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ORIGINAL PAPER

Sexual dimorphism in immune function changes during the annual cycle in house sparrows

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Abstract Difference between sexes in parasitism is a common phenomenon among birds, which may be related to differences between males and females in their investment into immune functions or as a consequence of differential exposure to parasites. Because life-history strategies change sex specifically during the annual cycle, immunological responses of the host aiming to reduce the impact of parasites may be sexually dimorphic. Despite the great complexity of the immune system, studies on immunoecology generally characterise the immune status through a few variables, often overlooking potentially important seasonal and gender effects. However, because of the differences in physiological and defence mechanisms among different arms of the immune system, we expect divergent responses of immune components to environmental seasonality. In male and female house sparrows (*Passer domesticus*), we measured the major components of the immune system (innate, acquired, cellular and humoral) during four important life-history stages across the year: (1) mating, (2) breeding, (3) moulting and (4) during the winter capture and also following introduction to captivity in aviary. Different individuals were sampled from the same population during the four life cycle stages. We found that three out of eight immune variables showed a significant life cycle stage × sex interaction. The difference in immune response between the sexes was significant in five immune variables during the mating stage, when females had consistently stronger immune function than males, while variables varied generally non-significantly with sex during the remaining three life cycle stages. Our results show that the immune system is highly variable between life cycle stages and sexes, highlighting the potential fine tuning of the immune system to specific physiological states and environmental conditions.

Keywords Acquired immunity · Annual cycle of immune responses · Cellular immunity · Humoral and innate immunity · *Passer domesticus* · Sex-differential immunity

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Introduction

Difference between sexes in parasite infestation rate is a well-established phenomenon in vertebrates (Folstad and Karter 1992; Poulin 1996; Zuk and McKean 1996; McCurdy et al. 1998), with males being generally more susceptible to parasites than females. The causes of sexual differences in parasite infections are multiple, but an established pattern—which might reveal underlying cause—is a difference between sexes in immune system defence (Zuk and McKean 1996; Møller et al. 1998). Indeed, several studies have found that females show increased investment into immune functions compared to males in the same population (e.g., Møller et al. 1998; Tschirren et al. 2003). However, in natural bird populations, studies investigating sex differences in immunocompetence or susceptibility to parasites are rare; these few studies are mainly in agreement with the general concept that differential immune investment by males and females might play a central role in the generation of sex-differential parasitism (e.g., McCurdy et al. 1998; Hasselquist 2007). Several hypotheses were proposed to explain the sexual differences in immune functions in birds (reviewed in Hasselquist 2007), among which the most commonly invoked is the "immunocompetence handicap" hypothesis (Folstad and Karter 1992). This hypothesis assumes that a males' costly sexual activity, determined by the increased concentration of immunosuppressive steroid hormones (Roberts et al. 2004; Romero et al. 2006), is responsible for the suppression of the immune system during the sexually most active period of the year. Hence, it predicts enhanced immune function in females compared to males, at least during mating and breeding. Therefore, the outcome of studies on the immune system may depend on the period of the annual cycle when the study is conducted (Møller et al. 1998; Lozano and Lank 2003; Martin et al. 2008), though previous studies generally have not accounted for seasonal effects in this way.

Providing a broad characterisation of the immune system of vertebrates is a non-trivial challenge in immunoecological studies due to the need for simple, real-time indicators of immunocompetence while at the same time facing the great complexity of the immune defence system (Schmid-Hempel and Ebert 2003; Adamo 2004; Viney et al. 2005; Owen and Clayton 2007; Martin et al. 2008). Despite the evidence for a diversity of immune defence components in birds, evolutionary ecologists often use a single measure of immune activity (like the PHA skin test or injection of sheep red blood cells) because of logistical constraints or the particular question being addressed (Kennedy and Nager 2006; Martin et al. 2006a; Tella et al. 2008). But, because of the potential trade-offs among the different immune branches (Nelson et al. 2002; Forsman et al. 2008; Martin et al. 2008), one measurement of immune response is unlikely to be enough to characterise the whole immune system. Hence, to compare sex-related differences in immunocompetence as many of the major components of immune function as possible should be measured (Matson et al. 2006a; Buehler et al. 2008; Forsman et al. 2008).

