the population under study typically begins in late May, and clutch sizes range

from 4 to 7 eggs with a mode of 6 eggs (mean 5.5 ± 0.6 SE). The female incubates alone and receives part of her food from her mate (Moreno *et al*. 2010). Young are brooded by the female only up to day 8 (hatching day = day 1) (Sanz & Moreno 1995). Both sexes feed the young. Young fledge within 14–16 days of hatching (Lundberg & Alatalo 1992). This occurs in the second half of June in our study area.

A sample of 78 broods of four to six chicks was used for this study. Of these nests, we sampled two chicks at random in 59 nests, one randomly sampled chick in 14 nests and finally three randomly sampled chicks in 5 nests, a total of 148 chicks. Only nestlings that produced a fecal sample to be used in another study (González-Braojos *et al*. 2012) were blood-sampled, which explains the different numbers of nestlings per nest included. Nestlings were measured and weighed at the ages of 7 and 13 days. Tarsus length was measured with a digital calliper to the nearest 0.01 mm, mass was obtained with a Pesola® spring balance (precision of 0.25 g) and wing length was measured with a stopped ruler to the nearest mm. Chicks were banded on day 7 with numbered aluminium. Blood was collected from nestlings of pied flycatchers on day 13 by puncturing the brachial vein and collecting two heparinised capillaries, blood being subsequently transferred into Eppendorf tubes and stored in a cooling box. In the lab, we centrifuged the Eppendorf tubes at 12000 rpm for 2 min during the day of collection. Plasma and cells were separated and stored at -20ºC until analyses in the lab.

Hemaggluttination-Hemolysis Assay

To estimate the levels of circulating NAbs and complement, we used the procedure developed by Matson *et al*. (2005). The agglutination part of the assay estimates the interaction between NAbs and antigens in rabbit blood, producing blood clumping. The lysis part of the assay estimates the action of complement from the amount of hemoglobin released from the lysis of rabbit erythrocytes. Quantification of

agglutination and lysis is achieved by serial dilution in polysterene 96-well assay plates, using the dilution step at which the agglutination or lysis reaction is stopped, i.e. column 3 is a score of 3 for hemolysis and column 8 is a score of 8 for agglutination (*see* Matson *et al*. 2005 for more details). Although lysis scores ranged from 1 to 3, hemolysis only occurred in 33 out of 108 responses (hemolysis is not a general phenomenon, *see* De Coster *et al*. 2010, Matson *et al*. 2005). Therefore scores of lysis were treated as a binary variable, i.e. "0" (score = 0, no lysis), or "1" (score > 0, lysis). We used fresh rabbit blood with Alsever's anticoagulant (HemoStat Laboratories, Dixon, USA), 96 round well assay plates and an HP Photosmart Essential 3.0 scanner that was set at professional mode, with document type colour film, 48 bit colour and 300 dpi. Whole rabbit blood was stored at 4ºC. After determination of the level of hematocrit, we diluted to obtain a solution of 1% of erythrocytes.

The protocol for hemolysis and hemagglutination is as follows. The plasma samples were thawed and homogenized using a vortex. Subsequently, 25 µl of plasma was pipetted into column 1 and 2 followed by the addition of 25 µl of 0.01 M phosphate buffered saline (PBS) in all wells, except column 1. The contents of the column 2 wells are serially diluted (1:2) through column 11. Well number 12 only contained the dilution of erythrocytes and PBS, thus serving as a negative control. Subsequently, 25 µl of the 1% solution of rabbit blood was added to all wells. The assay plate was then covered and shaken for 10 s followed by incubation for 90 min in a bath at 37ºC. The assay plate was then removed from the bath and left at an inclination of 45º at ambient temperature for 20 min. Plates are then scanned. Afterwards, plates were kept at room temperature for an additional 70 min and scanned for a second time to record maximum lytic activity. All tests were made blindly by S.G-B. As 40 samples were not usable due

to insufficient volume of blood plasma, we were able to measure samples of only 108 individuals.

