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Sexual dimorphism in susceptibility to parasites and cell-mediated immunity in great tit nestlings

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Summary

1. Parasites can affect host fitness, provoke host responses, and thereby mediate host life history evolution. As life history strategies are often sex-specific, immunological or behavioural responses of the host aiming to reduce the impact of parasites may be sexually dimorphic, e.g. as a consequence of sex differences in the resource allocation trade-off between parasite defence, morphological traits and body functions. Parasites may therefore affect males and females differently leading to sex specific patterns of parasite susceptibility.
2. In an experimental field study, we manipulated the ectoparasite load of great tit nests (*Parus major*) and investigated its effects on male and female nestlings. As susceptibility to parasites may be linked to the ability of the nestlings to fight off parasites immunologically, we further investigated sex differences in cell-mediated immunity using a phytohaemagglutinin (PHA) assay.
3. Body mass, metatarsus length and overall body size, but not feather length, showed a sexual dimorphism at the end of the nestling period. A significant interaction between the effects of sex and parasite treatment on the sexually dimorphic traits indicates that the parasite effect is sex-specific. While no differences in morphological traits were found in females raised in infested and uninfested nests, parasitized males were significantly smaller and lighter than males raised in uninfested nests. Further, we found a pronounced sexual dimorphism in the response to the PHA assay with males showing a reduced cellular immunity. The parasite treatment had a non-significant effect on the PHA response and the PHA response of males and females were not influenced differently by parasites.
4. Our study shows that sexual dimorphism in susceptibility to parasites and immunocompetence develops early in life, and suggests sex-specific strategies in the allocation of limited resources. Possible mechanisms of sex differences in susceptibility to parasites and immunocompetence during postnatal growth and the consequences for optimal sex allocation strategies of the parents are discussed.

Key-words: *Ceratophyllus gallinae*, immunocompetence, phytohaemagglutinin (PHA), resource allocation.

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Introduction

Parasites, through their effect on host fitness, select for adaptive responses and thereby mediate the life history evolution of their hosts (reviewed in Clayton & Moore 1997). Behavioural and/or immunological parasite defence that reduces the negative impact of parasites is thus assumed to be advantageous, but is also costly in terms of energy or metabolites (Lochmiller & Deerenberg 2000). Parasite defence may thus compete with other traits or functions for limited resources (e.g. the production of elaborate sexually selected traits, Folstad & Karter 1992), resulting in an allocation trade-off. This trade-off may be solved in a sex-specific way due to differential selection on males and females, leading to sexual dimorphism in morphology, physiology and behaviour.

Sex differences in immunocompetence and susceptibility to parasites have been found in mammals, including humans, with males being generally less

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immunocompetent and more susceptible to parasites than females (e.g. Olsen & Kovacs 1996; Poulin 1996; Schalk & Forbes 1997). In natural bird populations, studies investigating sex differences in immunocompetence or susceptibility to parasites are rare, not consistent and in most cases restricted to adults (e.g. McCurdy *et al*. 1998; Møller, Sorci & Erritzøe 1998; Moreno *et al*. 2001; Saino *et al*. 2002). In juveniles, evidence for sex differences in immunocompetence is limited to the study of Fargallo *et al*. (2002), which demonstrated a reduced cellular immune response in nestling male Eurasian kestrels (*Falco tinnunculus*) under limited food conditions. Similarly, evidence for sex differences in susceptibility to parasites is limited to Potti & Merino (1996), which showed in a subsample of recruited birds that the tarsus length of male nestling pied flycatchers (*Ficedula hypoleuca*) was reduced if raised in nests with high mite abundance (but see Potti 1999; Potti *et al*. 2002).

