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RESEARCH ARTICLE

Impact of nest sanitation on the immune system of parents and nestlings in a passerine bird

Jessica K. Evans[1,2,*], Simon C. Griffith[1], Kirk. C. Klasing[3] and Katherine L. Buchanan[2]

ABSTRACT

Bacterial communities are thought to have fundamental effects on the growth and development of nestling birds. The antigen exposure hypothesis suggests that, for both nestlings and adult birds, exposure to a diverse range of bacteria would select for stronger immune defences. However, there are relatively few studies that have tested the immune/bacterial relationships outside of domestic poultry. We therefore sought to examine indices of immunity (microbial killing ability in naive birds, which is a measure of innate immunity, and the antibody response to sheep red blood cells, which measures adaptive immunity) in both adult and nestling zebra finches (*Taeniopygia guttata*). We did this throughout breeding and between reproductive attempts in nests that were experimentally manipulated to change the intensity of bacterial exposure. Our results suggest that nest sanitation and bacterial load affected measures of the adaptive immune system, but not the innate immune parameters tested. Adult finches breeding in clean nests had a lower primary antibody response to sheep red blood cells, particularly males, and a greater difference between primary and secondary responses. Adult microbial killing of *Escherichia coli* decreased as parents moved from incubation to nestling rearing for both nest treatments; however, killing of *Candida albicans* remained consistent throughout. In nestlings, both innate microbial killing and the adaptive antibody response did not differ between nest environments. Together, these results suggest that exposure to microorganisms in the environment affects the adaptive immune system in nesting birds, with exposure upregulating the antibody response in adult birds.

KEY WORDS: Humoral, Cell-mediated, Microorganism, Sheep red blood cell, Avian immunity, Microbiome

INTRODUCTION

Bacteria have had a profound effect on the evolution of animals, impacting ontogeny, behaviour, physiology and immune function (Archie and Theis, 2011; Bäckhed et al., 2005; Lee and Mazmanian, 2010; McFall-Ngai et al., 2013). There are certain stages during the annual life cycle of birds where they may be exposed to greater bacterial diversity and abundance. The nesting behaviour of birds could expose them to elevated levels and diversity of bacteria, affecting both adults and nestlings. Exposure is likely to vary depending on species-specific sanitation practices and behaviours, as well as factors such as nest type and nest reuse (Brandl et al., 2014; Godard et al., 2007; Singleton and Harper, 1998). Bird species differ significantly in the level of nest sanitation: in some species, parents actively remove excrement from the nest; in others, young will defecate over the side of the nest; and in many species, excrement accumulates over the course of the breeding attempt (as reviewed by Guigueno and Sealy, 2012). The natural variation in nest hygiene across and within species can be attributed to interspecific differences in selection pressures such as predation risk. Nest sanitation can be further hindered by species that reuse their nests, which can increase bacterial exposure to eggs and nestlings, and a community of bacteria will develop over time (Walls et al., 2012; Wang et al., 2011). Nestlings are inoculated with microorganisms from the nest lining as well as through saliva and the delivery of food by parents, which begins soon after hatching (Benskin et al., 2009; Berger et al., 2003; Kyle and Kyle, 1993; Mills et al., 1999; Singleton and Harper, 1998). Both nestlings and adult immune function are likely to be affected by bacterial exposure in the nest (Hooper et al., 2012; Lee and Mazmanian, 2010). However, despite this variation in nesting environment and hygiene, the effect of nest cleanliness on the development of the avian immune system and the impact it has on adult immune function remains largely unaddressed.

The immune system is composed of interacting cells, tissues and proteins that form two distinct arms: the innate and adaptive immune responses. The innate immune system is the first line of defence and is rapid and non-specific but lacks memory of pathogens; therefore, it does not need prior experience of a pathogen to mount an attack, but subsequent challenges by the same pathogen result in a similar response to that of the first exposure (Sharma, 1991). Adaptive immunity, however, is very slow to reach protective levels upon an initial exposure to a pathogen, but class switching occurs and the memory B cells generated are stored, which permits a rapid and specific defence against subsequent exposures to the same pathogen. At hatching, birds are quite vulnerable to environmental pathogens, as the immune system has not matured (Apanius, 1998; Glick, 1983a,b; Rose et al., 1981). At this early stage of life there is a strong reliance on maternal antibodies (Brambell, 1970) and innate immune function (Levy, 2007), whilst the adaptive immune system gradually develops in response to microflora colonisation (Klasing, 2004; Klasing and Leshchinsky, 1999). Immune function is adaptively developed and regulated, and variation in immune defence in birds has been found between seasons (as reviewed by Nelson and Demas, 1996), life stages (Evans et al., 2015; Pap et al., 2010) and environments (Buehler et al., 2008, 2009).

The antigen exposure hypothesis suggests that in environments where there is a greater amount of microbial pathogen exposure there will be a stronger immune response and investment across generations (Horrocks et al., 2012a). However, microbial exposure during the development of the immune system can impact

[1]Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia. [2]Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, Geelong, VIC 3217, Australia. [3]Department of Animal Science, University of California, 1 Shields Avenue, Davis, CA 95616, USA.

*Author for correspondence (Jessica.evans@deakin.edu.au)

J.K.E., 0000-0003-0900-5636

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subsequent functioning greatly; for example, neonatal chickens and mice raised in germ-free laboratory environments have a reduced functional immune capacity into adulthood, compared with offspring raised in conventional environments (Bedrani et al., 2013; Lee and Mazmanian, 2010). To some extent, this has also been found in two species of wild bird, where microbial exposure was positively correlated with various measures of the innate immune response (Buehler et al., 2008; Horrocks et al., 2012a,b). The mechanisms behind such correlations remain unclear and further experimental studies outside of domestic poultry are required.

A second explanation for variation in immune function is the trade-off hypothesis (Sheldon and Verhulst, 1996), which predicts that consumption of limited nutritional resources by the immune system leads to trade-offs between immune function and other resource-demanding activities such as reproduction (Ilmonen et al., 2000; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Sheldon and Verhulst, 1996). Production of acute phase proteins and lymphocytes can be nutritionally costly (Lee and Klasing, 2004), and the hypertrophy of the liver, which rapidly produces acute phase proteins, is also significant (Iseri and Klasing, 2014). Decreased intake of food and inefficient digestion are other important consequences of a robust immune response (Iseri and Klasing, 2014). Examples of a trade-off between immune function and other physiological processes in passerine birds in the wild include an increase in metabolic energy expenditure following an experimental challenge by the injection of sheep red blood cells (SRBCs) (Ots et al., 2001) and increases in metabolic rate following injection of phytohaemagglutinin (PHA) (Martin et al., 2003). In the laboratory, a trade-off between immune function and reproduction has been demonstrated through a negative relationship between antibody titres and the number of offspring in nesting zebra finches, *Taeniopygia guttata* (Deerenberg et al., 1997). In another wild bird, the basophilic inflammation response to a PHA challenge resulted in reduced nestling growth rates (Soler et al., 2003).

