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Variation in innate immunity in relation to ectoparasite load, age and season: a field experiment in great tits (*Parus major*)

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SUMMARY

It remains largely unknown which factors affect the innate immune responses of free-living birds. Nevertheless, the degree of innate immunity may play a crucial role in an individual's survival as it procures the first defence against pathogens. We manipulated the ectoparasite load of great tit (*Parus major*) nests by infesting them with hen fleas (*Ceratophyllus gallinae*) before egg laying. We subsequently quantified natural antibody (NAb) concentration and complement activation in nestlings and adult females during breeding and post-breeding periods. NAb concentrations increased in nestlings and adult females breeding in flea-infested nest boxes during the nestling provisioning period, but not in breeding females during incubation. In contrast, parasite abundance did not affect levels of complement activity in females. NAb levels of nestlings were already fully developed at the end of the nestling stage, but complement activation was only observed post-fledging. Concentrations of NAbs and complement activation of adult females were significantly lower during the breeding season compared with post-breeding levels, but did not differ between incubation and chick rearing. Further experimental studies in species that vary in life-history strategies will allow us to unravel the mechanisms underlying the observed variation in innate immune defences.

Key words: birds, hole-nesting, immune function, natural antibodies, parasites.

INTRODUCTION

The immune system of an organism comprises its main defence mechanism against pathogens (Zuk and Stoehr, 2002). Despite the high fitness value of a well-functioning immune system, the type and effectiveness of immune defences vary among species (Lee, 2006), possibly reflecting evolutionary benefits and costs related to development, maintenance and use (Klasing and Leshchinsky, 1999; Sheldon and Verhulst, 1996). For instance, immune activity entails energetic and nutritional costs that may trade-off against other vital life-history functions such as growth and reproduction (Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996). When the immune response is self-directed, additional costs may arise from immuno-pathological responses such as hypersensitivity and autotoxicity (Råberg et al., 1998). The strongest immune response may therefore not be the most optimal one for a given organism under a particular set of environmental conditions (Råberg et al., 1998; Sheldon and Verhulst, 1996). Individual immune responses have also been shown to vary with environmental conditions during development or reproduction, such as parasite load (e.g. Christe et al., 2000; de Lope et al., 1998; Morales et al., 2004). Because of the deleterious effects of parasites on their hosts, infected organisms can be expected to enhance their immune function (Janeway et al., 1999). Parasites are particularly deleterious to newly hatched chicks, which have poorly developed adaptive immune defences. Hatchlings must thus rely on innate immunity and maternally transferred antibodies for specific immunity, but the latter solely protect against pathogens to which the mother was exposed prior to egg laying and are only effective during the first weeks after hatching (Klasing and Leshchinsky, 1999). The immune system of altricial nestlings is assumed to be less well developed than that of precocial ones given their shorter incubation period (Ricklefs, 1992), while parasite exposure is thought to be higher given their lower mobility (Ardia and Schat, 2008). Parasites may therefore comprise a strong selection pressure in altricial nestlings, especially of hole-breeding species that frequently re-use their nests (Møller and Erritzoe, 1996).

Natural antibodies (hereafter NAbs) and the complement system are two humoral components of constitutive innate immunity that provide the first line of defence against invading pathogens (Thornton et al., 1994). Even though an early defence against pathogen infection may play a crucial role in enhancing an organism's survival (Ochsenbein and Zinkernagel, 2000), innate immunity has been less well investigated than acquired immunity in free-living birds. Natural antibodies (mostly IgM) are present in individuals that have not been immunized (reviewed in Bandeira et al., 1988). Their function includes the binding of antigens, clearing of foreign substances and initiation of the complement system, which is composed of circulating serum proteins that are sequentially activated to result in cell lysis [see Boes (Boes, 2000) and references therein]. While NAb and complement levels are known to augment quickly during development (e.g. Matson et al., 2005; Mauck et al., 2005), the rate of increase might vary depending on developmental characteristics such as developmental strategy (altricial or precocial) and growth rate (Matson et al., 2005; Mauck et al., 2005). Moreover, seasonal changes in immune function have been shown for a variety of bird species (reviewed in Martin et al., 2008) albeit to different extents (e.g. Hasselquist et al., 1999; Ilmonen et al., 2002; Moreno et al., 1999; Zuk and Johnsen, 1998). In general, non-specific immune defences (e.g. inflammatory responses) are thought to be downregulated, and specific ones upregulated, during the most

