Innate humoural immunity is related to eggshell bacterial load of European birds: a comparative analysis

Juan José Soler, Juan Manuel Peralta-Sánchez, Einar Flensted-Jensen, Antonio Manuel Martín-Platero & Anders Pape Møller

Naturwissenschaften
The Science of Nature

ISSN 0028-1042
Volume 98
Number 9

Naturwissenschaften (2011)
98:807-813
DOI 10.1007/s00114-011-0830-z

Springer

Naturwissenschaften (2011) 98:807–813
DOI 10.1007/s00114-011-0830-z

SHORT COMMUNICATION

Innate humoural immunity is related to eggshell bacterial load of European birds: a comparative analysis

Juan José Soler · Juan Manuel Peralta-Sánchez · Einar Flensted-Jensen · Antonio Manuel Martín-Platero · Anders Pape Møller

Received: 15 June 2011 / Revised: 19 July 2011 / Accepted: 22 July 2011 / Published online: 3 August 2011

Abstract Fitness benefits associated with the development of a costly immune system would include not only self-protection against pathogenic microorganisms but also protection of host offspring if it reduces the probability and the rate of vertical transmission of microorganisms. This possibility predicts a negative relationship between probabilities of vertical transmission of symbionts and level of immune response that we here explore inter-specifically. We estimated eggshell bacterial loads by culturing heterotrophic bacteria, *Enterococcus*, *Staphylococcus* and *Enterobacteriaceae* on the eggshells of 29 species of birds as a proxy of vertical transmission of bacteria from mother to offspring. For this pool of species, we also estimated innate immune response (natural antibody and complement (lysis)) of adults, which constitute the main defence against bacterial infection. Multivariate general linear models revealed the predicted negative association between natural antibodies and density of bacteria on the eggshell of 19 species of birds for which we sampled the eggs in more than one nest. Univariate analyses revealed significant associations for heterotrophic bacteria and for *Enterobacteriaceae*, a group of bacteria that includes important pathogens of avian embryos. Therefore, these results suggest a possible trans-generational benefit of developing a strong immune system by reducing vertical transmission of pathogens.

Keywords Anti-bacterial defence · Bacteria · Complement · Natural antibodies · Trade-offs · Vertical transmission of microorganisms

Communicated by: Sven Thatje

Electronic supplementary material The online version of this article (doi:10.1007/s00114-011-0830-z) contains supplementary material, which is available to authorized users.

J. J. Soler · J. M. Peralta-Sánchez
Estación Experimental de Zonas Áridas (CSIC),
Almería, Spain

J. J. Soler (✉) · J. M. Peralta-Sánchez · A. M. Martín-Platero
Grupo Coevolución, Unidad asociada al CSIC,
Universidad de Granada,
Granada, Spain
e-mail: jsoler@eeza.csic.es

E. Flensted-Jensen
Cypresvej 1,
9700 Brønderslev, Denmark

A. M. Martín-Platero
Departamento de Microbiología, Facultad de Ciencias,
Universidad de Granada,
Granada, Spain

A. P. Møller
Laboratoire d'Ecologie, Systématique et Evolution,
CNRS UMR 8079, Université Paris-Sud,
Bâtiment 362,
91405 Orsay Cedex, France

Introduction

Immune responses protect individuals from pathogenic infections. Therefore, immunocompetence (i.e. the ability to efficiently overcome the fitness costs of parasitism through immune response) should be related to health status of hosts as has been shown previously (see Møller 1997). Immunity of adults could also affect the probability of pathogen transmission to their offspring because certain immunological factors that are directly transmitted from parents to offspring protect the latter against infections (Saino et al. 2002a, b; Gallizzi et al. 2008; Roth et al. 2010). Moreover, parents showing more efficient immune responses will harbour lower pathogen loads than others, which may result in low risk of vertically transmitted pathogens to their offspring. Consequently, elevated immunocompetence of adults will be of selective advantage under high risk of infection, not only because of its effects on self-maintenance (i.e. survival) (personal immunology *sensu* Cotter and Kilner (2010)) but also because it is diminishing the probability of infection of developing offspring (social immunology *sensu* Cotter and Kilner (2010)), both through enhanced immune response of offspring and by reducing the probability of vertical transmission of pathogens. Therefore, this scenario of social immunity of mothers predicts a negative relationship between adult immunocompetence and probability of pathogenic infections experienced by offspring.