In the zebra finch, faeces accumulate in the nest as nestlings grow and defecate, exposing them continuously to faecal bacteria (Benskin et al., 2009) and the community of bacteria that presumably builds up in the nest over the period of use. In this study, by experimentally manipulating nest bacterial communities and nest hygiene, we sought to determine whether bacterial load within the nesting environment impacts the innate and adaptive immune response in breeding adult zebra finches and their developing nestlings, and also examined potential trade-offs in nestling growth. Together, these data allow a robust experimental test for the environmental influences on immunity and the trade-off hypothesis, by testing both the innate and the cellular components of immunity. Fundamentally, we sought to test the hypothesis that exposure to microorganisms in the nest environment leads to robust immune defences, with the possibility that nest sanitation behaviour causes variation in the immune response.

MATERIALS AND METHODS

Birds

Adult zebra finches (*Taeniopygia guttata* Reichenbach 1862, *N*=42) were sourced from local breeders and maintained under standardised conditions of 14 h:10 h light:dark photoperiod, 21–24°C, with 40–50% relative humidity. Birds were initially kept in single-sex groups for 2 weeks, and then were able to choose a mate. The pairs were then kept in cages (Terenziani, Montichiari, Italy; 50 cm high×50 cm wide×100 cm long) and provided with seed (GoldenCob, Wodonga, VIC, Australia), cucumber, eggs, shell grit and water *ad libitum*. Pairs were provided with nest boxes and nesting material at the time of pairing. Nests were monitored daily after the onset of egg laying to determine the hatch date of nestlings and post-hatch timings. Nestlings were marked with individual coloured non-toxic nail varnish on the nail for identification. There were 10 pairs (20 birds) in the clean nest treatment and 11 pairs (22 birds) in the dirty treatment (see below). To ensure adequate power, we used sample sizes similar to those of previous studies that have assessed either adaptive immunity in zebra finches (Deerenberg et al., 1997) or microbial killing in birds (Evans et al., 2015).

Manipulation of nest hygiene

Nests were randomly allocated to two treatment groups that differed in their level of nest hygiene: ‘clean’ and ‘dirty’. For clean nests, nesting material was replaced with fresh sterilised (autoclaved) nesting material within 2 days of hatching, and was continuously replaced with sterilised material twice weekly until nestlings were 30 days of age, by which time the nestlings had completely fledged from the nest (ca. 20 days). In this treatment, the replacement of the nest material removed all nestling faeces at least twice weekly so that the nests remained clean. For dirty nests, 5 g of chicken faeces was added on the interior surface of the nest cup within 2 days of hatching, and 2 g of chicken faeces (from free-ranging chickens) was further added twice weekly for 30 days. This was in addition to the natural finch faeces deposited in the nest. Chicken faeces were pulverised and mixed with distilled water to create a thick paste for spreading around the nest cup. Dirty nests were also sham cleaned so that nestlings were disturbed for the same time period as nestlings from clean nests and the nesting cup was moved gently around the box. Gloves were used in the handling of clean and dirty nest material, nestlings and adults. Bacterial abundance within the nests was assessed using bacterial agar paddles (Hycheck paddles, Micromedia, Moe, VIC, Australia) the day after nesting material changes (clean) or the addition of chicken faeces (dirty) at around day 7 after hatching.

Blood sampling and microbial killing assay

Adult birds were bled at three different time points and assayed for microbial killing capacity: (i) during incubation (ca. 10 days after clutch completion); (ii) 10 days after nestlings hatched; and (iii) 20 days after nestlings hatched (see Fig. 1). Comparing across these periods gave an indication of how innate immune function changes across reproductive stages. In addition, blood samples from adults were taken at 27 and 34 days post-hatch for the purpose of antibody detection (after SRBC injection at 20 days).

Two separate groups of offspring were assayed for microbial killing. One group (G1; Fig. 1) were tested as nestlings, before fledging (18–20 days of age) and after fledging (25–27 days of age). The second group (G2; Fig. 1) were assayed as sexually mature birds, after 60 days of age to test whether rearing environment had an impact on innate immune function into adulthood (see Fig. 1). Each offspring had three blood samples taken, where blood was used for testing microbial killing and antibody detection, but not all samples were used for microbial killing (if the blood collected was not adequate to test both immune measures for each sampling). Therefore, nestlings were blood sampled and injected at either 11–13 days (20 nestlings total, 10 in dirty nests, 10 in clean) or 60 days (12 birds, 6 per treatment) with SRBCs, with two blood samples occurring over the following fortnight. Only primary (day 7 after injection) and secondary (day 14 after injection) blood samples

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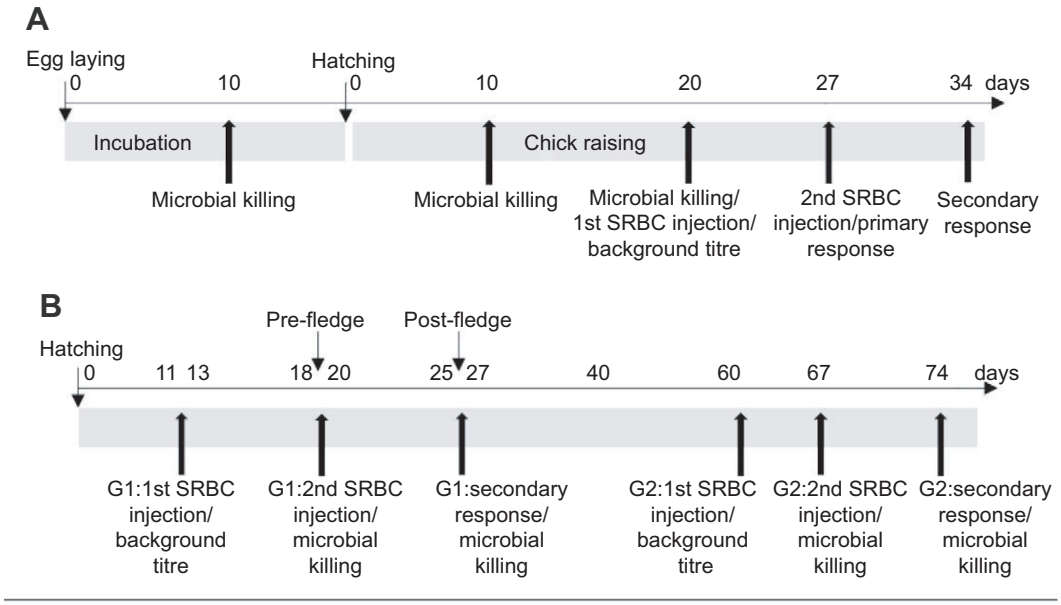

Fig. 1. Timeline of immune measurements in adults and their offspring. Timeline of blood sampling and immune measurements including microbial killing and secondary responses to sheep red blood cell (SRBC) injections in (A) adults and (B) offspring. The bold arrows along the timeline indicate a blood sample being taken and immune measurements at a given stage (3 blood samples taken in total for offspring, 5 for adults). The timings for adults were before offspring hatched (incubation) and after hatching (from day 0). Offspring timings were from hatching (day 0). Offspring were sampled at different ages in two groups (as nestlings or juveniles of 60 days) and are indicated as group 1 (G1) and group 2 (G2).

were used to assess microbial killing. Offspring in the nest were randomly allocated an age group of 9 days (results not reported here – collected for the purpose a parallel study on age-related changes), 11 days, 13 days or 60 days.