Concerning the measurement of the defence system, there are two ways to investigate variation in the immune function of birds over the annual cycle: (1) following the same individuals by repeated measurements or (2) sampling different individuals from the same (local) population at several occasions over the annual cycle. The first approach has several drawbacks, with two potential problems as the most important. First, repeated capturing of the same individuals may cause serious stress to these individuals, which, besides ethical considerations, can hinder the collection of biologically meaningful results. Second, repeated exposure to the same set of immune measures and antigens is likely to alter the individual's future immune responses (Hasselquist et al. 1999; Lee et al. 2006; Tella et al. 2008), and hence, the longitudinal pattern (annual pattern) may be confounded. The second approach consists of sampling different individuals each time during the study period from the population, which overcomes the presumed methodological and ethical problems associated with repeated samples from the same individual. However, this latter approach will lack detailed longitudinal knowledge about the sampled individuals. We decided to use the second approach and sampled each individual just once. This also meant that blood samples for measuring constitutive immune variables could be collected directly in the field, however the measurement of induced cell-mediated and humoral immune responses implicates that birds had to be taken into captivity and held temporally in enclosures. Taking all together, the objective of the present study was to determine the sex-specific seasonal changes in the immune activity by measuring the major components of the immune system (innate, acquired cellular and humoral immunity) of a common sedentary passerine bird, the house sparrow (*Passer domesticus*), over the whole annual cycle, specifically during mating, breeding, moulting and wintering.

Materials and methods

General methods, measurements and aviary conditions

We captured adult house sparrows using mist nets (Ecotone, Poland), which were not included in any immunological or other manipulation experiments in our long-term study of this species. These capture efforts took

place across four different life-history stages across the annual cycle (see below) at a farm situated near Cluj-Napoca (46°46′ N, 23°33′ E), Transylvania, central Romania, where we have been studying the biology of these birds since 2006. The birds were captured on the following dates: 2 June 2007 (breeding; N=31), 26 September 2007 (moulting; N=34), 20 December 2007 (wintering; N=32) and 9 April 2008 (mating; N=32). In June, house sparrows were still in their breeding phase, as indicated by the presence of brood patch in all females captured, and the majority of birds were between two breeding events (e.g., first and second breeding). However, we have no information about the exact breeding state of individual birds. In April, most of the house sparrow nest construction in our colony had just been completed and only some of the birds had started laying eggs. Hence, at this stage, the costs related to the incubation and chick-rearing are absent, and birds instead present intensive courtship behaviour. We therefore consider this period as representing the mating stage, and the behaviours during this period are clearly different from those carried out by the birds during the breeding stage in June. The sex ratio within capture seasons was ~1:1, and each individual bird was measured only once during the study period. Following capture, all birds were individually ringed, measured (wing and tarsus length) and weighed. Thereafter, they were transported into a large outdoor aviary (8 (L) ×5 (W)× 2.5 m (H)) situated at the Campus of the Babeş-Bolyai University, Cluj-Napoca. House sparrows were fed ad libitum with a mixture of seeds containing grinded corn, sunflower, oat and barley, and their food was supplemented with two grated boiled eggs on every even day and with two mealworms per bird on odd days throughout the housing. To increase the comfort of the birds, we provided shelter (small bushes), perches and nest boxes inside the aviary. Sand and fresh water were available ad libitum throughout the experiment. None of the birds died in the aviary, and following the experiments during the four seasons, all house sparrows were released in good health condition in the population of origin.

Immune measurement and challenge protocol

Because of the possible negative effect of cross-reaction on immune responses due to injecting multiple antigens, we randomly divided the 30 caught birds during each life cycle stages into two groups, making sure that each of these groups had a balanced sex ratio (see Table 1 for the schematic presentation of the experimental design). Within 5 min following the capture in the farm, we collected 75 μL blood from all individuals, which was used to measure the bacterial killing ability of the whole blood from the first group or for haemolysis–haemagglutination assay in the case of birds from the second group (for details, see below). Following collection, the plasma was separated by centrifugation of capillary tubes for 5 min at 5,000 rpm, then it was stored at −20°C until analysis. Because of the challenging nature of immunological tests, all other measures were performed in aviaries, where after injection with specific antigens, the birds could easily be recaptured at a specific time to assess the strength of acquired immune responses (see below). Following capture and blood sampling, we introduced the birds in the aviary where after 6 to 9 days of accommodation, the experiments were initiated. Before immunisation, we collected 75 μL blood in order to determine the pre-exposure immune responses against the specific antigens with which the birds were treated. House sparrows from the first group were injected with 100 μL 20% fresh sheep red blood cell (SRBC) suspension into the pectoralis muscles (see Pap et al. 2008). Birds from the second group received 100 μL of 1 mg mL^{-1} keyhole limpet haemocyanin (KLH) in sterile physiological (0.9% w/v) saline solution into the left wing web following the protocol of Martin et al. (2006b). After 10 days, we collected a second blood sample from the birds of both groups in order to measure the primary antibody humoral immune response against SRBCs and KLH. At this time and 10 days later, we injected the second and third dose of KLH solution into the left wing web of birds from the second