Immunoglobulin assay

To estimate IgY levels in plasma, we used the procedure developed by Martínez *et al*. (2003). In brief: ELISA plates (Maxi-sorp, Nunc, Rochester, NY, USA) were coated with serial dilutions of serum (100μl) in carbonate–bicarbonate buffer (0.1M, pH = 9.6, overnight at 4°C) in order to determine the linear range of the sigmoid curve. Later, the plates were blocked with defatted milk diluted in PBS-Tw buffer for 1 h at 37°C (200 μl). Antichicken conjugates (Sigma A-9046, MO, USA) were added at 1/250 dilution in PBS-Tw and incubated for 2 h at 37°C (100 μl). The dilution of antichicken antibody was selected after a previous study to achieve the maximum slope in the linear range. In addition, antichicken antibodies were diluted without any protein (i.e. BSA, gelatine, defatted milk, etc.) which avoids unspecific binding. After incubation with a substrate comprising ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) and concentrated hydrogen peroxide diluted to 1/1000 for 1 h at 37°C, absorbances were measured using a plate spectrophotometer at λ= 405 nm. Under these conditions, we achieved the maximum values of absorbance. Once the linear range of the sigmoidal curve was achieved for pied flycatcher nestlings, we chose the data obtained using the serum dilution nearest to the centre of the linear range. We could use 139 samples for this assay.

Phytohaemagglutinin (PHA) injection

PHA is a plant-derived mitogen that stimulates the recruitment of leucocytes involved in both adaptive and innate immune responses at the site of injection, producing a measurable tissue swelling (Martin *et al*. 2006, Forsman *et al*. 2010). This is commonly used in evolutionary ecology to estimate T-cell-mediated immunity, although it also

reflects other components of the immune system such as major histocompatibility complex molecules (Moreno *et al*. 1999, Morales *et al*. 2006). We used the protocol without control wing proposed by Smits *et al*. (1999). Nestlings of 12 days were injected with 0.02 mg of PHA in 0.02 ml of PBS in the left wing web, after measuring the thickness at the point of injection. Three measures of thickness were taken with a digital spessimeter with constant pressure (Mitutoyo 7/547, Tokyo, Japan) to the nearest 0.01 mm. After 24 h, three new measurements of the thickness of wing webs at the point of injection were taken (repeatabilities were 0.99). The immune response was estimated as the difference between the average initial and average final measurements. All injections and measurements were made by the same person (SG-B.). Only 138 nestlings could be correctly measured (4 were missing due to failure to inject the correct amount, another 4 were not injected by mistake and for 2 we could not obtain the pre-injection measure).

Statistical analyses

IgY levels were normalized by square root transformation. PHA response and hemagglutination were normally distributed. Scores of lysis were treated as a binary variable, i.e. "0" (score = 0, no lysis), or "1" (score > 0, lysis) (De Coster *et al*. 2010).

To explore the relationships among immunological variables at the individual level, we performed mixed model ANOVAs for each variable with brood ID as random factor and the other variables as covariates. For hemolysis, we used a GLIMMIX with a binomial distribution. To examine correlations between immunological variables at brood level, we used means per brood in linear correlations.

To estimate the association between biometrical variables on days 7 and 13 and their growth between these ages and each immunological variable, we used linear mixed models using Satterthwaite's correction for estimating degrees of freedom

(Satterthwaite 1946) with SAS 9.1 (SAS Institute Inc., 2002-2003, Cary, NC, USA). Three linear mixed models were run, with one normally distributed immune variable (IgY level, PHA response, hemagglutination) as dependent and hatching date, brood size, tarsus length, mass and wing length of nestlings at 7 or 13 days of age or the difference in measures between both ages as independent variables. In total three analyses for each variable of immunity and biometric variables were conducted (7 days, 13 days and the difference in measures between 7 and 13 days). Nest was included as a random factor.

To test lysis, we used a GLIMMIX with a binomial distribution, with nest as a random factor and using Satterthwaite's correction. In this analysis, we included the same variables as in earlier analyses.

Full models will be presented plus final models obtained by a backward deletion procedure until final models with only significant effects were obtained ($\alpha = 0.05$).

Degrees of freedom in the different analyses are not the same given the different numbers of samples available for the various immunity measures (see above).

Results

The variables of acquired and innate immunity, i.e. PHA response, IgY level and hemolysis-hemagglutination were not correlated at the individual level (Tables 1, 2) or at the brood level (Table 3). Hemolysis at the individual level was not correlated with any variable (PHA response: $F_{1,\ 93.7} = 0.44$, $p = 0.509$, IgY: $F_{1,\ 72.7} = 2.18$, $p = 0.144$, Hemagglutination: $F_{1,\ 88.3} = 0.02$, $p = 0.885$).