Here we tested this hypothesis in a comparative analysis exploring the association between adult immune response of birds and bacterial loads of eggshells in their nests. Eggshell bacterial load is a good proxy for probability of trans-shell bacterial infection of embryos as suggested in the poultry literature (Board and Fuller 1994) and experiments on wild populations (Cook et al. 2003, 2005a, b; Shawkey et al. 2009). Bacteria can be vertically transmitted to eggs by several routes, and the part of the egg in which a microorganism is deposited depends largely on whether it becomes localized in the ovary, oviduct or cloaca (Barrow 1994). For instance, microorganisms that naturally are located in the lower alimentary tract of hosts may contaminate egg surfaces as the egg passes through the cloaca, and the probability of having potentially pathogenic microorganisms transmitted to embryos in the cloaca will depend on the immunocompetence of avian hosts (Barrow 1994). In accordance with this possibility, evidence of a positive relationship between bacterial loads on eggshells and in the cloaca of maternal pied flycatchers *Ficedula hypoleuca* have been recently detected (Ruiz-de-Castañeda et al. 2011a, b). Moreover, pathogenic bacteria of embryos (e.g. *Salmonella* sp., *Pseudomonas* sp., *Staphylococcus* sp.) may infect skin and feathers of parents (Nde et al. 2007) and reach the eggshell and embryo during the laying and incubation period (Bruce and Drysdale 1994; Gunderson 2008). Since immune response of adults prevents the establishment of pathogenic bacteria in their digestive tract and/or on their skin (Salyers and Whitt 2002) and consequently on feathers, an efficient immune system of parents may result in reduced colonisation rate of eggshells by bacteria that are pathogenic for the embryo. Therefore, there are good reasons to predict a relationship between level of immune response of birds and the bacterial environment of their eggshells.

The main immune response against bacterial infection is mediated by innate humoural immunity (natural antibodies, NAbs) (Playfair and Bancroft 2004; Matson et al. 2005), and it is known that the bacterial killing ability associated with NAbs varies significantly among species (Matson et al. 2006). Thus, we predicted a negative interspecific relationship between level of NAbs and eggshell bacterial loads. An alternative scenario suggesting a positive relationship between immunity of adults and eggshell bacterial load depends on the possibility that eggshell bacterial loads are mainly due to pathogenically risky breeding environments experienced by adult birds. Species with higher risk of pathogenic infection are those with stronger immune responses, and they will also be those experiencing larger eggshell bacterial loads because bacteria are not controlled by adult immunity.

We tested these predictions by estimating natural antibodies and complement of 29 bird species as well as the eggshell density of heterotrophic bacteria in general and of potentially pathogenic bacteria such as *Enterobacteriaceae*, *Staphylococcus* and *Enterococcus* in particular.

Material and methods

Study site, nest locations and adult captures

Bacterial communities on eggshells of nests were sampled during the breeding seasons 2007–2008 at Kraghede and surroundings, Denmark (57°12′ N; 10°00′ E). For a detailed description of the study area, see Møller (1987). Innate immune responses were estimated at the same locations during 2005, and values were already published when we planned the present paper (Møller et al. 2008).

Most nests were located during nest building by intensively searching suitable habitat in the study area and by checking nest boxes. We deliberately attempted not to touch nests or disturb the surrounding vegetation to avoid increasing the risk of nest predation. When a nest was detected during egg laying, on the basis of typical clutch size of the species, we estimated date of clutch completion and visited the nests the following days and perform eggshell bacterial samplings 2 to 3 days after clutch completion. Nests were again visited at hatching. The number of nest checks was in this way minimized to reduce any unnecessary predation due to investigators.

Adult birds were captured with mist nets during extensive capture events during the breeding season April–June. Typically, we captured birds by placing mist nets in appropriate habitats, mainly during morning (sunrise until noon) and evening (18:00 until sunset) capture sessions. Captured adult birds were subsequently blood sampled by puncturing the brachial vein and collecting two heparinized capillaries of 75 μl blood that were centrifuged for 10 min at 4,000 rpm and plasma and cells separated and stored at −20°C until analysis in the laboratory.