energy-demanding periods of the year (e.g. the breeding season) and in the sex that invests most (e.g. females during reproduction), probably because of the lower energetic costs associated with specific defences (Klasing and Leshchinsky, 1999; Lee, 2006). The costs associated with an upregulation of the constitutive innate immune system are also thought to be rather low (Klasing, 2004; Lee, 2006). Nevertheless, it has been demonstrated that high reproductive outcome (Bayyari et al., 1997), low body condition (Pomeroy et al., 1997) and fast wing growth (Mauck et al., 2005) are related to low natural antibody and/or complement titres, suggesting a decrease in constitutive innate immunity during energy-demanding periods.

Whereas earlier studies mainly focused on induced acquired immune responses (e.g. Ardia and Rice, 2006; Kilpimaa et al., 2005; Tschirren et al., 2003), more recent ones attempted to integrate patterns of acquired and constitutive innate immunity (e.g. Buehler et al., 2008; Lee et al., 2006; Parejo and Silva, 2009). Although innate immunity varies greatly among species in relation to ecological requirements, distribution and abundance (Lee et al., 2006; Mendes et al., 2006), it is currently unknown whether it is equally affected by short-term variations in health status and parasite pressure like other immunity components. Previous experimental studies were performed under laboratory conditions which may not necessarily reflect natural populations (Baumgarth et al., 1999; Matson et al., 2005). Studies on natural populations, on the other hand, were mainly correlational and yielded heterogeneous relationships with pathogen abundance [positive (Lindström et al., 2004), negative (Parejo and Silva, 2009; Whiteman et al., 2006) or no relationship (Møller and Haussy, 2007)]. Here we studied the effect of parasite load, and age-related and seasonal variation in constitutive innate immune defences (natural antibodies and complement immunity) in a free-living population of great tits (*Parus major* L.), a hole-nesting, altricial bird species. We present the results of a field experiment that was designed to test: (i) whether individuals adjust their investment in innate immunity according to variations in environmental parasite pressure, by manipulating the ectoparasite load in the nest before egg laying; (ii) whether immature birds show lower innate immune responses than adult ones, by comparing NAb concentrations and complement activation of nestlings and adults of different age classes; and (iii) whether the innate immune response is suppressed during reproduction, by comparing NAb levels and complement activity of the same females between breeding and post-breeding periods.