Table 1. Mean and Standard Error (SE) of immune variables.

	n	Mean	SE
IgY levels (absorbance)	139	0.219	0.003
PHA (mm)	138	0.255	0.007
Hemagglutination (titre)	108	7.399	0.119
Hemolysis (titre)	108	0.550	0.093

Table 2. Mixed model ANOVA between different measures of immunity with brood ID as a random factor.

	df	*F*	*p*
IgY level			
PHA response	1, 80.8	0.09	0.766
Hemolysis	1, 79.4	0.01	0.926
Hemagglutination	1, 63.8	2.12	0.150
PHA response			
IgY level	1, 80.5	0.19	0.666
Hemolysis	1, 80.6	0.01	0.939
Hemagglutination	1, 64.7	0.02	0.886
Hemagglutination			
IgY level	1, 81	1.50	0.224
PHA response	1, 81	0.03	0.868
Hemolysis	1, 81	0.12	0.731

In the analyses of nestling measures on day 7, only hemagglutination was negatively correlated with tarsus length (Table 4), while other immune variables were not correlated with any biometrical measure. None of the immune variables were correlated with biometrical variables of nestlings at the age of 13 days or with growth between 7 and 13 days of age (all $p > 0.10$). Rank in the mass hierarchy on days 7 and 13 showed no associations with any immunity measure (all $p > 0.30$).

Table 3. Linear correlations between brood means of different measures of immunity; as hemolysis is not linear, we have used nonparametric Spearman rank correlations. We only included nests in which we had the three measures of immunity (n=58).

	IgY level	**PHA response**	**Agglutination**	**Lysis**
PHA response	$r = 0.038$, $p = 0.776$	----		
Agglutination	$r = 0.099$, $p = 0.455$	$r = 0.018$, $p = 0.890$	----	
Lysis	$r_s = -0.108$, $p = 0.418$	$r_s = 0.072$, $p = 0.590$	$r_s = 0.063$, $p = 0.638$	----

Table 4. Mixed linear model for IgY level, PHA response and hemagglutination as dependent variables and generalized mixed model for hemolysis. We included nest as a random factor and hatching date, brood size, wing length, body-mass and tarsus length on day 7 as covariables using the Satterthwaite correction for estimating degrees of freedom. Minimal models are obtained from full models by successive backward deletion of variables when the variance explained does not significantly improve the model ($\alpha = 0.05$).

	Estimate	*df*	*F*	*p*
IgY level				
Full model				
Hatching date	-0.000	1,75.4	0.23	0.621
Brood size	-0.009	1,80.6	5.97	0.052
Wing length on day 7	0.002	1,124	1.93	0.313
Body-mass on day 7	-0.007	1,119	1.40	0.157
Tarsus length on day 7	0.006	1,121	0.02	0.364
PHA response				
Full model				
Hatching date	-0.001	1,66.8	1.00	0.320
Brood size	-0.000	1,68.1	0.01	0.939
Wing length on day 7	-0.006	1,119	1.50	0.222
Body-mass on day 7	0.015	1,112	2.13	0.146
Tarsus length on day 7	0.013	1,120	0.84	0.362
Hemagglutination				
Full model				
Hatching date	0.007	1,94	0.07	0.790
Brood size	0.153	1,94	1.40	0.238
Wing length on day 7	0.046	1,94	0.34	0.563
Body-mass on day 7	-0.100	1,94	0.43	0.512
Tarsus length on day 7	-0.408	1,94	3.14	0.079
Minimal model				
Tarsus length on day 7	-0.367	1,98	5.40	**0.022**
Hemolysis				
Full model				
Hatching date	-0.045	1,66	0.47	0.497
Brood size	-0.282	1,58.9	0.90	0.348
Wing length on day 7	0.143	1,95.2	0.69	0.409
Body-mass on day 7	-0.316	1,93.1	0.87	0.353
Tarsus length on day 7	0.068	1,97.9	0.02	0.884

Discussion

We did not find any correlation between different measures of the innate and acquired immune systems at the individual and brood means levels. We only showed a negative correlation between tarsus length on day 7 and hemagglutination, while no other

association between measures of the immune system employed and biometrical variables of nestlings at ages of 7 and 13 days and their growth between these ages were found.

The first result underlines the problems involved in obtaining a general measure of immunocompetence, and emphasizes the importance of measuring different aspects of the immune system due to their statistical independence and their complexity, including numerous well-defined, but interacting components (Blount *et al*. 2003, Adamo 2004, Matson *et al*. 2005, Matson *et al*. 2006, Salvante 2006). Since different types of infections (viruses, bacteria, etc.) are controlled using different types of immune responses, a single measure of immunity is not sufficient to evaluate every kind of immune response (Adamo 2004). Moreover, correlations between various immunological variables and resistance to specific diseases appear to be generally pathogen-dependent (Adamo 2004).