Practical reasons may explain the lack of evidence for sex differences in immunocompetence and susceptibility to parasites in wild-living juvenile birds as appropriate molecular techniques for avian sex determination were established only a few years ago (Ellegren & Sheldon 1997; Griffiths *et al*. 1998). However, the investigation of sex differences in immunocompetence and susceptibility to parasites at early life stages may be important for sex allocation theory, as sexual dimorphism in the physiology of juveniles may influence the cost of rearing male and female offspring and thus their reproductive value (Fisher 1930; Trivers & Willard 1973; Clutton-Brock, Albon & Guinness 1985).

In our study we used a combination of both experimental parasite manipulation and direct assessment of the immune system in great tit nestlings (*Parus major* L.) to investigate sexual dimorphism in susceptibility to parasites and immunocompetence in wild living nestling birds. Great tits are small hole-nesting passerines whose nests are commonly infested with the haematophagous hen flea *Ceratophyllus gallinae* Schrank (Tripet & Richner 1997). Earlier studies showed that this ectoparasite impairs condition and survival of nestlings as well as adult reproductive success (Richner, Oppliger & Christe 1993; Oppliger, Richner & Christe 1994). In two subsequent years, we manipulated the ectoparasite load of great tit nests in the field and investigated sex-specific effects of the parasites on the nestlings. As sex differences in susceptibility to parasites may be linked to sex-specific levels of immunocompetence, we further assayed the cell-mediated immunity of nestlings by an *in-vivo* hypersensitivity response to an injection of phytohaemagglutinin (PHA).

Materials and methods

PARASITE TREATMENT AND GENERAL EXPERIMENTAL PROCEDURE

The study was performed in 2001 and 2002 in a great tit population breeding in nest boxes in the Forst, Switzerland (46°54′ N, 7°17′ E/46°57′ N, 7°21′ E). At the start of egg laying all nests were heat-treated in a microwave oven following Richner *et al*. (1993) to kill nest-based parasites. The nests were assigned randomly to be an experimental nest or a flea-donor nest, respectively. Flea-donor nests were infested with 40 female and 20 male hen fleas directly after heat-treatment while all experimental nests remained uninfested until hatching.

One day after hatching, the nest insets including the nest material but not the nestlings were exchanged between pairs of experimental (n = 153 experimental nests) and flea-donor nests. The flea-donor nests were assigned to experimental nests according to hatching date and brood size, and every second one was heat-treated before nest exchange. Thus, 50% of the experimental nests contained hen fleas whereas the other 50% did not contain hen fleas after the nest exchange. With this procedure potential parasite-induced differences in maternal investment into the eggs (Buechler *et al*. 2002), in breeding behaviour (Christe, Richner & Oppliger 1996a) or in nest desertion until hatching were avoided in the experimental nests. Additionally, we accounted with this procedure for the natural life cycle of hen fleas, which immigrate into the nests and start reproduction already during the egg-laying period of their hosts (Tripet & Richner 1999).

Eight days post-hatching nestlings were ringed with aluminium rings and approximately 20 μL of blood were taken from the brachial vein and transferred to 100 μL of EDTA buffer. The samples were frozen at –20 °C until molecular sex determination. Fifteen days post-hatching we measured the body mass, the length of the metatarsus and the length of the third primary feather of the nestlings (Svensson 1992).

CELL-MEDIATED IMMUNITY

As an assessment of the cellular immunocompetence, we performed a hypersensitivity assay using phytohaemagglutinin (PHA) as a mitogen. The phytohaemagglutinin assay provides a measure of the proliferative response of the circulating T lymphocytes to the injected mitogen (see Cheng & Lamont 1988 for details). Studies on poultry revealed that the PHA assay is a reliable indicator of *in vivo* cellular immunity (Goto *et al*. 1978; McCorkle, Olah & Glick 1980; Cheng & Lamont 1988), and it is used commonly to assess the cell-mediated immune response in birds (e.g. Lochmiller, Vestey & Boren 1993; Brinkhof *et al*. 1999; Tella *et al*. 2000; Fargallo *et al*. 2002).