Estimation of eggshell bacterial loads

We sampled eggs at the beginning of incubation (i.e. 2–3 days after clutch completion), which assured that independently of the species all sampled eggs were incubated and therefore possible interspecific differences in eggshell bacterial loads due to variation in the onset of incubation (Cook et al. 2003) were partially mitigated. We successfully collected information for 284 nests of 35 species of birds (see Appendix 1). For 29 of these species, information of innate immune response was available (see below), and for 19 species, more than one nest was sampled for of eggshell bacterial loads.

We sampled eggshells in sterile conditions mainly to prevent between-nest contamination. We wore latex gloves sterilised with ethanol and took bacterial samples by cleaning eggshells with a sterile swab slightly wet with sterile sodium phosphate buffer (0.2 M; pH 7.2). The entire clutch was cleaned with the same swab, which was preserved in an Eppendorf tube at 4°C containing the sterile buffer until lab analyses during the following 30 days. The duration of the storage period did not affect rank position of different species as shown by a comparison of ranked values of heterotrophic bacterial loads of 21 species from which in 2006 we collected samples that were stored less than 3 days ($N=120$) and others that were stored up to one month ($N=156$) (Kendall coefficient of concordance=0.95; average Spearman rank correlation=0.91, Friedmann ANOVA, chi-square=38.13, $P=0.009$). Estimates of bacterial load were standardized to total eggshell surface sampled by taking into account number of sampled eggs and surface (following Narushin 2005) of each egg in the nests.

In the lab, samples were collected from Eppendorf tubes after vigorously shaking the Eppendorf in vortex for at least three periods of 5 s. Serial decimal dilutions up to 10^{-6} were cultivated by spreading homogeneously 100 μl of sample (measured with a micropipette) in plates containing four different sterile solid growth media (Scharlau Chemie S.A. Barcelona). We used Tryptic Soy Agar (TSA), a broadly used general medium to grow heterotrophic bacteria and three specific media: Kenner Fecal Agar (KF) for growing bacteria belonging to the genus *Enterococcus*, Vogel–Johnson Agar (VJ) for bacteria of the genus *Staphylococcus* and Hecktoen Enteric Agar (HK) for Gram-negative bacteria of the family *Enterobacteriaceae*. *Enterobacteriaceae* and *Staphylococcus* sp. are saprophytic and opportunistic bacteria (Singleton and Harper 1998; Houston et al. 1997; Cook et al. 2005a) that live on skin, hair and feathers of mammals and birds (Krieg and Holt 1984). They commonly appear on avian eggshells and are known to be pathogenic for avian embryos (Bruce and Drysdale 1994). Enterococci, the third analysed group of bacteria, are also frequently found inside unhatched eggs (Bruce and Drysdale 1994) and are opportunistic pathogens (Franz et al. 1999), although some species might also have beneficial effects (Soler et al. 2008, 2010; Moreno et al. 2003). Moreover, heterotrophic bacterial loads of the eggshells are related to probability of trans-shell embryo infection (Bruce and Drysdale 1994; Cook et al. 2003, 2005b), and therefore, there are good reasons for considering the estimated eggshell bacterial loads as proxies of probability of trans-shell bacterial infection of embryos.

Plates were incubated at 37°C for 72 h, and afterwards, the number of colonies on each plate was counted. Bacterial density was estimated as colony forming units per square centimetre. See Peralta-Sánchez et al. (2010) for repeatability estimates of intraspecific variation.