For both adults and their offspring, blood samples were collected in the morning between 09:00 h and 11:00 h and within 10 min of the experimenter entering the room. Blood was collected from the brachial vein and no more than 200 µl was taken at a time (<2% body mass). Blood for microbial killing tests was used within 1 h of sampling.

To assess the variation in microbial killing ability, we followed the procedure of Millet et al. (2007) and Tieleman et al. (2005), under sterile working conditions. We used two different microbial strains: *Escherichia coli* (ATCC 8739) and *Candida albicans* (ATCC 10231). The use of *E. coli* in this assay tests killing that is complement dependent (Demas et al., 2011), involving circulating proteins in the blood that kill bacteria, and is a measure of humoral immune function. *Candida albicans* tests the ability of blood to kill via phagocytosis, a measure of cellular immune function. We reconstituted the lyophilized pellet (Microbiologics, St Cloud, MN, USA) in sterile PBS and made a working solution of approximately 300 colony forming units (CFU) per 100 µl (or 3000 CFU ml^{-1}). The working solution was made fresh daily. A control to test CFU was incubated with each batch of test plates. A negative control plate with only whole blood was also made for each bird to ensure sterility was maintained whilst sampling. Whole blood was diluted (1:10) in CO_2-independent medium (Gibco, Life Technologies, NY, USA) containing 4 mmol l^{-1} L-glutamine (Sigma Aldrich, Castle Hill, NSW, Australia) and the test microbe. Diluted blood was incubated for 30 min (for *E. coli*) or 2 h (for *C. albicans*) at 41°C. Following incubation, 50 µl of diluted blood was spread on soy tryptic agar plates in duplicate (total 100 µl) using sterile spreaders. The plates were inverted and incubated at 37°C overnight (*E. coli*) or for around 48 h (*C. albicans*) and the colonies were then counted visually across the whole plate (blind to the sample identity). All plates, except for negative controls, were prepared in duplicate. For both strains, we calculated the proportion killed as the average number of colonies on experimental plates relative to the average number of colonies on the CFU control plates {[1−(average number of CFU on experimental plate/average number of CFU on control plate)]×100}. No negative controls contained CFUs.

Vaccination

To test the adaptive antibody response, we used SRBCs, which possess a variety of antigens that are not considered pathogenic. The antibody response to SRBCs indicates the ability of the humoral immune system to mount a response to foreign antigens (Bacon, 1992; Deerenberg et al., 1997). To make the vaccine of SRBCs, sheep blood in Alsever's solution was spun at 2000 rpm (295 ***g***) for 15 min and the Alsever's solution discarded. The blood cells were washed 3 times in 1× phosphate buffered saline (PBS), and then prepared as 40% sheep red blood cells by volume in PBS. The preparation was made fresh daily. Adults were blood sampled 20 days after their offspring had hatched, to assess the initial level of natural antibodies to SRBCs and part of the blood sampled at this time was used for microbial killing. The two offspring groups also had background antibodies assessed at 11–13 days or 60 days. The blood collected from adults and nestlings for all background, primary and secondary responses was spun at 14,000 rpm in a benchtop centrifuge for 15 min with the plasma stored at −80°C for later analysis. Adults and offspring were weighed prior to each vaccination to enable administration of the correct dose of SRBCs (50 µl of 40% SRBCs per 13 g bird). At the time of blood sampling, the vaccination was administered intramuscularly into the left breast. Seven days after the first vaccination, blood was taken for analysis of the primary immune response and the plasma stored. However, nestlings sampled at this time (18–20 days) for the primary response did not show any titre when analysed and therefore only the secondary response is mentioned for nestlings throughout the rest of the paper. The second vaccination of 40%

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SRBC was then administered into the right breast. A blood sample to analyse the secondary immune response was taken 7 days after the second vaccination (blood sample at 34 days post-hatch for adults, 25–27 days of age for nestlings, 74 days of age for sexually mature offspring). The blood was spun and the plasma stored at −80°C. Plasma from each blood sample (initial, primary response, secondary response) was then analysed using an agglutination assay.

Agglutination assay

The agglutination assay was conducted to test the humoral component of the immune system and antibody response, and followed previous protocols (Deerenberg et al., 1997), although we used 15 µl of unheated plasma for each sample serially diluted in PBS. A 15 µl sample of 2% SRBC was added to each well, and the plate was gently tapped, covered with clingfilm and incubated in a waterbath for 1 h at 37°C. Agglutination was scored twice, blind to the sample identity, by the same person. Plates were then scanned on an Epson flatbed scanner (model V700). Lysis scores were recorded 1 h after incubation. The visualisation of lysis (cloudiness in wells) indicates the ability of complement to lyse the red blood cells.

Statistics

Data was analysed using SPSS (version 22). Normality of the data was assessed using Shapiro–Wilk tests. To test the effect of nest treatment on humoral and cellular innate immune measures across the breeding period, microbial killing data of *E. coli* and *C. albicans* were analysed separately using general linear mixed models [with restricted maximum likelihood (REML) estimation] where bird ID was used to account for repeated measures. Fixed factors in the model included treatment, stage of breeding and sex. Potential nest effects that may influence condition were included as covariates, such as number of nestlings per nest and body mass. For all models described, all two-way interaction terms and single variables were included initially in each model and then interactions were removed sequentially by highest *P*-value for those interactions with $P>0.10$. Repeatability of microbial killing was also tested. Models with both random intercept and random intercept with random slope were calculated and the best model was selected based on the Akaike information criterion (AIC) and degrees of freedom. Random intercept was chosen as the best model for the adult data. The estimated means (±s.e.m.) are presented throughout the Results (unless otherwise indicated) and actual means can be found in Table 2. Note that there are four microbial killing samples (two nests) and one additional sample (in *C. albicans* killing only) missing across nest treatments during incubation. This was due to either the unavailability of microbes (for a week during the work) or a failure to collect enough blood to measure all functions (the additional bird). To test the relatedness, relationship and immune strategies between the innate immune measures during breeding, a Pearson's product moment correlation between the killing of both microbes for adults from both treatments was assessed at incubation, and 10 and 20 days post-hatch. To test the effect of the nest treatment on the primary and secondary immune response, agglutination was assessed using general linear mixed model (with REML estimation) with nest ID used as a random effect to account for non-independence of data and potential nest effects. For both the primary and secondary immune response (and lysis), the analysis was run with main effects of treatment and sex, with the number of offspring and mass as covariates. Interactions were dealt with in the same manner as above. For the assessment of nest treatment on immune function of offspring, a general linear mixed model was used. To account for non-independence of data and potential nest effects, nest ID was included as a random factor in all linear mixed models. Model parameters used to assess whether there were differences in agglutination and microbial killing included treatment, sex, mass and number of nestlings. Sexually mature (60 day) offspring were tested separately using the same parameters. Growth rate was calculated from the percentage increase in mass between day 5 and day 10 [(day 10 mass−day 5 mass/day 5 mass)×100]. When testing relationships between growth rate and microbial killing, only the final sample of blood (day 25–27) was used as this was an age where the birds were big enough to allow a sufficient amount of blood to be taken for testing microbial killing for every individual. Parent–offspring immune relationships were assessed by taking the nest average of nestlings (aged 25–27 days) for microbial killing or agglutination (secondary response) and running a correlation between the father, mother or mid-parent (average of the mother and father) 20 day microbial killing data or secondary agglutination scores.