MATERIALS AND METHODS

Field procedures

The study was performed during two successive breeding and post-breeding seasons (2008 and 2009) in a 56 ha broadleaved forest near Ghent, Belgium (50°57′N, 3°43′E). Great tits bred in nest boxes, 387 of which had been placed at equal distances of 40 m during October–November 2007. When nest building was at an advanced stage, the ectoparasite load of the nest was manipulated for the first time with hen fleas (*Ceratophyllus gallinae* Schrank), a common ectoparasite of great tits. In 2009, all nests were first heat-treated to kill all nest organisms (Richner et al., 1993), after which half of them (N=24) were inoculated with 40 fleas; the remaining 24 nests were left parasite free. Uninfested nests received two additional heat treatments after the start of egg laying and after hatching. Infested nests were also transported to a microwave but were twice infested with 20 fleas per nest instead of being heat treated. In 2008, no heat-treatment experiments were performed since nest boxes were new, and infested nests were only inoculated once with 40 fleas at an advanced stage of nest building. During that year, 27 out of 62 nests were experimentally infested with fleas prior to egg laying while 35 others remained uninfested. Additionally, in both years a partial cross-fostering experiment between infested and uninfested nests was carried out 2 days after hatching; however, in the present study only data from not cross-fostered nestlings were analysed. In 2008 (but not 2009), females of 32 nests (16 infested and 16 uninfested) were captured at the nest after 2 days of incubation. Their clutches were collected for biochemical analysis and could thus no longer being investigated during the nestling stage. Consequently, 78 nests (30 nests in 2008 and 48 in 2009) were available after hatching. Of these, nine nests were deserted between 2 and 14 days after hatching, five in 2008 (1 infested, 4 uninfested) and four in 2009 (3 infested, 1 uninfested). All nestlings were ringed at the age of 5–6 days, and when nestlings were 7–10 days old all but two adult females (1 uninfested in 2008 and 1 infested in 2009) were captured at the nest while feeding them. Within 10 min of capture, 100–150 µl blood was collected in heparinized capillary tubes from all captured females (incubating and chick rearing) and from 15 day old nestlings *via* brachial vein puncture, stored under cool conditions and centrifugated (10,600 ***g*** for 5 min) during the same day. Plasma was separated from the cells and frozen at –20°C. Since great tit males do not incubate eggs and are more difficult to capture at the nest during the nestling stage, we only collected blood samples from female adults and nestlings. Furthermore, all captured birds were weighed, aged and individually ringed, and their tarsus length and wing length were measured following Svensson (Svensson, 1992). All empty nests were collected after clutch collection (2008) or on the day of fledging (2008, 2009) and stored at 4°C. Within 20 days of collection, each nest was placed in a Tullgren funnel (Pacejka et al., 1998) for 96 h for flea extraction, and numbers of flea larvae were counted. As predicted, mean (±s.e.m.) numbers of flea larvae were higher in infested nests compared with uninfested ones from which clutches had been collected at the beginning of incubation (infested: 139±50; uninfested: 59±23; one-sided t-test: t_{30}=1.72; P=0.048) and after fledging in 2009 (infested: 1168±228; uninfested: 110±27; one-sided t-test: t_{42}=2.74; P=0.005), while nest storage time did not significantly affect the number of flea larvae extracted (general linear model, GLM: $F_{1,99}$=1.71; P=0.19). Since the number of flea larvae was not different for the nests collected after fledging in 2008 (infested: 1330±378; uninfested: 1369±285; one-sided t-test: t_{23}=–0.08; P=0.47), we only included the data of 2009 for the analyses of parasite effects during chick rearing. A total of 26 adult females (i.e. older than hatching year; 14 from infested, 12 from uninfested nests) and 28 fledglings (14 from infested, 14 from uninfested nests) were recaptured with mist nets and blood sampled post-breeding (July–November 2008; July–August 2009). Recaptured fledglings are hereafter referred to as first-year birds. This study was carried out with permission from the Animal Ethics Committee of Ghent University (ECP 08/05).

Haemolysis–haemagglutination assay

To estimate the levels of circulating natural antibodies and complement we used the haemolysis–haemagglutination assay developed by Matson et al. (Matson et al., 2005). This assay is based on red blood cell agglutination and NAb-mediated complement activation. Quantification was done through serial dilutions (1:2) in phosphate-buffered saline of 50 µl plasma in a 96-well assay plate, which was then incubated with 25 µl of rabbit red blood cell suspension at 1% for 90 min at 37°C. NAb titres and complement activity were scored blindly from digitized images of the assay plates as the negative $\log_2$ of the highest dilution at which agglutination

(NAb) or lysis (complement) of red blood cells was observed. Half scores were assigned if the termination of the reaction was intermediate between two wells. To assess the level of repeatability among scores, agglutination and lysis titres of 55 samples were scored twice on separate days by the same person, yielding a Pearson correlation coefficient of 0.95 (P<0.0001) for agglutination and a perfect correlation (ρ=1; P<0.0001) for lysis. Sample sizes of agglutination and lysis titres are slightly smaller than the initial number of blood samples because samples were excluded from further analysis in the case of insufficient plasma or if an ambiguous reaction was observed (14 out of 413 plasma samples).