The nestlings were injected subcutaneously with 0·01 mg of PHA-P (Sigma Chemicals, Germany) dissolved in 0·02 mL of sterile phosphate-buffered saline (PBS) in the centre of the left wing-web (patagium) 14 days post-hatching. The thickness of the patagium at the injection site was measured with a micrometer (Mitotuyo, Type 2046FB-60) to the nearest 0·01 mm prior and 24 h (± 1 h) after injection. The micrometer applied a constant pressure on the wing web and the

measure stabilized with time. The thickness of the wing web 5 s after applying the micrometer was thus used as a standardized measurement of the wing web thickness. The difference between the wing-web thickness before and after PHA injection was used as an assessment of the cell-mediated immune response (referred to hereafter as wing web index; see Smits, Bortolotti & Tella (1999) for general information about the method).

All nestlings were PHA challenged in 2002 while in 2001 the six heaviest nestlings of each nest were assayed. The proportion of male and female nestlings in 2001 was not different among assayed and unassayed nestlings (females: $n = 33$, males: $n = 47$; $\chi^2 = 1{\cdot}59$, $P = 0{\cdot}21$). As the effects of sex on the PHA response were strong and consistent in both years (revealed by separate analyses of the two years) and as in 2002 the effects and the explained variance of the analysed variables did not change when including all or only the six heaviest nestlings, we can assume that assaying only the heaviest six nestlings in 2001 did not bias our results.

The accuracy of our measurement was assessed among a sample of 113 nestlings where the wing-web index was measured twice. Statistical analysis revealed that our measurements were highly repeatable ($r = 0{\cdot}993$, $P < 0{\cdot}0001$, $n = 113$) (Lessells & Boag 1987).

MOLECULAR SEXING

The sex of the nestlings was determined following the protocol of Griffiths *et al*. (1998). DNA was extracted from a subsample of the nestlings' blood using a commercial kit (Wizard® Genomic DNA Isolation Kit, Promega, Switzerland) following the manufacturer's protocol. PCR amplification was carried out in a total volume of 10 μL. The final reaction conditions were as follows: 1 μL *Taq* buffer, 2·5 mM $MgCl_2$, 0·25 U HotStar-*Taq* DNA polymerase (Qiagen, Basel, Switzerland), 0·2 mM of each dNTP (Amersham Pharmacia Biotech Inc.) and 1 μM each of primers P2 and P8; 1 μL of genomic DNA was used as template. PCR was performed in a GeneAmp 2400 or 9700 Thermocycler (Applied Biosystems) with the following temperature profile: initial denaturation at 95 °C for 15 min; 40 cycles of 94 °C for 30 s, 52 °C for 15 s and 72 °C for 75 s. The programme was completed by an additional extension step at 72 °C for 7 min. PCR products were separated with electrophoresis at 80 V for 30 min on a 2% agarose gel stained with ethidium bromide. Eighty-eight nestlings were not sexed because they died before blood was obtained.

STATISTICAL ANALYSES

Nested ANOVAS were used to analyse sex differences in morphological traits and immunocompetence. Year (random effect), parasite treatment and sex were included as factors into the model, and the nest (random effect) was included as a nested factor, nested within year and parasite treatment. Non-significant interactions were backward eliminated. Mortality was analysed by logistic regression analysis using the GLIMMIX macro with a logit link (SAS). All tests were two-tailed with a significance level set at $P = 0{\cdot}05$. Residuals of the models were tested for normality and equal variances. Means ± SE are given. Statistical analyses were performed using JMP IN 4·0 (Sall & Lehmann 1996) except for the mortality analysis where we used SAS (Littell *et al*. 1996).

Results

SEXUAL DIMORPHISM IN MORPHOLOGY

Sexual dimorphism was found in body mass (males: 15·41 g ± 0·09, females: 14·82 g ± 0·09) and in metatarsus length (males: 19·4 mm ± 0·04, females: 19·0 mm ± 0·03) 15 days post-hatching (Table 1). However, there was no difference in the feather length between male and female nestlings at the end of the nestling period (males: 34·4 mm ± 0·18, females: 34·2 mm ± 0·17) (Table 1).