Ethics statement

All experimental procedures were approved by the Deakin University Animal Ethics Committee (permit no. G40-2013).

RESULTS

Nest treatment

Data from the agar paddles confirmed that the experimental treatment increased the abundance of bacteria in dirty nests: there was a significantly higher number of colonies collected with agar paddles swabbing dirty nests compared with clean nests (independent samples *t*-test; $t=12.91$, d.f.=19, $P<0.001$), with dirty and clean nests having a mean of 155.64±10.22 and 14.6±2.09 colonies per 7 cm^2, respectively.

Table 1. Statistics for adult immune measures

	F	d.f.	*P*
Microbial killing: *E. coli*			
Nest treatment	0.28	1,121	0.87
Stage (incubation, 10 days, 20 days)	10.38	2,121	**<0.001**
Sex	0.29	1,121	0.59
Mass	0.15	1,121	0.70
Number of offspring	0.82	1,121	0.37
Microbial killing: *C. albicans*			
Nest treatment	0.15	1,120	0.70
Stage (incubation, 10 days, 20 days)	1.70	2,120	0.19
Sex	2.54	1,120	0.12
Mass	1.72	1,120	0.20
Number of offspring	0.83	1,120	0.37
Primary response			
Nest treatment	4.24	1,40	**0.05**
Sex	0.002	1,40	0.97
Mass	1.48	1,40	0.25
Number of offspring	0.000	1,40	0.997
Treatment×sex	4.32	1,40	**0.05**
Secondary response			
Nest treatment	0.001	1,40	0.98
Sex	2.65	1,40	0.12
Mass	0.17	1,40	0.90
Number of offspring	0.15	1,40	0.71
Treatment×sex	5.48	1,40	**0.03**
Lysis			
Nest treatment	1.15	1,40	0.29
Sex	0.08	1,40	0.78
Mass	0.01	1,40	0.91
Number of offspring	0.22	1,40	0.64

Significant *P*-values are in bold.

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Table 2. Mean microbial killing of *E. coli* and *C. albicans* during breeding

	Clean nest (% killed)			Dirty nest (% killed)		
Stage	Males	Females	Total	Males	Females	Total
Escherichia coli						
Incubation	76.5±8.5 (*N*=9)	65.6±8.7 (*N*=9)	71.1±6.0 (*N*=18)	66.7±7.3 (*N*=10)	64.0±9.5 (*N*=10)	65.3±5.9 (*N*=20)
10 days post-hatch	60.1±11.5 (*N*=10)	55.0±9.5 (*N*=10)	57.5±7.3 (*N*=20)	55.7±7.0 (*N*=11)	58.7±10.5 (*N*=11)	57.3±6.2 (*N*=22)
20 days post-hatch	56.3±12.7 (*N*=10)	35.2±11.3 (*N*=10)	45.7±8.6 (*N*=20)	41.6±12.4 (*N*=11)	45.7±8.7 (*N*=11)	43.8±7.3 (*N*=22)
Candida albicans						
Incubation	40.6±7.7 (*N*=8)	51.6±4.1 (*N*=9)	46.4±4.3 (*N*=17)	44.3±5.2 (*N*=10)	50.1±4.1 (*N*=10)	47.2±3.38 (*N*=20)
10 days post-hatch	38.0±3.5 (*N*=10)	38.6±6.1 (*N*=10)	38.3±3.4 (*N*=20)	38.8±7.5 (*N*=11)	50.2±6.2 (*N*=11)	44.5±4.9 (*N*=22)
20 days post-hatch	34.3±6.0 (*N*=10)	47.1±2.9 (*N*=10)	40.7±3.6 (*N*=20)	45.8±4.7 (*N*=11)	41.3±6.8 (*N*=11)	43.5±4.1 (*N*=22)

Data are means±s.e.m.

Adult immune function

Microbial killing

Microbial killing of *E. coli*

The microbial killing capacity via complement proteins in the whole blood of adult zebra finches against *E. coli* was significantly repeatable across individuals and across reproductive stages (r=0.42±0.056). However, microbial killing of *E. coli* in adults attending clean and dirty nests at different stages was not significantly different (56.01±6.07% versus 57.35±5.72%; see Tables 1 and 2), suggesting circulating proteins involved in complement-mediated immunity did not differ in birds attending nests with varying levels of sanitation. Despite the repeatability of microbial killing measures within individuals over time, there was a decrease in microbial killing capacity from incubation throughout nestling rearing (Tables 1 and 2, Fig. 2). *Post hoc* tests indicated that microbial killing of *E. coli* was significantly higher for adults at incubation compared with 20 days after their offspring hatched (P<0.001), and *E. coli* killing 10 days after their offspring hatched was significantly higher than at 20 days after their offspring hatched (P=0.02). There was no significant difference in the microbial killing of *E. coli* in adult males and females and it was not related to an individual's mass or the number of offspring they were rearing.

Microbial killing of *C. albicans*

The microbial killing capacity and level of phagocytosis of adult zebra finch blood against *C. albicans* was also significantly repeatable for an individual across stages of incubation, 10 days after offspring hatched and 20 days after offspring hatched (r=0.35±0.059). However, there was no significant treatment effect, with no difference in killing of *C. albicans* in adults attending clean versus dirty nests (Tables 1 and 2, Fig. 2; mean of 42.86±3.13% versus 44.38±2.94%). This suggests that mechanisms inducing phagocytosis may not be stimulated by variation in bacteria in the nesting environment. Furthermore, the killing capacity of adults against *C. albicans* did not differ between incubation, 10 days post-hatch or 20 days post-hatch (Tables 1 and 2, Fig. 2). Overall, there were no significant differences in the killing of *C. albicans* between males and females, and killing capacity was not related to the number of nestlings that they were rearing or to adult body mass.

Relationship between *E. coli* and *C. albicans*

For adults, there was no significant relationship between the killing of *E. coli* and *C. albicans* at incubation (r=−0.14, N=37, P=0.41) or at 10 days after their offspring hatched (r=0.26, N=42, P=0.09). However, at 20 days after their offspring hatched, there was a significant positive relationship between the microbial killing capacity for *E. coli* and *C. albicans* (r=0.35, N=42, P=0.02).

Agglutination

Background analysis

The initial blood sample for all adults and offspring had a zero agglutination score.