Statistical analysis

NAb levels were compared among parasite treatments in adult females by means of GLM and in nestlings by means of linear mixed models (LMM). Laying date was included as a variable to account for temporal variation in parasite abundance during the breeding season. Differences in NAb concentration between age classes (nestlings, 1 year old adults, and older adults) were also analysed with LMM. P-values for *post-hoc* pairwise differences between age classes were Tukey adjusted. Nest identity was included as a random factor in the LMMs to account for similarities between nestlings (and female parent) from the same nest. Degrees of freedom were estimated following Kenward and Roger (Kenward and Roger, 1997). Since incubating females were captured during 2008 only, differences in NAb concentrations between incubating and chick-rearing females were calculated based on this year (GLM). Although lysis scores ranged from 0 to 3, haemolysis only occurred in 59 out of 399 responses (both first-year birds and adults). Therefore, scores of lysis were treated as a continuous or binary variable, i.e. '0' (score 0; no lysis) or '1' (score >0; lysis), and asymptotic or exact tests were applied depending on the distribution and number of responses showing haemolysis in each analysis. We applied logistic regression (LR) models to compare the proportion of individuals that showed complement activation between parasite treatments (controlled for laying date) and breeding stages. Differences in the proportion of nestlings and female adults showing complement activation were tested by means of exact inference for logistic regression in LogXact 4.1 (Cytel Software Corporation 1989–2000, Cambridge, MA, USA), thereby averaging complement activity of nestlings from the same nest and pooling 1 year old and older adult females.

Post-breeding, age-related differences (first-year birds, 1 year old adults and older adults) were analysed with GLM (NAb levels) and LR (complement activation). We used LMM (with bird identity modelled as random factor) to compare adult NAb levels and complement activity between breeding and post-breeding periods based on female individuals captured during both seasons. In first-year birds, variation in NAb levels before and after fledging was based on the same procedure, whereas paired differences in complement activity were analysed with an exact McNemar's test in StatXact 4.0.1 (Cytel Software Corporation 1989–1999). All models included body condition, calculated as the residuals from a regression of body mass on tarsus length, while yearly and seasonal variation in immune response was accounted for by including year (2008, 2009) and recapture date in all relevant models. We also included treatment group as an additional factor in all analyses of the effects of age and season. Fixed variables of interest were estimated from the most parsimonious models after stepwise backward selection (nest or bird identity were always retained as random effects). Unless stated otherwise, body condition, laying date, recapture date, year and treatment group did not significantly account for variation in NAb levels and complement activity (all P>0.07). Response variables were logarithmic transformed when residuals from GLMs deviated from normality. All statistical analyses were performed in SAS 9.1 (SAS Institute Inc. 2002–2003, Cary, NC, USA) unless stated otherwise.

RESULTS

Parasite treatment

NAb levels (GLM, $F_{1,28}$=0.17, P=0.68; Fig. 1A) and complement activation (LR, χ^2_1=0.29, P=0.59; Fig. 1B) of incubating females did not differ between treatment groups. However, NAb levels of chick-rearing females were higher in infested nests compared with uninfested ones after logarithmic transformation, although this difference was only marginally significant (GLM, $F_{1,41}$=3.67, P=0.063; Fig. 1A). Complement activation, in contrast, did not differ among parasite treatments (LR, χ^2_1=0.97, P=0.32; Fig. 1B). Nestlings, too, showed a trend towards higher NAb concentrations under parasite treatment (LMM, $F_{1,38.9}$=3.89, P=0.056; Fig. 1A), while complement activation was absent in both treatments (Fig. 1B).