Estimation of NAbs and complement

To estimate the levels of circulating NAbs and complement, we used the procedure developed by Matson et al. (2005) and modified by Møller and Haussy (2007). The agglutination part of the assay estimates the interaction between natural antibodies and antigens in rabbit blood, producing blood clumping. The lysis part of the assay estimates the action of complement from the amount of haemoglobin released from the lysis of rabbit erythrocytes. Quantification of agglutination and lysis is achieved by serial dilution in polystyrene 96-well assay plates, with the dilution step at which the agglutination or lysis reaction is stopped. We used fresh rabbit blood with Alsever's anticoagulant, 96 round well assay plates and an EPSON 4490 photo scanner that was set at professional mode, with document type colour film, 48-bit colour and 300 dpi. Whole rabbit blood was stored at 4°C. After determination of the level of haematocrit, we diluted to obtain a solution of 1% of erythrocytes. See Møller and Haussy (2007) for details of the procedures and Soler et al. (2007) for repeatability estimates of intraspecific variation and some others sources of errors.

Statistical analyses

For estimating year-independent interspecific variation in eggshell bacterial density, we standardized (mean=0 and SD=1) species mean values ($\log_{10}$-transformed) for each year and estimated means per species. However, these mean values did not approach normal distributions (Kolmogorov–Smirnov tests for continuous variables, $P<0.05$), and thus, we used rank values of bacterial density in our analyses.

These values, however, cannot a priori be considered statistically independent observations due to similarity in values among closely related species caused by common descent, and phylogenetic relationships between species should be taken into account in comparative analyses

(Harvey and Pagel 1991). Therefore, we tested residuals of the regressions between estimated eggshell bacterial loads and immune response using the λ statistic of Pagel (1999; Freckleton et al. 2002) and a composite phylogeny derived from Jønsson and Fjeldså (2006), while polytomies of basal nodes were solved following Sibley and Ahlquist (1990) (Appendix 1). Branch lengths were arbitrarily assigned to one, but constraining tips to be contemporaneous. We found that the estimated values of λ were equal to zero for models explaining variation in heterotrophic bacteria, *Enterococcus* and *Staphylococcus* ($\lambda<6.61\times10^5$, test for $\lambda=0$: chi-square<0.001; $P=1$) and non-significantly different from zero for that of *Enterobacteriaceae* ($\lambda=0.34$, test for $\lambda=0$: chi-square$=0.10$; $P=0.75$), indicating that there was no phylogenetic signal, and therefore, the analyses did not need to be corrected for phylogenetic effects (see Sæther et al. 2011).

The predicted relationships between eggshell bacterial loads and avian immune responses were tested by means of multiple general lineal models with eggshell bacterial loads (i.e. heterotrophic bacteria, *Enterococcus*, *Staphylococcus* and *Enterobacteriaceae*) as dependent variables and innate immune responses (NAbs and complement) as independent factors. Body mass was also included as independent variable since it is known to affect immune response and risk of pathogenic infections of animals (e.g. Møller et al. 2004). In addition, we corrected our comparative analyses for heterogeneity in data quality due to the large variation in sample sizes among species by weighting the comparative analyses for number of sampled nests (Garamszegi and Møller 2010). The weighted comparative analyses were performed with species with more than a single nest sampled because inclusion of single observations might considerably increase sampling error (Garamszegi and Møller 2010). However, results were qualitatively identical (i.e. in terms of variables that reached statistical significance) when using the complete data set in Appendix 2 (results not shown).

Results

Species with higher NAb responses were those with lower density of bacteria on their eggshells (Wilks $\lambda=0.45$, $F=3.71$, $df=4,12$, $P=0.034$) after controlling for the non-significant effects of body mass (Wilks $\lambda=0.65$, $F=1.63$, $df=4,12$, $P=0.23$) and complement (Wilks $\lambda=0.75$, $F=0.98$, $df=4,12$, $P=0.45$). This general effect was mainly due to the significant relationship detected for heterotrophic bacteria and *Enterobacteriaceae* (Table 1; Fig. 1). Excluding non-significant independent variables from the model did not qualitatively affect the detected negative relationships between eggshell bacterial loads and NAbs (results not shown).