As body mass, metatarsus and feather length were intercorrelated (Pearson correlations: metatarsus length and body mass: $r = 0{\cdot}558$, $P < 0{\cdot}0001$, metatarsus length and feather length: $r = 0{\cdot}549$, $P < 0{\cdot}0001$, body mass and feather length: $r = 0{\cdot}606$ p < 0·0001; $n = 741$), we calculated the first principal component of those measurements using principal component analysis as an overall measure of the nestlings body size (hereafter referred to as body size PC1). Body size PC1 explained 71·41% of the total variance (factor loadings: body mass = 0·585, metatarsus = 0·565, feather length = 0·582). Male nestlings had significantly higher body size PC1 values than females (Table 1).

EFFECTS OF PARASITES

The parasite treatment affected male and female nestlings differently as shown by a significant sex–parasite interaction on body size PC1 and body mass and a tendency towards statistical significance in metatarsus length (Table 1). While body size PC1 did not differ between females growing up in infested or uninfested nests, male nestlings had significantly lower body size PC1 values in infested nests, showing that males were more heavily impaired by parasites than females (Table 1, Fig. 1). The same pattern was also found in body mass and in metatarsus length. Interestingly, the differential effects of parasites on male and female nestlings were found only in sexually dimorphic traits, but not in feather length, where no sexual dimorphism was observed (Table 1).

CELL-MEDIATED IMMUNITY

The cell-mediated immune response as assessed by the wing-web index was significantly lower in male than in female nestlings (males: 0·657 mm ± 0·012, females:

Table 1. Differential effects of parasites on morphological traits of male and female nestling great tits at the end of the nestling period. Mixed-model nested ANOVA with year as random effect, parasite treatment and sex as fixed effects and the nest nested in year and parasite treatment as random nested factor. Non–significant interactions were backward eliminated

Source of variation	SS	d.f.	*F*	*P*
Body size PC1				
Year	0·904	1, 112	0·959	0·329
Nest (year, parasite treatment)	801·803	112, 624	7·614	< 0·0001
Parasite treatment	0·446	1, 112	0·473	0·493
Sex	52·680	1, 624	55·938	< 0·0001
Sex × parasite treatment	4·513	1, 624	4·792	0·029
Error	587·653	624		
Body mass				
Year	2·713	1, 112	2·203	0·141
Nest (year, parasite treatment)	1377·997	112, 624	9·993	< 0·0001
Parasite treatment	0·697	1, 112	0·566	0·454
Sex	61·846	1, 624	50·230	< 0·0001
Sex × parasite treatment	7·587	1, 624	6·162	0·013
Error	768·311	624		
Metatarsus length				
Year	3·304	1, 112	11·080	0·001
Nest (year, parasite treatment)	131·294	112, 624	3·931	< 0·0001
Parasite treatment	0·068	1, 112	0·227	0·635
Sex	38·043	1, 624	127·574	< 0·0001
Sex × parasite treatment	0·934	1, 624	3·132	0·077
Error	186·078	624		
Feather length				
Year	26·755	1, 112	4·979	0·028
Nest (year, parasite treatment)	3896·106	112, 624	6·474	< 0·0001
Parasite treatment	1·321	1, 112	0·246	0·621
Sex	0·011	1, 624	0·002	0·964
Sex × parasite treatment	6·618	1, 624	1·232	0·268
Error	3353·070	624		

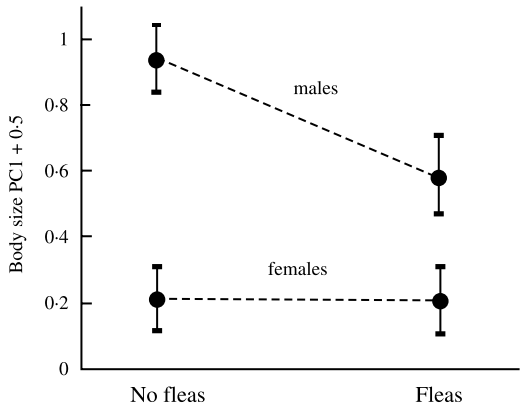

Fig. 1. Differential effects of parasites on body size PC1 of male and female nestling great tits.