Table 1 Schematic presentation of the experimental design

Schedule	Action[a]	Measures in the first group (n_{male}=33, n_{female}=33)	Measures in the second group (n_{male}=30, n_{female}=33)
Day 0	Birds captured in the wild	*E. coli* bacteria killing ability	Agglutination
		S. aureus bacteria killing ability	Lysis
Day 9	SRBC (1) and KLH (2) injection		
Day 19	KLH injection (2)	SRBC humoral immune response	KLH humoral immune response
Day 29	PHA (1) and KLH (2) injection		
Day 30		PHA swelling response	KLH swelling response
Day 35	Birds released back in the wild		

[a] Numbers in parentheses denote the group number in which the immune challenge was applied

experimental group to induce the replication of T memory cells and to increase the swelling response. On the same day as the third KLH injection was applied, birds from the first experimental group were injected with 100 μL of 1 mg mL^{-1} phytohaemagglutinin (PHA) solution into the left wing web in order to measure the induced cellular immune response (Martin et al. 2006a; Tella et al. 2008; Pap et al. 2009). Twenty four hours later, with a pressure-sensitive calliper, we measured the swelling response to KLH in the first group and PHA in the second group, respectively. We used this experimental design and parallel capture of birds from the two groups to diminish the stress, thus reducing the confounding negative effect of handling on immune response (see Matson et al. 2006b).

Constitutive innate defence

Constitutive innate immunity, a mixture of humoral (e.g., natural antibodies, complement and acute phase proteins) and cellular (macrophages, heterophils, eosinophils and thrombocytes) components, provides the first line of immune defence against invading microorganisms. We measured the constitutive innate immunity by assessing the bacteria killing ability of the whole blood (Tieleman et al. 2005) and by quantifying the levels of the natural antibodies (NAbs) and complement using the haemolysis–haemagglutination assay (Matson et al. 2005).

Bacteria killing ability of the whole blood In order to reduce the negative effect of stress on immune responses, we collected 75 μL blood under sterile conditions from the brachial vein within 5 min after capture in the farm (Tieleman et al. 2005; Matson et al. 2006b). The whole blood samples were transferred in sterile 1.5-mL Eppendorf tubes and within 40 min transported to the laboratory, being processed in sterile conditions under a laminar flow hood (Biohazard safety clean bench LCB-1203B, Daihan Labtech Co., Ltd., Korea). We tested the bactericidal activity of the blood against standard laboratory strains of *Escherichia coli* (ATCC no. 10536) and *Staphylococcus aureus* (ATCC no. 6538P). In case of *E. coli* and *S. aureus*, we diluted 20 and 50 μL blood, respectively, up to 200-μL solutions using CO_2-independent media (no. 18045, Gibco-Invitrogen, CA, USA) enriched with 4 mM L-Glutamine (no. 25030, Gibco-Invitrogen) and 5% heat-inactivated Newborn Calf Serum (no. N4762, Sigma). To each diluted sample, we added 20 μL of the bacterial working solutions, these being obtained by lyophilised pellets (Epower Assayed Microorganism Preparation; Microbiologics Inc., Saint Cloud, MN, USA), which were reconstituted according to the manufacturer instructions in sterile PBS. Before running the tests, after reconstituting the lyophilised pellets, the working solution was adjusted and controlled to have ~150 colonies after plating 75 μL of 1:10 diluted bacterial solution. The blood–bacteria mixture was incubated for 20 min (in case of *E. coli*) and for 1 h (in case of *S. aureus*), both at 41°C. After incubation, we mixed the diluted samples in duplicates and spread 75 μL aliquots onto tryptic soy agar plates (no. 22091, Fluka), after which the plates were again incubated overnight at 37°C. To obtain the initial number of bacteria that we had before starting to interact with the blood, we diluted 200 μL media alone with bacterial species and immediately plated without incubation. To verify if we collected the blood samples sterile, we made blood sterility control plates, adding 200 μL media to the remaining blood, plated them and incubated under the same conditions. On the following day, the colony-forming units were counted, and the microbial activity of the whole blood was defined as the percent of the killed bacteria, which was calculated as 1−(average of the viable bacteria after incubation/the initial number of bacteria). The average was calculated from two plates per bird.