Age-related variation

NAb levels significantly differed among age classes (LMM, $F_{2,329}$=64.33, P<0.0001): NAb levels of nestlings were significantly greater than those of breeding females (*post-hoc* test; 1 year old females: t_{332}=–8.04, P<0.0001; older females: t_{326}=–9.04, P<0.0001; Fig. 2A), while 1 year old and older females did not differ in immune response (t_{337}=0.56, P=0.84; Fig. 2A). During the breeding season, complement activation appeared in 34% of the adults but in none of the nestlings (LR, P<0.0001; Fig. 2B). However, 70% of the

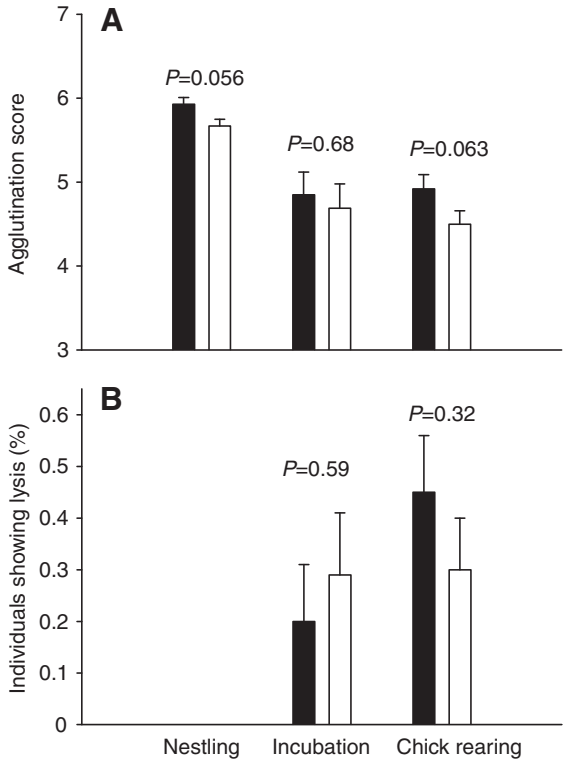

Fig. 1. Effects of parasite treatment on innate immunity of nestlings (N=44 nests) and breeding females during incubation (N=30) and chick rearing (N=43). (A) Mean (+s.e.m.) agglutination score, indicating NAb levels, and (B) proportion (+s.e.m.) of individuals showing lysis, indicating complement activation, in infested (filled bars) and uninfested (open bars) nests. P-values for differences between parasite treatments are shown.

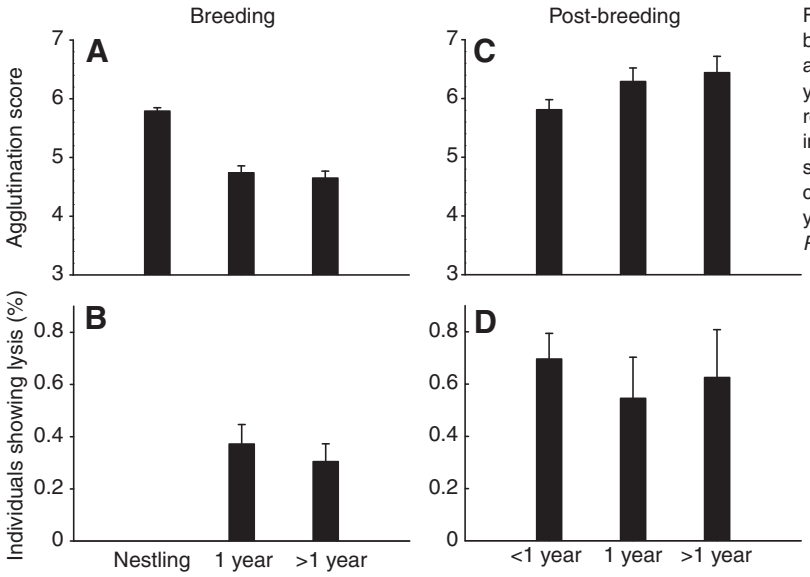

Fig. 2. Differences between age classes in innate immunity during breeding (N=69 nests for nestlings and N=44 and 47 for 1 year old and older females, respectively) and post-breeding (N=24 for first-year birds and N=14 and 9 for 1 year old and older females, respectively) periods. (A,C) Mean (±s.e.m.) agglutination score, indicating NAb levels, and (B,D) proportion (±s.e.m.) of individuals showing lysis, indicating complement activation. For both *post-hoc* comparisons of NAb and complement activation between first-year birds and adult age classes during the breeding season P<0.0001. Other P-values were non-significant (P>0.10).