Primary response

Nest treatment (clean/dirty nests) had a significant effect on agglutination score for blood samples taken on day 7 after injection of SRBCs (Table 1, Fig. 3). Agglutination scores were higher in adults attending dirty nests (3.21±0.48; Fig. 3) compared with clean nests (1.52±0.48), indicating that antibody titres were higher in adults from dirty nests. There was also an interaction between sex and nest treatment. Further analysis suggested that titres were higher in males from dirty compared with clean nests ($F_{1,20}$=7.61, P=0.01;

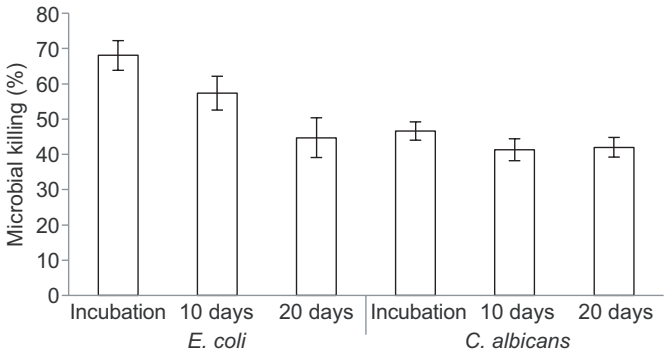

Fig. 2. Adult microbial killing during breeding and nestling rearing. Microbial killing capacity was assessed at: incubation (*E. coli*: N=38, *C. albicans* N=37); 10 days after offspring hatched (N=42); and 20 days after offspring hatched (N=42). Data are means±s.e.m.

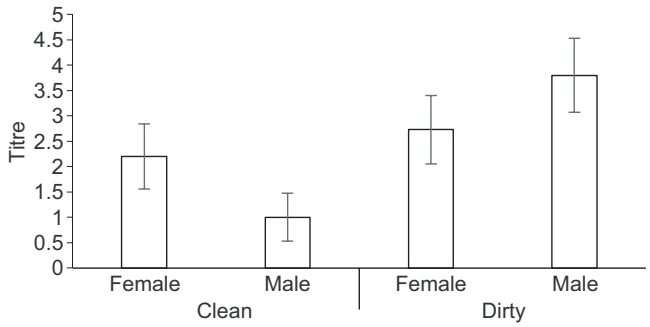

Fig. 3. Adult primary agglutination titres between nest treatments. Treatments were clean (female titres, N=10; male titres, N=10) and dirty (female titres, N=11; male titres, N=11). Data are means±s.e.m.

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Fig. 4. Adult secondary agglutination titres between nest treatments. Treatments were clean (female titres, *N*=10; male titres, *N*=10) and dirty (female titres, *N*=11; male titres, *N*=11). Data are means±s.e.m.

Fig. 3). There were no significant differences between females from the different treatments ($F_{1,20}$=0.46, P=0.51), or sexes within each treatment (clean: $F_{1,20}$=1.60, P=0.23; dirty: $F_{1,20}$=3.03, P=0.12). There was no significant effect of adult body mass, the number of nestlings they were rearing or overall sex differences.

Secondary response

Agglutination titres for blood collected on day 14 after injection of SRBCs were not significantly different between nest treatments (Table 1, Fig. 4), with scores of 6.48±0.57 and 6.05±0.56 for adults attending clean and dirty nests, respectively. There was no significant relationship between agglutination score and adult sex, body mass and the number of nestlings that they were rearing. There was a significant interaction between sex and treatment (Table 1), with further analysis indicating that males had significantly lower agglutination than females in the clean treatment ($F_{1,20}$=10.79, P=0.01). However, there were no sex differences within the dirty treatment ($F_{1,20}$=0.25, P=0.63), and no differences in males between treatments ($F_{1,20}$=0.69, P=0.42) or between females ($F_{1,20}$=0.77, P=0.39). There was a significant positive relationship between agglutination and lysis ($F_{1,40}$=7.39, P=0.01), indicating a relationship between antibody titre and complement. Of 41 birds given a second challenge with SRBCs, only one female was a non-responder. Of these, 15 did not show any lysis. However, there was no significant relationship between lysis and nest treatment, sex, mass or the number of offspring they were rearing.

Difference in primary and secondary response

There was a correlation between the primary and secondary immune response (Pearson correlation 0.78, P<0.001, N=21) in adults attending dirty nests; however, there was no such relationship within clean nests (Pearson correlation −0.13, P=0.96, N=20). This indicates that adults attending dirty nests with lower agglutination titres in the primary response usually had lower titres in the secondary response. However, birds attending clean nests that had low (or zero) agglutination titres in the primary response did not have low titres in the secondary response, and matched the titre scores of those in dirty nests. The relative upregulation of the secondary response was calculated as the primary titre minus the secondary titre. There was a significant difference in upregulation of the secondary response between clean and dirty nests ($F_{1,40}$=5.3, P=0.026; Fig. 5), with adults attending clean nests having a greater titre difference (4.75±0.7) compared with those attending dirty nests (2.95±0.4). There were no differences between males and females in the agglutination titre differences ($F_{1,40}$=1.3, P=0.27).

Fig. 5. Differences in adult primary and secondary responses between nest treatments. The agglutination titre difference was analysed between clean (*N*=20) and dirty (*N*=22) nests. Data are means±s.e.m.

Table 3. Statistics for offspring immune measures

	F	d.f.	*P*
Before fledging – microbial killing			
Nest treatment	1.78	1,19	0.23
Mass	1.22	1,19	0.30
Sex	0.91	1,19	0.36
Number of siblings	0.32	1,19	0.59
After fledging – microbial killing			
Nest treatment	0.03	1,15	0.86
Mass	2.70	1,15	0.15
Sex	3.36	1,15	0.13
Number of siblings	1.17	1,15	0.32
Secondary response (agglutination)			
Nest treatment	0.06	1,20	0.82
Sex	0.66	2,20	0.55
Number of siblings	0.09	1,20	0.78
Mass	0.06	1,20	0.81
60 day old offspring			
Nest treatment (killing *E. coli*)	1.2	1,12	0.30
Nest treatment (killing *C. albicans*)	0.39	1,12	0.55
Nest treatment (secondary response)	0.18	1,12	0.68

Offspring immune function

Microbial killing of *E. coli*

In nestlings, microbial killing was tested before fledging (18–20 days) and after fledging (25–27 days). Before fledging, nestlings did not differ in their ability to kill *E. coli* between nest treatments (Table 3), with estimated means for birds from clean and dirty nests of −7.7±5.7% and 3.9±5.5%, respectively. There was no difference in microbial killing between males and females, and microbial killing was not affected by body mass or the number of siblings a nestling had. After fledging, there was also no significant difference between the immune response of nestlings from clean and dirty nests (Table 3; killing: clean 2.58±9.31%, dirty 12.59±9.83%), and there were no differences across the two sexes (Table 3). There was no significant effect of body mass on nestling microbial killing or the number of siblings in the nest.

Agglutination response

The secondary response of nestlings (secondary response at 25–27 days, N=20) showed no significant difference in agglutination titres between nest treatments (Table 3, Fig. 6). There were no differences in titres between males and females, nor was titre affected by the number of siblings or mass at the time of sampling (Table 3).