Haemolysis–haemagglutination assay We assessed the humoral innate immunity, namely the levels of the NAbs and complement, by using the haemolysis–haemagglutination assay as described by Matson et al. (2005) adjusted to the limited volume of blood. After pipetting 15 μL sterile physiological (0.9% *w*/*v*) saline solution in each well of a U-shaped 96-well microtitre plate, 15 μL plasma was pipetted into the first column. The content of the first column wells was serially diluted (1:2) until the 11th column, resulting in a dilution series for each sample from 1/2 to 1/2048. We used the last column of the plate as negative controls, containing saline solution only. We then added 15 μL of 1% freshly collected rabbit red blood cells suspension to all wells and incubated at 37°C for 90 min. After incubation, in order to increase the visualisation of agglutination, the plates were tilted at a 45° angle at room temperature. Agglutination and lysis, which reflect the activity of the natural antibodies and the interaction between NAb and complement (Matson et al. 2006a; Buehler et al. 2008), was recorded after 20 and 90 min, respectively. Haemagglutination is characterised by the appearance of clumped red blood cells, as a result of antibodies binding multiple antigens, while during haemolysis, the red blood cells are destroyed. Titres of the NAbs and complement were given as the $\log_2$ of the reciprocal of the highest dilution of plasma showing positive haemagglutination or lysis, respectively. The haemolysis–haemagglutination assay was performed by the same person (GÁC) randomly with respect to the identity of the bird after 1 to 2 months following blood sampling during the four different periods.

Adaptive humoral immune defence

To measure the adaptive humoral immune defence, birds from the first and second groups received different antigens. Thus, we assessed the primary antibody titre against SRBCs of the birds from the first group, and also the primary adaptive humoral immune response against KLH for the birds from the second group. In case of the first experimental group, antibody titres against SRBCs were measured using a base-2 serial dilution haemagglutination test conducted with 15 μL of heat-inactivated plasma (30 min on 56°C) on U-shaped 96-well microtitre plates (Pap et al. 2008). Samples were serially diluted starting with 15 μL PBS, and to each well, we added 15 μL of a 1% suspension of SRBC in PBS. Plates were incubated at 37°C for 1 h. Titres are given as the $\log_2$ of the reciprocal of the highest dilution of plasma showing positive haemagglutination. To measure KLH-specific antibodies in house sparrow plasma, we used an enzyme-linked immunosorbent assay (ELISA) developed for red-winged blackbirds (*Agelaius phoeniceus*; Hasselquist et al. 1999) that works well also for house sparrows (Lindström et al. 2005; Lee et al. 2006; Martin et al. 2006b). The KLH antigen was first bound to the sides of each well of a 96-well ELISA plate (Costar, Cambridge, MA, USA) when incubated overnight at 4°C. After washing the plates, we added diluted plasma samples (diluted 1:2,000 in PBS, Tween 20 and 1% milk powder) from house sparrows to each well, allowing antigen-specific antibodies to bind to the antigen attached to the well when incubated overnight at 4°C. After washing the plates, we added a secondary rabbit anti-red-winged blackbird Ig antiserum (diluted 1:1,000; recognises both IgY and IgM) to each well and allowed it to bind to antigen-bound house sparrow antibodies for 1 h at 37°C. After washing the plates, we added a goat-anti-rabbit antibody labelled with peroxidase (1:2,000 dilution; Sigma no. A6154) to each well and allowed plates to incubate for 45 min at 37°C. Finally, after washing, we added 2,2-azino-bis-3-ethylbenzthialzoline-6-sulphonic acid (Sigma A1888) and peroxidase to the wells (to allow detection of the magnitude of antigen-bound sparrow antibodies, which is proportional to the colour change in each well). Plates were then immediately transferred to a Molecular Devices V_{max} kinetic reaction ELISA reader. Plates were read every 30 s for 14 min using a 405-nm wavelength filter. Antibody concentrations were calculated according to the slope of substrate conversion over time in units of 10^{-3} optical densities (OD) per minute (m_{OD} min^{-1}), with a higher slope indicating a higher titre of anti-KLH antibodies in the sample. All samples were run in duplicate, and the average of the two readings was our measure of antibody levels in the plasma. For more details of the general ELISA methods, see Hasselquist et al. (1999), Ilmonen et al. (2000), and Owen-Ashley et al. (2004).

Primary and secondary T-cell-mediated immune defence

Birds from the first group were injected with PHA in order to measure the primary induced cellular immune response against this natural lectin (Tella et al. 2008; Pap et al. 2009), while individuals from the second group received KLH to assess the secondary T-cell-mediated immune response according to the experimental protocol. Approximately 24 h after the inoculation of the antigens, we measured the swelling response of birds from both groups with a pressure sensitive calliper.

Statistical analyses

All dependent variables were normally distributed, as revealed by the non-significant effect of the Kolmogorov–Smirnov tests ($P>0.1$ in all cases). The effects of life cycle stage, sex and the interaction between the two variables on immune responses were tested separately for each dependent variables with general linear models, and the differences between life cycle stages and sexes were analysed using planned comparisons of least squares means. The effect of explanatory variables on the probability of birds to respond to SRBC immunisation was tested by using the generalised linear model with binomial error distribution and log-link function, where the probability of the responsiveness was introduced as the dependent factor. All analyses were carried out in the Statistica 7.0 software package (StatSoft 2004).