Table 1 Results from multiple general lineal models of the relationship between eggshell bacterial loads estimated for heterotrophic bacteria (TSA), *Enterococcus* (KF), *Staphylococcus* (VJ) and *Enterobacteriaceae* (HK) and level of innate immune response (natural antibodies (NAbs) and complement (lysis))

		(Beta SE)	t_{18}	P
TSA (ranks)	Body mass	0.51 (0.20)	2.52	0.023
	NAbs	−0.80 (0.22)	3.73	0.002
	Lysis	0.25 (0.21)	1.20	0.25
KF (ranks)	Body mass	0.29 (0.27)	1.09	0.29
	NAbs	−0.02 (0.29)	0.08	0.94
	Lysis	−0.04 (0.28)	0.15	0.89
VJ (ranks)	Body mass	0.06 (0.27)	0.22	0.55
	NAbs	−0.12 (0.29)	0.43	0.49
	Lysis	−0.30 (0.27)	1.09	0.29
HK (ranks)	Body mass	0.48 (0.22)	2.20	0.044
	NAbs	−0.62 (0.24)	2.61	0.020
	Lysis	0.01 (0.23)	0.06	0.95

Coefficients are shown for analysis including all species with more than one nest sampled ($N=19$)

Discussion

The predicted interspecific relationship between eggshell bacterial and NAbs was found for heterotrophic bacteria and for *Enterobacteriaceae*, but not for eggshell bacterial density estimated from other specific culture media. Specific culture media are restrictive and growth of specific bacteria is only detected from a considerable concentration of microorganisms in the sample (Gerhardt 1981). Thus, when comparing these estimates with density estimates from heterotrophic media, those from specific media are underestimated and sometimes result in false negatives (Giraffa and Neviani 2001; Giraffa 2004). Bacterial densities estimated for *Enterococcus* and *Staphylococcus* were much lower than those of heterotrophic bacteria and *Enterobacteriaceae* and that might account for the lack of the predicted relationship in the former counts.

Bacteria on eggshells can be vertically transmitted from the cloaca of the female at laying (Barrow 1994), and consequently, factors affecting maternal cloacal bacteria can ultimately lead to transmission of bacteria onto the eggshells (Ruiz-de-Castañeda et al. 2011a). Females with poor immunocompetence may harbour potential pathogens in their digestive tract that would be vertically transmitted to their offspring when forming and laying eggs. In accordance with this possibility, Ruiz-de-Castañeda et al. (2011b) have recently shown that pied flycatcher females with rod-shaped Gram-negative bacteria in their cloacae laid eggs that also had these bacteria. Thus, at the interspecific level, immunity against bacterial infection of adults should be