Growth rates

The growth rate of nestlings was not affected by nest treatment ($F_{1,23}$=0.53, P=0.48), sex ($F_{2,23}$=2.36, P=0.13) or number of

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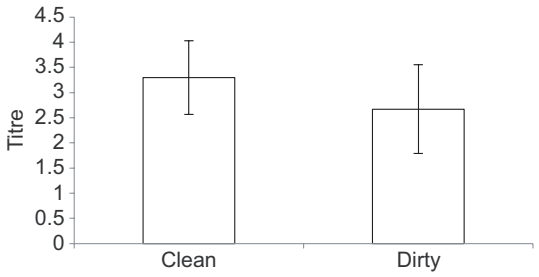

Fig. 6. Nestling secondary total agglutination titres between nest treatments. *N*=11 birds per nest treatment. Data are means±s.e.m.

siblings ($F_{1,23}$=3.50, P=0.08). No relationship was found between growth rate and microbial killing of *E. coli* ($F_{1,23}$=1.88, P=0.19) or between growth rate and agglutination score ($F_{1,23}$=0.70, P=0.80).

Immune function of offspring at 60 days of age

Nest treatment did not impact killing of *E. coli* in the 60 day age group (Table 3). Similarly, microbial killing of *C. albicans* was not affected by nest treatment (Table 3). There was no significant difference between offspring that were tested in adulthood from the two nest treatment groups with respect to agglutination (Table 3), with birds from clean nests having mean agglutination titres to SRBCs of 5±1.45 and birds from dirty nests having agglutination titres of 5.75±1.02.

Parent and offspring relationships

Whilst controlling for treatment group, we found no evidence of any correlation between nestling innate immune function (with *E. coli* killing at 23–27 days of age) and the immune function of their parents (at 20 days after offspring hatching; see Table S1). To compare adaptive immune function, we tested the average secondary titres of offspring from the same nest and parental secondary response, and found no correlation between the mother, father or mid-parent value (i.e. the average of the parental values). Similarly, there were no relationships between the offspring values at 60 days old and parental killing of *E. coli*, *C. albicans* or agglutination (see Table S1).

DISCUSSION

We found that increasing nest bacterial load up-regulated the adaptive humoral response in adults that attended dirty nests, predominantly the primary response, which can be attributed to the difference in males between treatments. However, manipulation of nest bacteria did not impact either the complement/humoral components of immunity (as tested by the killing of *E. coli*) or the cellular component (phagocytosis, as tested by the killing of *C. albicans*) in either adults or their offspring. Therefore, our results suggest that nest sanitation affects aspects of adaptive immunity, but not the innate immune measures taken in our study of adult zebra finches. Previous studies testing the impact of environmental pathogens, including bacteria, have mostly focused on innate mechanisms, rather than adaptive immunity (Horrocks et al., 2012a,b). However, despite these findings in adults, we found no difference in the adaptive immunity of nestlings as a result of our experimental nest treatments. This latter result is interesting and somewhat surprising, as we would expect nestlings to be impacted by microbes in the nest, where they spend more time in the nest than adults. Also surprising is that our measures of innate immunity were not impacted by the nest manipulations in either adults or nestlings. Reducing the hygiene of the nest, and in turn increasing the birds' exposure to a higher abundance of presumably novel bacteria, has been hypothesised to up-regulate microbial killing mechanisms such as phagocytosis and complement proteins, which are relevant for non-specific defence against microorganisms and are mechanisms that should be flexible in varying conditions (Buehler et al., 2008; Millet et al., 2007).

Although not expected, in adults there was a significant difference between those in the clean and dirty nest treatments for the primary immune response, but not the secondary response, although this result can largely be attributed to the low titre of males in clean nests. In the secondary response, males also had a lower titre than females, but only in the clean treatment. This result could indicate that the adaptive immune system was upregulated in adults, particularly males, attending dirty nests versus clean nests. This also suggests that males may be more immunologically affected by the nest treatment than females. This could be potentially due to differences in incubation or nest-cleaning behaviours and the amount of time spent on the nest; however, this was not measured. A previous study of zebra finches by McGraw and Ardia (2005) found that males had a significantly higher humoral response to SRBCs, but females had a higher cell-mediated immune response. However, the clean nest treatment seems to have had the opposite effect in our study. To account for individual differences in adaptive immune function, we tested the difference in titre scores between the primary and secondary antibody response. We found that adults with clean nests had a greater relative secondary response than those with dirty nests. Because the absolute level of the secondary response did not differ across treatment, the relatively greater secondary response in adults with clean nests can be attributed to low initial titre scores in the primary response. It is possible that the primary immune response in adults exposed to dirty nests was higher as a result of upregulation to defend against more frequent microbial challenges (priming effect), whereas birds from clean nests had fewer challenges and consequently less priming. The lack of an effect of nest sanitation on the secondary response was not expected, but indicates that the primary exposure resulted in similar rates of class switching and production of memory B cells, even though the initial rate of IgM production was low in adults attending clean nests. It is possible that the increased titre indicates that the adults were sick from introduced pathogenic bacteria; however, if this was the case, the titre in the background agglutination analysis would be higher, and it was not. This could also be the case for nestlings; however, the lack of difference in immune measures, as well as growth rate, may suggest otherwise. It should also be noted that we changed the nest material in one treatment but not in the other, and that may have affected the results. Although we tried to control for this by sham-cleaning the dirty nest, by moving the nest cup around and handling nestlings for the same period of time, it may be a confounding factor.

Microbial killing of *E. coli* decreased in adults throughout nestling rearing in this study, regardless of nest treatment. This finding is consistent with a previous study by Evans et al. (2015) that demonstrated a decrease in microbial killing of *E. coli* between incubation and nestling rearing in little penguins (*Eudyptula minor*). In a broader context, Pap et al. (2010) found that microbial killing of *E. coli* was at its lowest during breeding compared with the rest of the annual cycle in house sparrows (*Passer domesticus*). As there was no difference in microbial killing as a result of nest treatments in our current study, it is possible that the decrease is due to a physiological trade-off or potentially a decrease in nutritional intake as a result of the challenge of supporting growing nestlings. It has previously been demonstrated that there is a decrease in constitutive

innate immunity during energy-demanding periods (De Coster et al., 2010; Nebel et al., 2012; Pap et al., 2010). Breeding and care of offspring require a reallocation of resources, as these tasks are nutritionally demanding (Moreno, 2004) and are among the most energetically expensive events for birds (Tinbergen and Williams, 2002). The microbial killing of *C. albicans* did not follow the same trend as the killing of *E. coli*, and remained constant throughout nestling rearing. This result was not expected as other studies have found variation in *C. albicans* killing according to the environment (Buehler et al., 2008). It is possible that the chicken faeces added to the nest did not contain novel bacteria and thus did not result in challenges that triggered an immune response. However, this seems unlikely as the chicken faeces came from free-ranging chickens raised in a mixed eucalypt forest, fed a variety of pellets and vegetables, and thus represents a very different environment from that to which our captive zebra finches had been exposed. Interestingly, the killing of *E. coli* and *C. albicans* correlated in adults after 20 days post-hatch and there was a trend at 10 days post-hatch, but not during incubation. The correlation between the killing of *E. coli* and *C. albicans* was observed previously outside of the breeding period (Versteegh et al., 2012). Potentially, the differences in this correlation between incubation and nestling rearing could be attributed to differing immune strategies and balancing between nestling rearing, potential stressors (recapture/nest manipulations/frequent blood samples) and pathogen pressure. Future studies should measure indices of greater activation of the immune system due to microbial manipulation by examining levels of pro-inflammatory cytokines and of Ig specific to the microbes added.