Results

The differences between males and females in immune response varied during the annual cycle, as three out of eight immune variables showed a significant life cycle stage × sex interaction, and five out of the eight immune variables differed between males and females during the mating stage (Table 2, Fig. 1). In all five cases, females had stronger immune responses than males, while no significant sex difference was observed during the remaining three life cycle stages except for the natural antibody concentration that was higher in females during the moulting stage (Fig. 1). Of the eight measured immunological variables, six showed a significant variation among life cycle stages, and the two remaining variables were close to significant ($P=0.07$ and 0.08; for statistical details, see Table 2, Fig. 1).

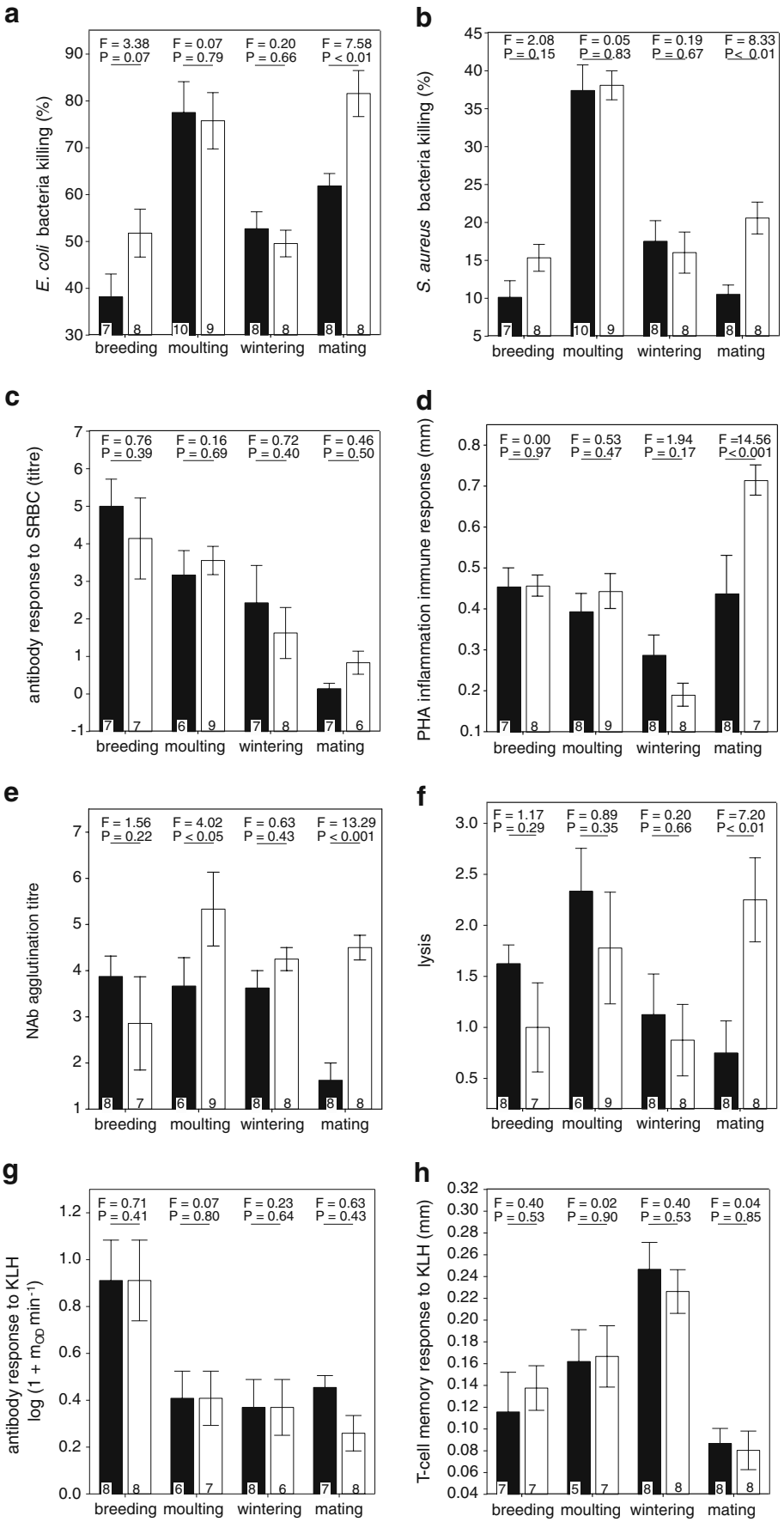
Immune responses measured in the first group The bactericidal activity of the blood against *E. coli*, measured at capture, was significantly higher in females than in males during the mating stage, and the bacteria killing ability was highest during the moulting and mating stages (Fig. 1a). The bacteria killing ability of the blood against *S. aureus*