recaptured fledglings post-breeding did show complement activation (McNemar's test, P<0.0001, N=23), while NAb levels did not differ before and after fledging (LMM, mean difference ± s.e.m.: 0.10±0.20, $F_{1,46}$=0.28, P=0.60). Immune responses, corrected for a significant year effect in complement (P=0.003), did not differ among age classes outside the breeding season (NAb: GLM, $F_{2,44}$=2.41, P=0.10; complement: LR, χ^2_2=1.61; P=0.45; Fig. 2C,D).

Seasonal variation

Average immune responses did not significantly differ between the onset of incubation and during chick rearing (NAb: mean difference ± s.e.m.: 0.19±0.28, GLM, $F_{1,50}$=0.45, P=0.50; complement: mean difference: 19%, LR, χ^2_1=1.95, P=0.16). However, individual immune responses were significantly higher during post-breeding compared with breeding season in adult females captured during both periods (LMM: NAb: $F_{1,19.3}$=42.15, P<0.0001; complement: $F_{1,28.6}$=6.09, P=0.020; Fig. 3A,B). The effect of treatment was not significant post-breeding (LMM: NAb: $F_{1,16.1}$=0.95, P=0.34; complement: $F_{1,22.1}$=0.21, P=0.65) and was therefore left out of the models.

NAb levels and complement activation were not correlated for incubating or chick-rearing females, nor were they correlated outside the breeding season (all $|\rho|$<0.20; P>0.41). Additionally, neither measure of innate immunity was correlated for first-year birds after fledging (both ρ<0.08; P>0.72).

DISCUSSION

Adult females feeding their young in nests that had been infested with parasites tended to have higher NAb levels. In contrast, infestation with parasites did not affect adult NAb levels during incubation nor did it affect complement activation during either period. An increased parasite pressure in the nest also resulted in higher NAb concentrations in nestlings, albeit not significantly. While NAb levels and complement activation of breeding females were not statistically different during incubation and chick rearing, both components of innate immunity significantly increased post-breeding. After fledging, complement activation significantly increased in first-year birds, while NAb concentrations remained similar to those measured at the nestling stage.

To the best of our knowledge, these results provide the first experimental evidence for a causal relationship between environmental parasite pressure and NAb level in a free-living bird population. Earlier studies showed heterogeneous patterns in correlative relationships between natural parasite load and innate immunity (Lindström et al., 2004; Møller and Haussy, 2007; Parejo and Silva, 2009; Whiteman et al., 2006), no experimental evidence of pathogenic effects on NAb concentration, and only small effects on complement [e.g. *Salmonella* infection in chickens *Gallus domesticus* (Matson et al., 2005) and influenza infection in mice, (Baumgarth et al., 1999)]. This supports the notion that NAb and complement activation are less strongly affected by antigen exposure

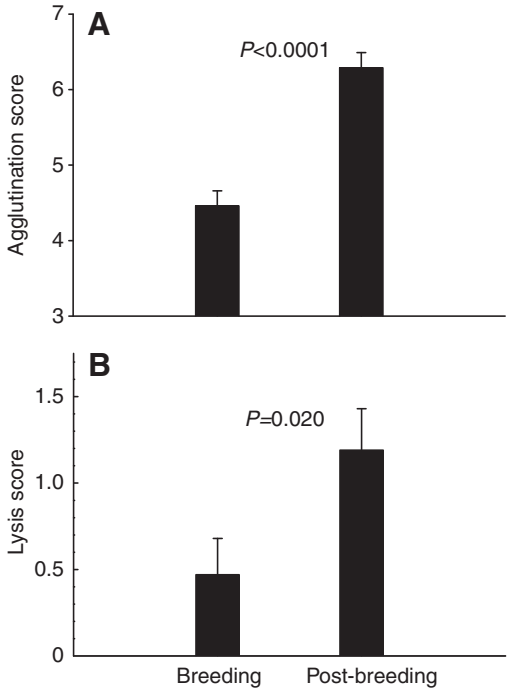

Fig. 3. Differences between innate immunity of the same females during breeding and post-breeding periods. Mean (±s.e.m.) (A) agglutination (N=24), indicating NAb levels, and (B) lysis score (N=19), indicating complement activation. P-values refer to differences between the two periods.