Our experimental manipulations of nest sanitation had little impact on either the innate or adaptive immune measures of nestlings taken in our study. Exposure to a diverse range of microorganisms after hatching is important for the maturation of immune defence, particularly in the first few weeks of development. In the first few weeks after hatching, nestlings rely on innate immune function and maternal antibodies for protection against pathogens, during which time the adaptive immune response matures. The adaptive response begins to become functional at 11–14 days and can take around 6 weeks to mature in many species (Klasing and Leshchinsky, 1999). Furthermore, the nest treatment had no observed effects on mature offspring. There are a number of possible interpretations. It is possible that the immune system does not require much microbial exposure to maximise the development of the immune system, given that exposure to only a few bacteria can lead to full population of the skin and intestinal mucosa. This is a possibility as we chose chicken faeces to introduce novel bacteria into the nest. However, exposure to diverse ranges of microbiota in early life has been shown to increase activation of immune-related genes in a wide variety of animals (Hooper et al., 2012; Mazmanian et al., 2005; Mulder et al., 2011). Potentially, the number of bacteria being sampled by the bursa for driving the diversification of the B cells is sufficient to drive lymphocyte differentiation in both clean and dirty nests. Alternatively, other sources of bacteria from the skin and saliva of parents (Kyle and Kyle, 1993; Mills et al., 1999) could be important in driving this diversification. There is also the possibility that small sample sizes may have contributed to a lack of difference between the nest treatments. Finally, it is possible that the offspring primary response in relation to nest hygiene was similar to that of adults (primary response); however, because of the age of the nestlings, we were unable to obtain that measurement and, as such, future studies could test this possibility.

We did not find any evidence of a relationship between the immune response in adults and that in their nestlings, unlike an earlier study that found a link with the mother's immune function (Stambaugh et al., 2011). However, a more comprehensive and experimental study by Pitala et al. (2007) found that the immune response was not heritable in 1626 collared flycatcher (*Ficedula albicollis*) nestlings that had mostly been cross-fostered. Evidence of variation in heritability exists in tree swallows (*Tachycineta bicolor*), where out of three different geographic locations, only one location showed heritability of immune response to PHA (Ardia and Rice, 2006). Our sample size and approach was rather small to address this question, and the source of variation in offspring immunity remains to be determined.

In conclusion, manipulation of bacterial load and sanitation in the nest did not impact measures of the innate immune function of either nestlings or adults. But, increasing the microbial load of the nest increased the relative magnitude of the secondary immune response above the primary response to SRBCs in adults. However, nestling immune function was not influenced by nest sanitation and future studies investigating the bacterial impact on immune function should test this further. Decreases in microbial killing of *E. coli*, a functional test of complement activity, during the nutritionally expensive period of nestling rearing may provide evidence for the trade-off hypothesis; however, a long-term study of birds, both in captivity and in the wild, is necessary to confirm this observation. Future studies should include analysis of the types of bacteria in the nest paired with immune function tests to get a further understanding of what might affect variation in immune function.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
J.K.E. designed the study, carried out all experiments, analysed the data, interpreted the findings and wrote the manuscript. K.C.K., S.C.G. and K.L.B. designed the study, interpreted the findings and contributed to the writing of the manuscript.

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References
Apanius, V. (1998). Ontogeny of immune function. *Oxf. Ornithol. Ser.* **8**, 203-222.
Archie, E. A. and Theis, K. R. (2011). Animal behaviour meets microbial ecology. *Anim. Behav.* **82**, 425-436.
Ardia, D. R. and Rice, E. B. (2006). Variation in heritability of immune function in the tree swallow. *Evol. Ecol.* **20**, 491-500.
Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A. and Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science* **307**, 1915-1920.
Bacon, L. D. (1992). Measurement of immune competence in chickens. *Poult. Sci. Rev.* **4**, 187-195.
Bedrani, L., Helloin, E., Guyot, N., Réhault-Godbert, S. and Nys, Y. (2013). Passive maternal exposure to environmental microbes selectively modulates the innate defences of chicken egg white by increasing some of its antibacterial activities. *BMC Microbiol.* **13**, 128.
Benskin, C. M. H., Wilson, K., Jones, K. and Hartley, I. R. (2009). Bacterial pathogens in wild birds: a review of the frequency and effects of infection. *Biol. Rev.* **84**, 349-373.
Berger, S., Disko, R. and Gwinner, H. (2003). Bacteria in starling nests. *J. Ornithol.* **144**, 317-322.