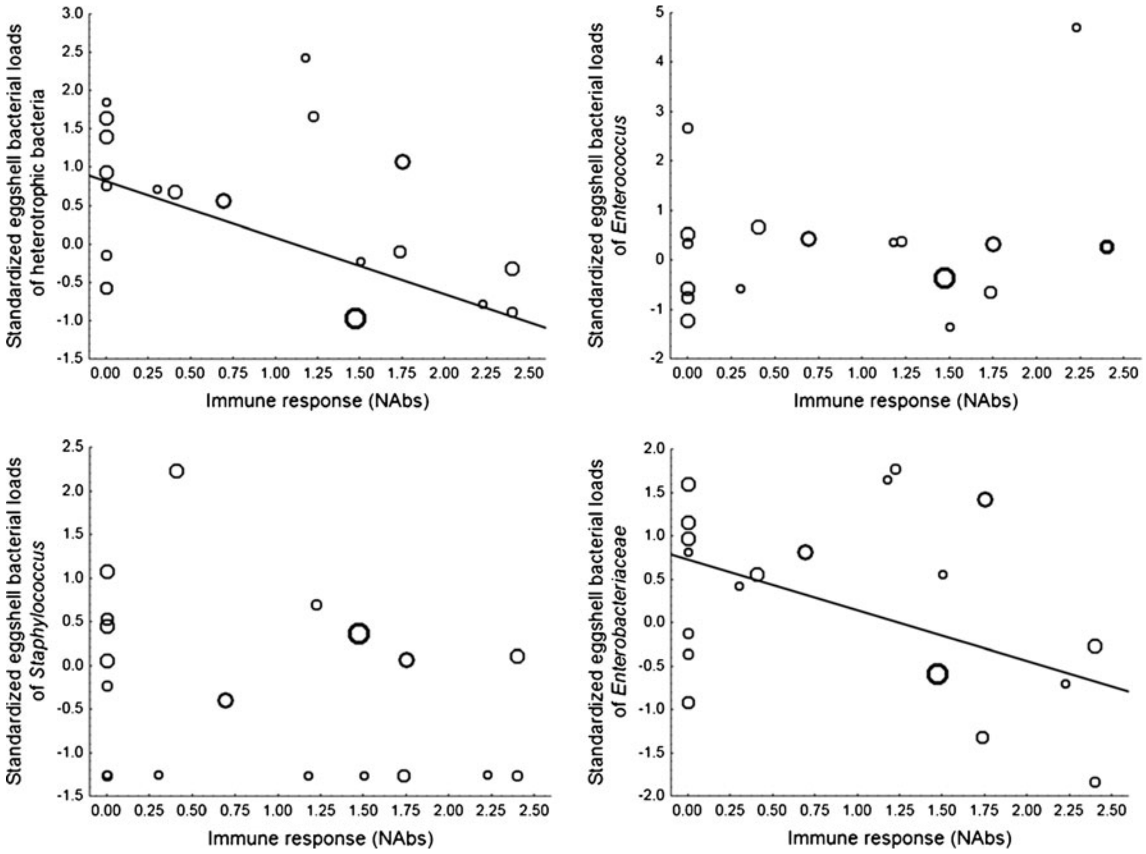

Fig. 1 Relationships between innate immune response mediated by natural antibodies (*NAbs*) and eggshell bacterial loads estimated for heterotrophic bacteria, *Enterococcus*, *Staphylococcus* and *Enterobacteriaceae*. The size of *symbols* is proportional to the log of sample size. *Regression lines* are shown for statistically significant relationships

negatively related to bacterial density of eggshells soon after start of incubation. As a measure of immunity against bacterial infection, we estimated NAbs, which constitute the innate first line of defence against pathogens including bacteria (Playfair and Bancroft 2004) and is relatively insensitive to external influences (i.e. level of infection). In agreement with the predicted scenario, we found evidence of a negative relationship for heterotrophic bacteria and for *Enterobacteriaceae*, a group of bacteria that include pathogens such as *Salmonella* and *Escherichia coli* that are commonly known as pathogen of avian embryos (Bruce and Drysdale 1994).

An alternative hypothesis predicting a relationship between eggshell bacterial density and adult immune response is that both variables were the consequence of risk of pathogenic infections experienced by both eggs and adults. At the interspecific level, immunity is positively related to intensity of parasitic microorganism-mediated selection (see Møller and Erritzøe 1996; Møller et al. 2001, 2008). If eggshell bacterial loads reflect the pathogenic environment, we should find a positive rather than a negative association between NAbs and eggshell bacterial load because both variables should increase in pathogenically risky environments. Thus, we consider the detected negative relationship to be an indirect consequence of parasite-mediated selection due to maternal effects (i.e. innate immunity (NAbs)) reducing vertical transmission of micro-organisms. However, the present study is not experimental, and the detected relationship between immunity of adults and eggshell bacterial load could be explained by third unknown variables related to the variables of interest. For instance, it is possible that species with strong immune responses invested more in nest sanitation or used anti-bacterial natural components that affected bacterial environmental conditions of nests (Clark and Mason 1985, 1988; Mennerat et al. 2009). Another possibility is that species with higher level of NAbs could use these antibodies during and after egg laying on eggshells, killing and/or reducing growth of colonizing bacteria. Further research including experimental modification of eggshell bacterial load is needed to determine the mechanism underlying the detected relationship.

The poultry literature, but also empirical studies of wild birds, indicates that eggshell bacterial loads are good predictors of hatching failure, and consequently, the detected negative interspecific relationship between NAbs immune responses eggshell bacterial densities suggests that innate immunity of adults may confer advantages to offspring by diminishing the probability of trans-shell bacterial infection. Thus, our results suggest possible trans-generational benefits of developing a strong immune system in pathogenically risky environments.

Acknowledgements We thank E. Lopez-Hernández for technical assistance in the lab. This study was funded by a graduate research grant from Junta de Andalucia to JMP and by Ministerio de Ciencia e Innovación/FEDER (project CGL2010-19233-C03-01) and the Junta de Andalucía (P09-RNM-4557).