Table 2 Results of the general linear models (GLMs) on the effect of life cycle stage and sex on *E. coli* and *S. aureus* bacteria killing capacity, antibody response to KLH and SRBC, PHA inflammation immune response, agglutination and lysis to rabbit blood cells, and T-cell memory swelling to KLH in the house sparrow

Variable	MS	F_{df}	P
Immune responses in the 1st group measured at capture			
E. coli killing capacity			
Life cycle stages	3,931.4	$19.17_{3,58}$	<0.0001
Sex	824.6	$4.02_{1,58}$	0.0496
Life cycle stages × sex	528.2	$2.58_{3,58}$	0.06
S. aureus killing capacity			
Life cycle stages	2,356.5	$48.48_{3,58}$	<0.0001
Sex	213.6	$4.39_{1,58}$	0.0404
Life cycle stages × sex	106.9	$2.20_{3,58}$	0.10
Immune responses in the 1st group measured in the aviary			
Antibody response to SRBC			
Life cycle stages	41.7	$12.38_{3,49}$	<0.0001
Sex	0.3	$0.09_{1,49}$	0.77
Life cycle stages × sex	2.2	$0.66_{3,49}$	0.58
PHA inflammation immune response			
Life cycle stages	0.3	$15.39_{3,55}$	<0.0001
Sex	0.1	$2.66_{1,55}$	0.11
Life cycle stages × sex	0.1	$4.87_{3,55}$	0.0045
Immune responses in the 2nd group measured at capture			
Agglutination to rabbit blood cells			
Life cycle stages	6.1	$2.45_{3,54}$	0.07
Sex	16.5	$6.62_{1,54}$	0.0129
Life cycle stages × sex	10.5	$4.21_{3,54}$	0.0096
Lysis to rabbit blood cells			
Life cycle stages	3.0	$2.36_{3,54}$	0.08
Sex	0.0	$0.00_{1,54}$	0.95
Life cycle stages × sex	3.9	$3.14_{3,54}$	0.0325
Immune responses in the 2nd group measured in the aviary			
Antibody response to KLH			
Life cycle stages	1.3	$5.30_{3,50}$	0.0030
Sex	0.0	$0.01_{1,50}$	0.91
Life cycle stages × sex	0.1	$0.54_{3,50}$	0.66
T-cell memory response to KLH			
Life cycle stages	0.1	$15.96_{3,50}$	<0.0001
Sex	0.0	$0.00_{1,50}$	1.00
Life cycle stages × sex	0.0	$0.28_{3,50}$	0.84

measured at capture was higher in females than males during the mating stage, and the bactericidal activity of the blood was highest during the moulting stage (Fig. 1b). No difference was observed between the sexes in humoral immune response against SRBC measured on birds when kept in aviaries (Fig. 1c). The antibody titre against SRBC was highest during the breeding stage, followed by a steady decrease until the mating stage the next spring. In total, 75% (n=57) of the birds responded to SRBC injection. We found similar results for the SRBC immune response of the birds when the probability of the responsiveness was introduced as the dependent factor (life cycle stage: $\chi^2_{3,49} = 19.93$, $P<0.001$, sex: $\chi^2_{1,49} = 0.40$, $P=0.53$, life cycle stage × sex: $\chi^2_{2,49} = 5.14$, $P=0.08$). The PHA inflammation immune response, measured on birds when kept in aviaries, was lowest in winter and it then increased, resulting in a significant difference between the sexes at the mating stage with female PHA responses being stronger than that of males (Fig. 1d).

Fig. 1 **a**–**h** Seasonal change in immune variables of male (*filled bars*) and female (*open bars*) house sparrows. Mean ± SE. The *values above bars* indicate the results from the planned comparison within life cycle stage and between sexes, and the *numbers within bars* are sample sizes ▶