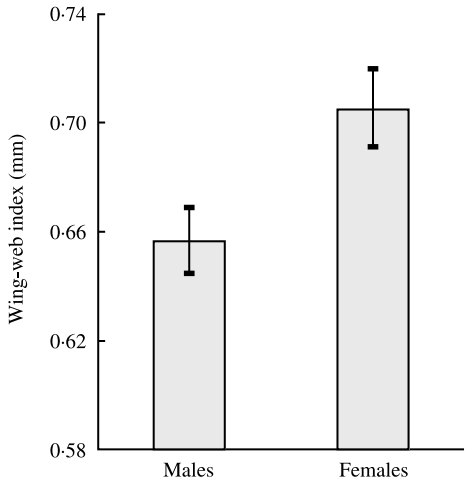

Fig. 2. Sex differences in cell mediated immunity of great tit nestlings as assessed by the PHA assay.

0·705 mm ± 0·014, Table 2, Fig. 2). As there was a positive correlation between body size PC1 and the wing-web index (Pearson correlation: $r = 0{\cdot}201$, $P < 0{\cdot}0001$, $n = 661$), body size PC1 was included into the model as a covariate. After inclusion of body size PC1, the wing-web index remained significantly different between the sexes, indicating that sex differences in PHA response were independent of morphological differences between the sexes. Nestlings from infested nests tended to have a reduced immune response compared to nestlings from uninfested nests (mean wing-web index of nestlings in uninfested nests: 0·694 mm ± 0·013, nestlings in infested nests: 0·665 mm ± 0·013), however, these differences were not statistically significant (Table 2). Furthermore, the interaction between sex and parasite treatment did not explain a significant amount of the variation of the wing-web index (Table 2).

Table 2. Sex differences in immunocompetence in great tit nestlings at the end of the nestling period as assessed by the PHA assay. Mixed-model nested ANCOVA with year as random effect, parasite treatment and sex as fixed effects, the nest nested in year and parasite treatment as random nested factor and body size PC1 as covariate. Non-significant interactions were backward eliminated

Source of variation	SS	d.f.	*F*	*P*
Wing-web index				
Year	4·359	1, 110	206·179	< 0·0001
Nest (year, parasite treatment)	6·121	110, 545	2·632	< 0·0001
Parasite treatment	0·040	1, 110	1·871	0·174
Sex	0·807	1, 545	38·183	< 0·0001
Sex × parasite treatment	0·016	1, 545	0·751	0·387
Body size PC1	2·011	1, 545	95·101	< 0·0001
Error	11·522	545		

MORTALITY

Complete nest mortality occurred in 16 infested and 21 uninfested nests ($\chi^2 = 1{\cdot}37$, $P = 0{\cdot}24$). In the remaining 116 nests 193 of 934 nestlings died. Nestling mortality was not significantly different between nestlings of infested ($n = 113$) and uninfested ($n = 80$) nests ($F_{1,114} = 0{\cdot}18$, $P = 0{\cdot}68$), or between female ($n = 54$) and male ($n = 51$) nestlings ($F_{1,728} = 0{\cdot}08$, $P = 0{\cdot}78$). Further, there was no significant sex–parasite interaction on mortality ($F_{1,728} = 0{\cdot}02$, $P = 0{\cdot}89$).

Discussion

Great tit nests are commonly infested with hen fleas (Tripet & Richner 1997), an ectoparasite that impairs the fitness of its host by reducing condition, growth and survival (Richner *et al*. 1993). Our study reveals that hen fleas do not affect the two sexes equally. While male nestlings from infested nests showed a reduced size and body mass, no such effects were found in female nestlings. Thus, the overall negative effect of fleas on nestling great tits is mainly due to the worse performance of males in infested nests.