than other components of the immune system (Baumgarth et al., 1999; Matson et al., 2005). Yet, this study, which measured experimentally-induced immune responses over a longer time period in free-ranging individuals subjected to natural ambient conditions, suggests that NAbs too are affected by antigens. Although the observed increase in NAb concentration in response to fleas was rather small, the fact that both adult females and nestlings tended to be affected suggests its biological relevance. While complement proteins are produced constitutively, they need to be activated sequentially to become effective. Since we measured NAb-mediated complement activation, which is one of the pathways for the activation of complement, cell lysis by the complement system may be necessarily delayed compared with upregulation of NAb production. Despite concerns that lysis titres might not always reflect complement activity in plasma, but rather result from antibody-mediated cytotoxicity (Huber et al., 2008), the non-significant correlation between agglutination and lysis titres suggests the opposite. Additionally, even if lysis titres would not accurately reflect complement activity, associations between lysis titres and the annual cycle (Buehler et al., 2008), body mass differences among nestlings (Parejo et al., 2007) and parasite abundance (Parejo and Silva, 2009) suggest that lysis titres are biologically relevant. The higher NAb response associated with our experimental manipulation in breeding females during chick rearing compared with incubation is probably related to a lower and shorter exposure to parasites during incubation. First, in accordance with previous findings that fleas accumulate in great tit nests over the breeding season (Harper et al., 1992), flea abundance was lower during incubation than during chick rearing (see earlier for flea abundance levels). Fleas might only invoke an immune response if their abundance, and thus the infection of the host, is intense enough. Second, because NAb concentrations were determined during the first days of incubation, exposure of breeding females to fleas only started a few days before. In contrast, females that were captured during food provisioning had already suffered exposure for 3–4 weeks, which might have been a sufficiently long period to increase NAb levels. After the breeding season, infested females no longer showed elevated NAb levels, suggesting the lack of carry-over effects of flea infestation on NAb concentrations.

The fact that NAb concentrations of nestlings, but not complement activation, equalled post-fledging concentrations suggests that NAb production in first-year birds had already matured by the end of their nestling stage. In contrast, the activation of the complement cascade was further increased post-fledging. While the production of NAbs has been shown to increase with development in other bird species too, adults appear to develop their maximal concentrations at later ages. For example, tree swallows (*Tachycineta bicolor*) exhibited lower NAb levels and complement activity than adults at the end of the nestling period (Palacios et al., 2009), 2 month old red knots (*Calidris canutus*) showed lower NAb levels than adults (Buehler et al., 2009), and chickens reached mature NAb levels between the age of 40 and 65 weeks (Star et al., 2007). As opposed to acquired immunity, NAbs are already present in non-immunized animals (reviewed in Bandeira et al., 1988), and therefore constitute an ideal defence mechanism for chicks that are exposed to a plenitude of antigens after hatching. Given that the great tit is a hole-nesting species, the results from this study suggest that deleterious effects of parasite infections associated with such a life history may be counteracted by the accelerated development of innate immunity (see also Møller and Erritzoe, 1996). This hypothesis is supported by the increased NAb levels in great tit nestlings of experimentally infested nests (this study). Alternatively, NAbs present in nestlings may be (partly) maternally transmitted through the egg yolk, rather than produced endogenously, as is the case for other immunoglobulins (Klasing and Leshchinsky, 1999). However, we consider this unlikely because the dominant type of maternally transmitted antibody is IgY, the homologue of mammalian IgG (Klasing and Leshchinsky, 1999), while NAbs mainly consist of IgM (Boes, 2000). Furthermore, maternally transferred antibodies in the offspring of many bird species are quickly catabolized and replaced by endogenously synthesized antibodies (Apanius and Nisbet, 2006; Grindstaff et al., 2006; Pihlaja et al., 2006), hence NAb levels measured in 15 day old nestlings can be assumed to reflect endogenous antibodies. Yet, it remains possible that maternal transfer of antibodies affects endogenous NAb production of nestlings (see Grindstaff et al., 2006; Moreno et al., 2008; Reid et al., 2006). The fact that female great tits exposed to hen fleas deposit higher levels of IgY into their eggs (Buechler et al., 2002), possibly triggering the higher flea resistance in nestlings originating from nests that already contained fleas during egg laying (Heeb et al., 1998), supports such relationship.

