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

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**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 *Enterobac-*

*teriaceae* 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.

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## 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 humoral 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  $\mu$ l blood that were centrifuged for 10 min at 4,000 rpm and plasma and cells separated and stored at  $-20^{\circ}\text{C}$  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, Friedman 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  $\mu$ l 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  $\lambda$  statistic of Pagel (1999; Freckleton et al. 2002) and a composite phylogeny derived from Jönsson 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  $\lambda$  were equal to zero for models explaining variation in heterotrophic bacteria, *Enterococcus* and *Staphylococcus* ( $\lambda < 6.61 \times 10^5$ , 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))

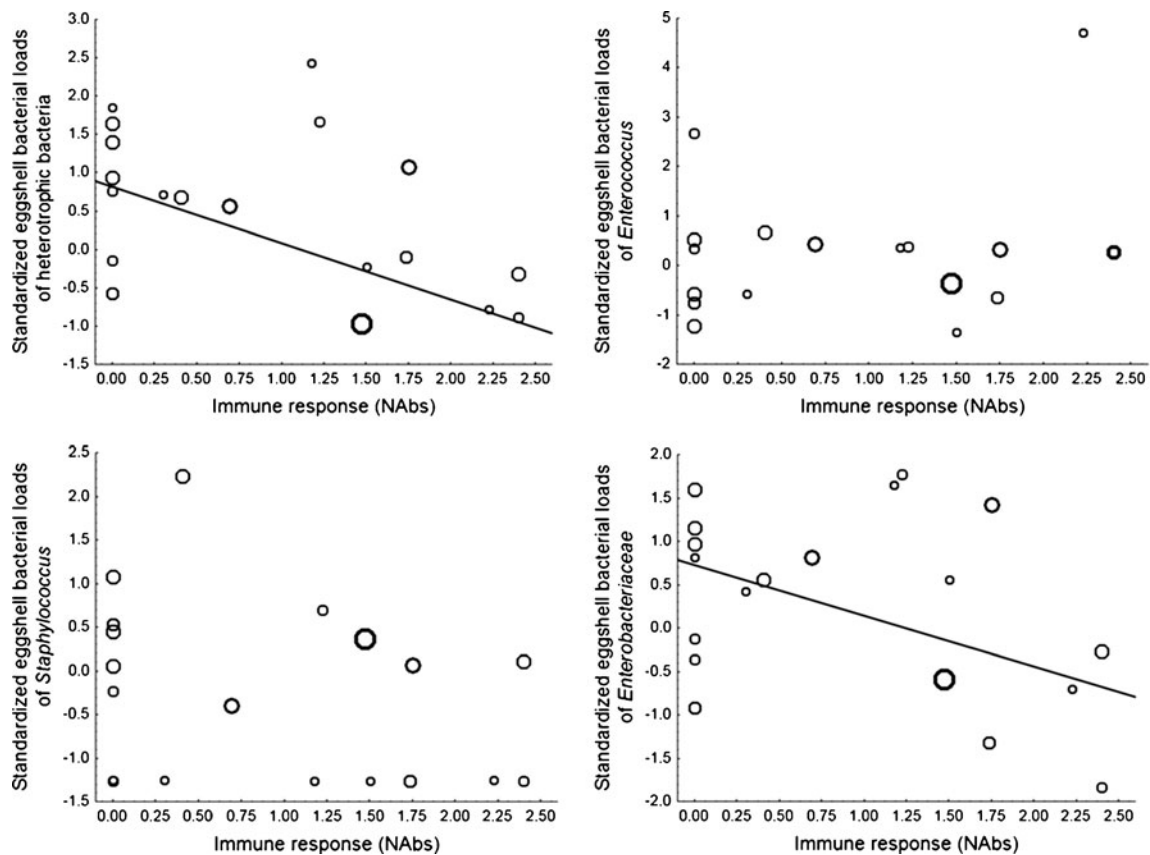
		(Beta SE)	$t_{18}$	$P$
TSA (ranks)	Body mass	0.51 (0.20)	2.52	0.023
	NAbs	-0.80 (0.22)	3.73	0.002
	Lysis	0.25 (0.21)	1.20	0.25
KF (ranks)	Body mass	0.29 (0.27)	1.09	0.29
	NAbs	-0.02 (0.29)	0.08	0.94
	Lysis	-0.04 (0.28)	0.15	0.89
VJ (ranks)	Body mass	0.06 (0.27)	0.22	0.55
	NAbs	-0.12 (0.29)	0.43	0.49
	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 *Enterobacter-*

*iaceae*. 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 microorganisms. 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.

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