There is conflicting evidence concerning the relationships between different measures of immunity in adult birds. In relation to humoral and cell-mediated immunity, some authors have reported a positive correlation between these two arms (Møller *et al*. 2001, Morales *et al*. 2004), while others have shown the opposite trend (González *et al*. 1999, Johnsen & Zuk 1999, Møller & Petrie 2002, Buchanan *et al*. 2003, Arriero 2009). In fact, this discrepancy has been found in females of the same species at three sites and is attributed to differences in condition between sites (Ardia 2007). Other studies have included measures of innate immunity (hemagglutination-hemolysis, plasma bactericidal activity, etc.); some have not found correlations between these different arms of the immune system (Matson *et al*. 2006, Mendes *et al*. 2006). However, Forsman *et al*. (2008) found that the humoral immune response was negatively related to the PHA response and positively related to plasma bactericidal

activity (*Escherichia coli* killing capacity) in house wren nestlings. However, the associations between different measures of immunity were not significant among individual nestlings within broods. Palacios *et al*. (2009) showed that innate immune components would develop earlier than adaptive components in nestling tree swallows. The present study shows a lack of correlation between a measure of innate immunity (hemagglutination/hemolysis) and two measures of adaptive immunity (PHA response and IgY), which implies a lack of constraints, synergism or trade-offs in these particular measures in nestlings. This may be due to the differences in onset and rate of development of different components of the immune system which may preclude any association at the individual level (Palacios *et al*. 2009). However, an absence of trade-offs between different arms of the immune system may be expected under good conditions for nestlings with respect to climate, nutrition or infection.

Innate immunity is particularly expensive to growing young because the inflammatory response induces anorexia and diverts nutrients needed for growth to the acute phase response (Klasing 1994, Klasing & Leshchinsky 1999). In nestling birds, some studies have shown trade-offs between the nutrients required for growth and those needed to mount an immune response (Klasing & Leshchinsky 1999, Szép & Møller 1999, Soler *et al*. 2003, Brommer 2004, Pihlaja *et al*. 2006). Mauck *et al*. (2005) showed that in Leach's storm-petrel chicks, agglutination titres increased with chick age and that there was an inverse relationship between NAbs and growth rate. It is not clear that the simple maintenance of immune function is costly in the absence of infection (Klasing 1998), though maintenance is apparently costlier for the innate immune system than for the adaptive immune system (Råberg *et al*. 2002). The higher cost of the innate immune system may explain our result that the only association between nestling biometry and immunity was found for Nabs. This trade-off was only detected at 7 days

of age. Nestlings are experiencing the fastest skeletal growth before 10 days of age (Lundberg & Alatalo 1992), which possibly explains why the association with NAbs was not found at 13 days.

Several studies of nestlings have reported a positive association of body mass with PHA response (Saino *et al.* 1997, Brinkhof *et al.* 1999, Hõrak *et al.* 2000, Westneat *et al.* 2004, Moreno *et al.* 2005, Morales *et al.* 2006, Roulin *et al.* 2007, Forsman *et al.* 2010). However other studies have found no association of body mass with cell-mediated immune response in nestlings (Hõrak *et al.* 1999, Martin *et al.* 2001, Tella *et al.* 2002, Moreno *et al.* 2005, Roulin *et al.* 2007). Brzek and Konarzewski (2007) found that the association between PHA response and body mass was negative when food was scarce, positive when resources were abundant while there was no significant correlation under intermediate conditions. However, we found significant positive associations between nestling mass and PHA response in other years (1999, mean mass on day 13 (SE): 13.68 g (0.18), and 2000, mean mass (SE): 13.73 g (0.11)) in the same study population, although not between these two variables in another year (2002, mean mass (SE): 13.20 g (0.14)) (Moreno *et al.* 2005). Thus, positive growth-immunity associations are only found when nestlings attain higher masses before fledging, which suggests that growth-immunity trade-offs may only be detectable under very poor conditions for breeding, with no significant association at intermediate conditions. In 2009, the mean mass (SE) was 13.35 g (0.10) which is similar to 2002 and intermediate with respect to nestling growth (in 2010 nestlings that fledged only attained 12.53 g (0.30)). It is therefore not surprising that we did not detect any association of nestling mass with PHA response in the present study. In general, we found weak evidence for associations between nestling growth and development of immune responses in the specific conditions experienced in the study year.

To conclude, we could not confirm the existence of a general axis of immunocompetence among individual nestlings or among broods, nor evidence of resource-based trade-offs among different components of the immune system. This absence of associations could be due to differences in the onset and rate of development of the different immunity components. We only detected associations between early skeletal growth and innate immunity, which may reflect the higher costs of developing this arm of the immune system in altricial nestlings.

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