a
E. coli bacteria killing (%)
F = 3.38 P = 0.07
F = 0.07 P = 0.79
F = 0.20 P = 0.66
F = 7.58 P < 0.01
b
S. aureus bacteria killing (%)
F = 2.08 P = 0.15
F = 0.05 P = 0.83
F = 0.19 P = 0.67
F = 8.33 P < 0.01
c
antibody response to SRBC (titre)
F = 0.76 P = 0.39
F = 0.16 P = 0.69
F = 0.72 P = 0.40
F = 0.46 P = 0.50
d
PHA inflammation immune response (mm)
F = 0.00 P = 0.97
F = 0.53 P = 0.47
F = 1.94 P = 0.17
F = 14.56 P < 0.001
e
NAb agglutination titre
F = 1.56 P = 0.22
F = 4.02 P < 0.05
F = 0.63 P = 0.43
F = 13.29 P < 0.001
f
lysis
F = 1.17 P = 0.29
F = 0.89 P = 0.35
F = 0.20 P = 0.66
F = 7.20 P < 0.01
g
antibody response to KLH log (1 + m$_{OD}$ min^{-1})
F = 0.71 P = 0.41
F = 0.07 P = 0.80
F = 0.23 P = 0.64
F = 0.63 P = 0.43
h
T-cell memory response to KLH (mm)
F = 0.40 P = 0.53
F = 0.02 P = 0.90
F = 0.40 P = 0.53
F = 0.04 P = 0.85
breeding
moulting
wintering
mating

Immune responses measured in the second group The variation of agglutination and lysis against rabbit red blood cells, measured at capture, was higher in females than in males during the mating and moulting stages, as indicated by the significant life cycle stages × sex interaction, while the response intensity showed no clear pattern between life cycle stages (Fig. 1e, f). This result was a consequence of a significant change in agglutination and lysis activity in males over the annual cycle (agglutination: $F_{3,26}=5.98$, $P<0.01$; lysis: $F_{3,26}=3.86$, $P<0.05$), while no significant annual change was observed in females (agglutination: $F_{3,28}=2.38$, $P=0.09$; lysis: $F_{3,28}=2.05$, $P=0.13$). No difference was observed between the sexes in humoral immune responses against KLH, measured on birds when held in aviaries, and the immune response was highest during the breeding stage; it then dropped significantly to the moulting stage and then remained unchanged for the rest of the year (Fig. 1g). The T memory cell-dependent wing-web swelling response to KLH of birds held in aviaries showed no sex differences at any annual stage, and it showed the highest responses during the winter stage and the lowest responses during the mating stage (Fig. 1h).

Because of the difference in body mass between the sexes in the house sparrow (Anderson 2006), we reanalysed the sex-specific seasonal change in immune variables controlling for body mass as a covariate. However, due to the absence or low effect of body mass on immune variables (*E. coli* bacteria killing: $F_{1,56}=0.04$, $P=0.85$; *S. aureus* bacteria killing: $F_{1,56}=0.50$, $P=0.48$; antibody response to SRBC: $F_{1,45}=0.82$, $P=0.37$; PHA inflammation immune response: $F_{1,51}=1.42$, $P=0.24$; agglutination: $F_{1,50}=5.22$, $P=0.03$; lysis: $F_{1,50}=1.16$, $P=0.29$; antibody response to KLH: $F_{1,48}=0.00$, $P=0.96$; T-cell memory swelling to KLH: $F_{1,48}=0.02$, $P=0.90$), the results remained qualitatively unchanged (data not shown). Therefore, we conclude that the observed sex-specific seasonal change in immune functions is not confounded by the difference in body mass between the sexes.

Discussion

Sex differences in immune function

We found evidence for sex-specific seasonal variation in immune functions in the house sparrow, as three out of eight immune variables showed a significant life cycle stage × sex interaction, and five out of the eight immune variables differed between males and females during the mating stage. The dimorphism in the concentration of immunosuppressive steroid hormones, like testosterone and corticosterone (Roberts et al. 2004; Romero et al. 2006), is most pronounced during mating and breeding (Romero 2002; Anderson 2006), which may explain the reduced immune function of males related to females. The consistently reduced immune function of males related to females during mating strongly supports the 'immunocompetence handicap' hypothesis (Folstad and Karter 1992), which states that during the mating and breeding season, the physiological and hormonal costs related to reproductive activity of males may render them more exposed to infection due to the reduced investment in immune function or immunosuppression. However, because three out of eight immunological variables did not differ between the sexes, our data also suggest that different components of the immune system can be modulated differently over the annual cycle in the two sexes. Moreover, our data show that only some components of the immune system are susceptible to physiological changes related to the sexual activity of males during breeding. The differential effect of steroid hormones on various components of the immune system (Casto et al. 2001; Roberts et al. 2004) may explain the variability in sexual dimorphism of the immune variables characterising different components of the immune system. For example, the increased concentration of testosterone and corticosterone steroid hormones during breeding (Romero 2002; Anderson 2006) may differentially suppress components of the immune system. In fact, Casto et al. (2001) have found that testosterone implanted captive dark-eyed junco (*Junco hyemalis*) males increased both the testosterone and corticosterone hormones level, resulting in a significant reduction of humoral immune response against SRBCs related to the control group, while no effect was observed on PHA inflammation immune response. Because the primary function of the immune system is defence, a next step to reveal the mechanism responsible for the differences in immune responses between the sexes should be to determine the role of different components of immunity in protection against the seasonally emerging specific parasite challenges (Adamo 2004). We are aware of only a few studies on wild birds, where the immunological defence and infestation by specific parasites have been correlated (e.g., Saks et al. 2006; Pap et al. 2009; Parejo and Silva 2009). It is worth mentioning that because of the methodological constraints, induced immune responses could be measured only under captive conditions, while four variables of the constitutive immune defence were assessed under more natural conditions, i.e., directly at capture. Hence, the absolute value for the measures of the adaptive immune responses may be partly affected by housing the birds in captivity. Due to the captive conditions, the sexual and parental activity of males and females changed, which could modulate the circulating hormone levels, thereby affecting the measures of immune function assessed following the introduction of the birds to the