References

Barrow PA (1994) The microflora of the alimentary tract and avian pathogens: translocation and vertical transmission. In: Board RG, Fuller R (eds) Microbiology of avian eggs. Chapman & Hall, London, pp 117–138

Board RG, Fuller R (1994) Microbiology of the avian egg. Chapman & Hall, London

Bruce J, Drysdale EM (1994) Trans-shell transmission. In: Board RG, Fuller R (eds) Microbiology of avian eggs. Chapman & Hall, London, pp 63–91

Clark L, Mason JR (1985) Use of nest material as insecticidal and anti-pathogenic agents by the European starling. Oecologia 67:169–176

Clark L, Mason JR (1988) Effect of biologically active plants used as nest material and the derived benefit to starling nestlings. Oecologia 77:174–180

Cook MI, Beissinger SR, Toranzos GA, Rodriguez RA, Arendt WJ (2003) Trans-shell infection by pathogenic micro-organisms reduces the shelf life of non-incubated bird's eggs: a constraint on the onset of incubation? Proc R Soc Lond B 270:2233–2240

Cook MI, Beissinger SR, Toranzos GA, Arendt WJ (2005a) Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection. Ecol Letters 8:532–537

Cook MI, Beissinger SR, Toranzos GA, Rodriguez RA, Arendt WJ (2005b) Microbial infection affects egg viability and incubation behavior in a tropical passerine. Behav Ecol 16:30–36

Cotter SC, Kilner RM (2010) Personal immunity versus social immunity. Behav Ecol 21:663–668

Franz CMAP, Holzapfel WH, Stiles ME (1999) Enterococci at the crossroads of food safety? Int J Food Microbiol 47:1–24

Freckleton RP, Harvey PH, Pagel M (2002) Phylogenetic analysis and comparative data: a test and review of evidence. Am Nat 160:712–726

Gallizzi K, Guenon B, Richner H (2008) Maternally transmitted parasite defence can be beneficial in the absence of parasites. Oikos 117:223–230

Garamszegi LZ, Møller AP (2010) Effects of sample size and intraspecific variation in phylogenetic comparative studies: a meta-analytic review. Biol Rev 4:797–805

Gerhardt P (1981) Manual of methods for general bacteriology. American Society for Microbiology, Washington, DC

Giraffa G (2004) Studying the dynamics of microbial populations during food fermentation. FEMS Microbiol Rev 28:251–260

Giraffa G, Neviani E (2001) DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. Int J Food Microbiol 67:19–34

Gunderson AR (2008) Feather degrading bacteria: a new frontier in avian and host–parasite research? Auk 125:972–979

Harvey PH, Pagel MD (1991) The comparative method in evolutionary biology. Oxford University Press, Oxford

Houston CS, Saunders JR, Crawford RD (1997) Aerobic bacterial flora of addled raptor eggs in Saskatchewan. J Wildl Dis 33:328–331

Jønsson KA, Fjeldså J (2006) A phylogenetic supertree of oscine passerine birds (Aves: Passeri). Zool Scripta 35:149–186

Krieg NR, Holt JG (1984) Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore

Matson KD, Ricklefs RE, Klasing KC (2005) A hemolysis-hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. Dev Comp Imm 29:275–286

Matson KD, Tieleman BI, Klasing KC (2006) Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds. Physiol Biochem Zool 79:556–564

Mennerat A, Mirleau P, Blondel J, Perret P, Lambrechts M, Heeb P (2009) Aromatic plants in nests of the blue tit *Cyanistes caeruleus* protect chicks from bacteria. Oecologia 161:849–855

Møller AP (1987) Egg predation as a selective factor for nest design: an experiment. Oikos 50:91–94

Møller AP (1997) Parasitism and the evolution of host life history. In: Clayton DH, Moore J (eds) Host–parasite evolution: general principles and avian models. Oxford University Press, Oxford, pp 105–127

Møller AP, Erritzøe J (1996) Parasite virulence and host immune defense: host immune response is related to nest reuse in birds. Evolution 50:2066–2072

Møller AP, Haussy C (2007) Fitness consequences of variation in natural antibodies and complement in the Barn Swallow *Hirundo rustica*. Funct Ecol 21:363–371