In great tit nestlings, the bigger sex – the male – has been shown to suffer less from mortality or reduced growth under harsh conditions (Dhondt 1970; Smith, Kallander & Nilsson 1989) due probably to the male's higher competitive ability (Oddie 2000). In our population, male nestlings had bigger overall body size, higher body mass and a longer metatarsus (see also Smith *et al*. 1989). According to Oddie (2000), we would thus predict a better performance of males in infested nests as fleas increase the nutritional demands of the nestlings and thus the competition for food (Christe, Richner & Oppliger 1996b). Contrary to this prediction, we found that males were more heavily impaired by hen fleas than their female nest mates, a pattern also found in other size-dimorphic bird and mammal species where the larger sex is commonly more susceptible (Clutton-Brock *et al*. 1985; Clutton-Brock 1991).

Differential exposure of the two sexes (Reimchen 2001) may not explain the observed sex differences in parasite susceptibility, because fleas are nest-based parasites, and equal exposure of male and female nestlings can be expected. Alternatively, fleas may either preferably feed on males, feed in higher numbers on males as they are bigger targets, or females may be more efficient in behavioural or immunological defence (reviewed in Zuk & McKean 1996). Our results support the last hypothesis as we found that male nestlings had a significantly lower cell-meditated immune function, as measured by the response against PHA.

Sex differences in immunocompetence have been investigated rarely in wild-living nestling birds. Fargallo *et al*. (2002) estimated sex differences in immune response against PHA in nestling Eurasian kestrels under different food conditions and revealed – similar to our study – a lower immune response of male nestlings; however, only under limited food conditions. In Eurasian kestrels male nestlings are substantially smaller than females, whereas in our study species males are the bigger sex, suggesting that characters associated with male sex per se rather than differences in body size reduce the male's ability to mount an immune response.

Sex differences in immunocompetence have commonly been explained by modulating effects of sex hormones (Grossman 1989; Olsen & Kovacs 1996; Gaillard & Spinedi 1998). However, Silverin & Sharp (1996) did not find significant sex differences in the testosterone level of nestling and juvenile great tits except in the first two days after hatching. Thus, if testosterone leads to the observed sex differences in immunocompetence these effects might occur very early in life or even at a prenatal stage in the egg (Schwabl 1996).

Carotenoids have positive effects in promoting and supporting the immune system (Bendich & Olson 1989). However, carotenoids used for immune defence are not available for pigmentation. Thus, sex differences in immunocompetence may also arise due to an allocation trade-off between the immune system and signalling traits for rare carotenoids (Olson & Owens 1998; von Schantz *et al*. 1999; Tschirren, Fitze & Richner 2003), which might be solved differently in males and females. As great tits show a sexual dimorphism in carotenoid-based colouration – with males being more colourful –already as nestlings (Slagsvold & Lifjeld 1985; personal observation), colouration might be more

important for males than for females (e.g. in social signalling and social interactions after fledging), leading to the observed differences in carotenoid allocation and thus immunocompetence.

Both differential effects of parasites on male and female offspring and sexual dimorphism in immune response may have direct consequences on optimal sex allocation (Fisher 1930). Under natural conditions, fleas immigrate into the nests during nest building or are already present in the old nest material. Females may thus be able to predict the flea abundance during the nestling period and adaptively bias the sex ratio of offspring towards the less susceptible sex if high flea abundance is anticipated. Alternatively, females may allocate antibodies (Gasparini *et al*. 2001; Buechler *et al*. 2002; Gasparini *et al*. 2002), hormones (Schwabl 1996; Petrie *et al*. 2001) or other metabolites, e.g. carotenoids (Blount, Houston & Møller 2000) differently to male and female eggs, depending on parasite abundance, in order to optimize reproductive output.

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