Constitutive innate immune responses of female adults were lower during the breeding season than post-breeding. In contrast, complement activation but not NAb concentration varied throughout the year in captive red knots (Buehler et al., 2008). However, these birds were not reproducing, which probably explains the different outcome. At least four, not mutually exclusive, hypotheses might explain the lower innate immune responses of breeding females compared with post-breeding levels.

First, innate immunity during reproduction may be energetically constrained as a result of resource allocation trade-offs with other energetically costly activities such as incubation and chick rearing (Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996) and the cost of reproductive state *per se* (Bentley et al., 1998). The observation that the energy expenditure of female great tits is considerably higher during egg production and chick feeding than during winter, but does not differ between egg laying and chick rearing (Nilsson and Råberg, 2001) [see Thomson et al. (Thomson et al., 1998) for an example on kittiwakes *Rissa tridactyla*] supports this hypothesis. Resource allocation trade-offs have previously been shown for other components of the immune system (reviewed in Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996) suggesting a similar reduction in investment in each component of the immune system. Alternatively, if the different components of the immune system do not show the same response, lower innate immune responses during breeding might be associated with higher investment in other components of the immune system (Norris and Evans, 2000). Therefore, the overall immunocompetence might remain constant during breeding and post-breeding seasons.

Second, as has been shown in other components of the immune system (e.g. Duffy et al., 2000; Mougeot et al., 2004), innate immune responses during reproduction might be suppressed due to increased levels of testosterone. In support of this hypothesis, testosterone levels were inversely correlated with two measures of innate immunity (total IgG and complement activity) in free-living dark-eyed juncos, *Junco hyemalis* (Greives et al., 2006). Furthermore, in many bird species, female plasma testosterone levels vary during the year and peak early during the breeding season, as in males (Ketterson et al., 2005). However, this hypothesis cannot explain why female innate immune responses were also lower during the late breeding season (i.e. chick-rearing stage).

Third, a reduction of immunity during reproduction might result from glucocorticosteroid-induced immunosuppression rather than from a trade-off with costly breeding activities *per se*. Because of

the short breeding season in the temperate region and the high investment in each breeding attempt, the reproductive season is thought to be the most energy demanding for temperate species (Hasselquist, 2007). Under such conditions, birds are known to enhance their glucocorticosteroid secretion and thereby reallocate resources to induce physiological and behavioural changes (Wingfield et al., 1998). A suppression of the (innate) immune system might allow the reallocation of resources towards physiological processes that enhance their survival. Also, the higher risk of immunopathology in periods of increased workload might make a downregulation of the immune system beneficial (Råberg et al., 1998). Yet, an empirical study of the effects of corticosterone on the immune system in barn owl (*Tyto alba*) nestlings could only partly support this hypothesis as corticosterone administration resulted in a reduction of humoral acquired immunity but did not change constitutive innate immunity (Stier et al., 2009).

Fourth, rather than decreasing during reproduction, innate immune responses may increase post-breeding in response to environmental cues. Indeed, while great tits are highly exposed to fleas during the breeding season only, the possibility cannot be excluded that they are exposed to other parasites post-breeding.

In conclusion, we have shown that innate immune responses appear to vary in relation to ectoparasite load, age and season. However, the proximate and ultimate mechanisms underlying this variation, and the consequences thereof, are still poorly understood. Therefore, further experimental study of taxa that span a wide range of life-history strategies is required.

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