Møller AP, Merino S, Brown CR, Robertson RJ (2001) Immune defense and host sociality: a comparative study of swallows and martins. Am Nat 158:136–145

Møller AP, Martin-Vivaldi M, Soler JJ (2004) Parasitism, host immune defence and dispersal. J Evol Biol 17:603–612

Møller AP, Nielsen JT, Garamszegi LZ (2008) Risk taking by singing males. Behav Ecol 19:41–53

Moreno J, Briones V, Merino S, Ballesteros C, Sanz JJ, Tomás G (2003) Beneficial effects of cloacal bacteria on growth and fledging size in nestling pied flycatchers (*Ficedula hypoleuca*) in Spain. Auk 120:784–790

Narushin VG (2005) Production, modeling, and education: egg geometry calculation using the measurements of length and breadth. Poultry Sci 84:482–484

Nde CW, Mcevoy JM, Sherwood JS, Logue CM (2007) Cross contamination of turkey carcasses by *Salmonella* species during defeathering. Poultry Sci 86:162–167

Pagel M (1999) Inferring the historical patterns of biological evolution. Nature 401:877–884

Peralta-Sánchez JM, Møller AP, Martín-Platero AM, Soler JJ (2010) Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: an experimental study. Funct Ecol 24:426–433

Playfair J, Bancroft G (2004) Infection and immunity. Oxford University Press, Oxford

Roth O, Joop G, Eggert H, Hilbert J, Daniel J, Schmid-Hempel P, Kurtz J (2010) Paternally derived immune priming for offspring in the red flour beetle, *Tribolium castaneum*. J Anim Ecol 79:403–413

Ruiz-de-Castañeda R, Vela AI, Lobato E, Briones V, Moreno J (2011a) Bacterial loads on eggshells of the pied flycatcher: environmental and maternal factors. Condor 113:200–208

Ruiz-de-Castañeda R, Vela AI, Lobato E, Briones V, Moreno J (2011b) Prevalence of potentially pathogenic culturable bacteria on eggshells and in cloacae of female Pied Flycatchers in a temperate habitat in central Spain. J Field Ornithol 82:215–224

Sæther SA, Grøtan V, Engen S, Noble DG, Freckleton RP (2011) Rarity, life history and scaling of the dynamics in time and space of British birds. J Anim Ecol 80:215–224

Saino N, Dall'ara P, Martinelli R, Møller AP (2002a) Early maternal effects and antibacterial immune factors in the eggs, nestlings and adults of the barn swallow. J Evol Biol 15:735–743

Saino N, Ferrari RP, Martinelli R, Romano M, Rubolini D, Møller AP (2002b) Early maternal effects mediated by immunity depend on sexual ornamentation of the male partner. Proc R Soc Lond B 269:1005–1009

Salyers AA, Whitt DD (2002) Bacterial pathogenesis. A molecular approach. ASM, Washington, DC

Shawkey MD, Firestone MK, Brodie EL, Beissinger SR (2009) Avian incubation inhibits growth and diversification of bacterial assemblages on eggs. PLoS One 4:e4522

Sibley CG, Ahlquist JE (1990) Phylogeny and classification of birds: a study in molecular evolution. Yale University Press, New Haven

Singleton DR, Harper RG (1998) Bacteria in old house wren nests. J Field Ornithol 69:71–74

Soler JJ, Martín-Vivaldi M, Haussy C, Møller AP (2007) Intra- and interspecific relationships between nest size and immunity. Behav Ecol 18:781–791

Soler JJ, Martín-Vivaldi M, Ruiz-Rodríguez M, Valdivia E, Martín-Platero AM, Martínez-Bueno M, Peralta-Sánchez JM, Méndez M (2008) Symbiotic association between hoopoes and antibiotic-producing bacteria that live in their uropygial gland. Funct Ecol 22:864–871

Soler JJ, Martín-Vivaldi M, Peralta-Sánchez JM, Ruiz-Rodríguez M (2010) Antibiotic-producing bacteria as a possible defence of birds against pathogenic microorganisms. Open Ornithology Journal 3:93–100