aviary. However, the aim of this study was to compare longitudinal data over the annual cycle, and because the birds were treated the same way at each life cycle stage and sampling, this comparison should be valid, although we are aware that the immune measures taken on captive birds have to be interpreted with caution. But, in line with the observed differences between the sexes in constitutive immune responses measured under natural conditions, at least one component of the induced immune response (PHA swelling) was significantly lower in males related to females, suggesting that under aviary conditions, the physiological differences between the sexes persist.

Seasonal change in immune functions

The seasonal change in immune function of birds can be explained by several hypotheses, which often relies on contradictory predictions about the immune activity among life cycle stages (Nelson et al. 2002; Hasselquist 2007; Martin et al. 2008). For example, the hypothesis about the physiological trade-off between the reproductive and immune system (the 'short summer–high stress' hypothesis; Hasselquist 2007; Martin et al. 2008) predicts that immunocompetence peaks in autumn and winter when work load is low, and the immune system is suppressed or down-regulated during the mating and breeding when the cost related to reproduction is at maximum (Sheldon and Verhulst 1996; Nelson et al. 2002; Hasselquist 2007). On the contrary, the 'parasite exposure' hypothesis predicts that immunocompetence should increase during the breeding season in temperate birds, when the risk of infection is high (Møller et al. 2003; Hasselquist 2007). Our results on the seasonal change in immune function suggest that the variation in immunocompetence of individual house sparrows depends on the variables measured, and that different measures characterise different components of the immune system. Bacterial killing assay, which is a direct measure of constitutive innate immunity in birds (Tieleman et al. 2005; Millet et al. 2007), characterise the capacity of blood to limit bacterial infection and increase significantly as a result of intestinal coccidian infestation, a common parasite in our house sparrow population (Pap et al. 2009; Pap et al. pers. obs.). In line with the adaptive regulation of a costly immune defence (Hasselquist 2007; Martin et al. 2008), we found that *S. aureus* killing was reduced during the physiological demanding mating and breeding stages, while the less costly bactericidal defence against *E. coli* (Millet et al. 2007) was reduced only during the breeding stage. Bacterial killing capacity of whole blood against *E. coli* is less costly then against *S. aureus* because killing of the gram-negative strain (i.e., *E. coli*) is mostly complement dependent while cell-mediated effectors contribute little. In contrast, killing of *S. aureus* is complement independent and requires phagocytic cell-mediated immune response, which is more costly compared with humoral effectors (Millet et al. 2007). The complement cascade (lysis to rabbit blood cells) increased and remained unchanged during mating in females and males, respectively, while the NAbs decreased in females. The antibody responses against KLH and SRBC characterise the humoral immune response of birds to specific antigens, which are standard complex antigens commonly used in immunological studies (e.g., Hasselquist et al. 1999; Ots et al. 2001; Blount et al. 2003; Eraud et al. 2005; Martin et al. 2006b; Ardia 2007). Both immune measures followed a similar seasonal pattern, with the highest response during breeding, suggesting common factors causing humoral immunity to be upregulated at this stage of the annual cycle. PHA immune response was lowest in winter followed by an increase during the mating stage, while the opposite pattern was observed in the wing-web swelling measuring the cellular memory response to KLH.

Concluding remarks

In conclusion, we found that three out of eight immune variables characterising either innate or acquired immunity showed a significant life cycle stage × sex interaction, and five immune variables differed between males and females during the mating stage, with females having stronger immune responses than males, while generally similar levels of immune responses between the sexes were observed at the three other stages of the year. Six out of the eight immune variables showed a significant seasonal change during the annual cycle. Taken together, this study shows that the immune system is highly variable between the sexes and different annual life cycle stages, and that there is considerable potential for fine tuning of the immune system to specific physiological states (that could differ between the sexes) and environmental conditions.

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