Brambell, F. W. R. (1970). *The Transmission of Passive Immunity from Mother to Young*. Amsterdam: North-Holland Publishing Co.
Brandl, H. B., van Dongen, W. F. D., Darolová, A., Krištofík, J., Majtan, J. and Hoi, H. (2014). Composition of bacterial assemblages in different components of reed warbler nests and a possible role of egg incubation in pathogen regulation. *PLoS ONE* **9**, e114861.
Buehler, D. M., Piersma, T. and Tieleman, B. I. (2008). Captive and free-living red knots Calidris canutus exhibit differences in non-induced immunity that suggest different immune strategies in different environments. *J. Avian Biol.* **39**, 560-566.
Buehler, D. M., Tieleman, B. I. and Piersma, T. (2009). Age and environment affect constitutive immune function in Red Knots (Calidris canutus). *J. Ornithol.* **150**, 815-825.
De Coster, G., De Neve, L., Martin-Galvez, D., Therry, L. and Lens, L. (2010). Variation in innate immunity in relation to ectoparasite load, age and season: a field experiment in great tits (Parus major). *J. Exp. Biol.* **213**, 3012-3018.
Deerenberg, C., Arpanius, V., Daan, S. and Bos, N. (1997). Reproductive effort decreases antibody responsiveness. *Proc. R. Soc. B Biol. Sci.* **264**, 1021-1029.
Demas, G. E., Zysling, D. A., Beechler, B. R., Muehlenbein, M. P. and French, S. S. (2011). Beyond phytohaemagglutinin: assessing vertebrate immune function across ecological contexts. *J. Anim. Ecol.* **80**, 710-730.
Evans, J. K., Dann, P. and Frankel, T. (2015). Variation in innate immune function during incubation, chick-rearing and moult in Little Penguins (Eudyptula minor). *Emu* **115**, 63-71.
Glick, B. (1983a). *Bursa of Fabricius*. New York; London: Academic Press.
Glick, B. (1983b). The saga of the bursa of fabricius. *Bioscience* **33**, 187-191.
Godard, R. D., Wilson, C. M., Frick, J. W., Siegel, P. B. and Bowers, B. B. (2007). The effects of exposure and microbes on hatchability of eggs in open-cup and cavity nests. *J. Avian Biol.* **38**, 709-716.
Guigueno, M. F. and Sealy, S. G. (2012). Nest sanitation in passerine birds: implications for egg rejection in hosts of brood parasites. *J. Ornithol.* **153**, 35-52.
Hooper, L. V., Littman, D. R. and Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science* **336**, 1268-1273.
Horrocks, N. P. C., Hegemann, A., Matson, K. D., Hine, K., Jaquier, S., Shobrak, M., Williams, J. B., Tinbergen, J. M. and Tieleman, B. I. (2012a). Immune indexes of larks from desert and temperate regions show weak associations with life history but stronger links to environmental variation in microbial abundance. *Physiol. Biochem. Zool.* **85**, 504-515.
Horrocks, N. P. C., Matson, K. D., Shobrak, M., Tinbergen, J. M. and Tieleman, B. I. (2012b). Seasonal patterns in immune indices reflect microbial loads on birds but not microbes in the wider environment. *Ecosphere* **3**, 14.
Ilmonen, P., Taarna, T. and Hasselquist, D. (2000). Experimentally activated immune defence in female pied flycatchers results in reduced breeding success. *Proc. R. Soc. B Biol. Sci.* **267**, 665-670.
Iseri, V. J. and Klasing, K. C. (2014). Changes in the amount of lysine in protective proteins and immune cells after a systemic response to dead Escherichia coli: implications for the nutritional costs of immunity. *Integr. Comp. Biol.* **54**, 922-930.
Klasing, K. C. (2004). The costs of immunity. *Acta Zool. Sin.* **50**, 961-969.
Klasing, K. C. and Leshchinsky, T. V. (1999). Functions, costs, and benefits of the immune system during development and growth. In *In 22nd International Ornithological Congress* (ed. N. J. Adams and R. H. Slotow), pp. 2817-2835. Durban, South Africa: BirdLife South Africa.
Kyle, P. D. and Kyle, G. Z. (1993). An evaluation of the role of microbial flora in the salivary transfer technique for hand-rearing Chimney Swifts. *Wildl. Rehabil.* **8**, 65-71.
Lee, K. A. and Klasing, K. C. (2004). A role for immunology in invasion biology. *Trends Ecol. Evol.* **19**, 523-529.
Lee, Y. K. and Mazmanian, S. K. (2010). Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science* **330**, 1768-1773.
Levy, O. (2007). Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat. Rev. Immunol.* **7**, 379-390.
Lochmiller, R. L. and Deerenberg, C. (2000). Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88**, 87-98.
Martin, L. B., Scheuerlein, A. and Wikelski, M. (2003). Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? *Proc. R. Soc. B Biol. Sci.* **270**, 153-158.
Mazmanian, S. K,, Liu, C. H., Tzianabos, A. and Kasper, D. (2005). An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* **122**, 107-118.
McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F. et al. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci. USA* **110**, 3229-3236.
McGraw, K. J. and Ardia, D. R. (2005). Sex differences in carotenoid status and immune performance in zebra finches. *Evol. Ecol. Res.* **7**, 251-262.
Millet, S., Bennett, J., Lee, K. A., Hau, M. and Klasing, K. C. (2007). Quantifying and comparing constitutive immunity across avian species. *Dev. Comp. Immunol.* **31**, 188-201.
Mills, T. K., Lombardo, M. P. and Thorpe, P. A. (1999). Microbial colonization of the cloacae of nestling tree swallows. *Auk* **116**, 947-956.
Moreno, J. (2004). Moult-breeding overlap and fecundity limitation in tropical birds: a link with immunity? *Ardeola* **51**, 471-476.
Mulder, I. E., Schmidt, B., Lewis, M., Delday, M., Stokes, C. R., Bailey, M., Aminov, R. I., Gill, B. P., Pluske, J. R., Mayer, C.-D. et al. (2011). Restricting microbial exposure in early life negates the immune benefits associated with gut colonization in environments of high microbial diversity. *PLoS ONE* **6**, e28279.
Nebel, S., Bauchinger, U., Buehler, D. M., Langlois, L. A., Boyles, M., Gerson, A. R., Price, E. R., McWilliams, S. R. and Guglielmo, C. G. (2012). Constitutive immune function in European starlings, Sturnus vulgaris, is decreased immediately after an endurance flight in a wind tunnel. *J. Exp. Biol.* **215**, 272-278.
Nelson, R. J. and Demas, G. E. (1996). Seasonal changes in immune function. *Q. Rev. Biol.* **71**, 511-548.
Norris, K. and Evans, M. R. (2000). Ecological immunology: life history trade-offs and immune defense in birds. *Behav. Ecol.* **11**, 19-26.
Ots, I., Kerimov, A. B., Ivankina, E. V., Ilyina, T. A. and Horak, P. (2001). Immune challenge affects basal metabolic activity in wintering great tits. *Proc. R. Soc. B Biol. Sci.* **268**, 1175-1181.
Pap, P. L., Czirják, G. A., Vágási, C. I., Barta, Z. and Hasselquist, D. (2010). Sexual dimorphism in immune function changes during the annual cycle in house sparrows. *Naturwissenschaften* **97**, 891-901.
Pitala, N., Gustafsson, L., Sendecka, J. and Brommer, J. E. (2007). Nestling immune response to phytohaemagglutinin is not heritable in collared flycatchers. *Biol. Lett.* **3**, 418-421.
Rose, M. E., King, A. S. and McLelland, J. (1981). *Lymphatic System*. London; New York etc: Academic Press.
Sharma, J. M. (1991). Overview of the avian immune system. *Vet. Immunol. Immunopathol.* **30**, 13-17.
Sheldon, B. C. and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**, 317-321.
Singleton, D. R. and Harper, R. G. (1998). Bacteria in old House Wren nests. *J. Field Ornithol.* **69**, 71-74.
Soler, J. J., de Neve, L., Perez-Contreras, T., Soler, M. and Sorci, G. (2003). Trade-off between immunocompetence and growth in magpies: an experimental study. *Proc. R. Soc. B Biol. Sci.* **270**, 241-248.
Stambaugh, T., Houdek, B. J., Lombardo, M. P., Thorpe, P. A. and Hahn, D. C. (2011). Innate immune response development in nestling tree swallows. *Wilson J. Ornithol.* **123**, 779-787.
Tieleman, B. I., Williams, J. B., Ricklefs, R. E. and Klasing, K. C. (2005). Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. *Proc. R. Soc. B Biol. Sci.* **272**, 1715-1720.
Tinbergen, J. M. and Williams, J. B. (2002). Energetics of incubation. *Avian Incub. Behav. Environ. Evol.* **13**, 299-313.
Versteegh, M. A., Schwabl, I., Jaquier, S. and Tieleman, B. I. (2012). Do immunological, endocrine and metabolic traits fall on a single Pace-of-Life axis? Covariation and constraints among physiological systems. *J. Evol. Biol.* **25**, 1864-1876.
Walls, J. G., Hepp, G. R. and Eckhardt, L. G. (2012). Effects of nest reuse and onset of incubation on microbial growth and viability of wood duck eggs. *Condor* **114**, 720-725.
Wang, J. M., Firestone, M. K. and Beissinger, S. R. (2011). Microbial and environmental effects on avian egg viability: do tropical mechanisms act in a temperate environment? *Ecology* **92**, 1137-1145.