Immune response of nestling warblers varies with extra-pair paternity and temperature

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Abstract

Extra-pair mating is widespread in birds, but its adaptive function remains unclear. It is often suggested that females obtain superior genes for their offspring as a consequence of extra-pair mating, but the evidence is limited. In this study, we examined the hypothesis that extra-pair mating provides females with offspring that have superior immune responses. We found that the T-cell-mediated immune response of extra-pair young was stronger than that of within-pair young in common yellowthroats (*Geothlypis trichas*). This paternity effect occurred when we compared all nestlings in the population, as well as in comparisons of both paternal and maternal half-siblings. Paternal half-siblings had a stronger immune response when they were produced with extra-pair females than with the male's social mate, which suggests that the greater immune response of extra-pair young was caused by non-additive (compatible) genetic effects. However, these patterns were only significant in the colder of 2 years. Immune response was related positively to air temperature and nestlings had a stronger immune response in the warmer year. We suggest that such environmental variation could obscure the genetic benefits of extra-pair mating.

Keywords: extra-pair mating, *Geothlypis trichas*, immune response, male ornaments, sexual selection

Introduction

Males with larger or more brightly coloured ornaments may be preferred by females as mates because the ornament signals a benefit to the female. These benefits may include the production of genetically superior offspring (indirect benefits) or direct benefits that affect the survival or reproductive success of the female, such as male assistance with parental care (Andersson 1994; Kokko 2002). Although indirect benefits are predicted to be small (Kirkpatrick & Barton 1997), empirical studies of mate choice suggest that females are choosy even in species in which males only contribute genes (nonresource-based mating systems; Neff & Pitcher 2005). Examples include female choice in lekking species (Welch *et al.* 1998; Ekblom *et al.* 2004), insects without parental care or nuptial gifts (Hedrick 1988), and extra-pair mating in birds (Yezerinac & Weatherhead 1997; Thusius *et al.* 2001).

Extra-pair mating is common in socially monogamous birds (Griffith *et al.* 2002). As a result, females often produce young sired by both within-pair and extra-pair males (e.g. broods of mixed paternity). This provides an excellent opportunity to examine the indirect genetic benefits of female choice, while controlling for the nongenetic effects of rearing environment and maternal effects (Sheldon *et al.* 1997). When extra-pair and within-pair young (maternal half-siblings) are raised in the same nest, the differences between them are most likely due to genetic differences between the sires (Sheldon *et al.* 1997). Recent studies of extra-pair mating in birds have revealed significant phenotypic or genotypic differences between extra-pair and within-pair offspring (Griffith *et al.* 2002; Foerster *et al.* 2003), and some of these studies have also shown a correlation between extra-pair mating and male ornaments (e.g. Hasselquist *et al.* 1996; Kempenaers *et al.* 1997; Sheldon *et al.* 1997). However, it remains unclear if and how male ornaments signal potential benefits to females. One possibility is that, within a species, males with larger ornaments may be healthier than males with smaller ornaments, because they carry genes for resistance to disease or parasite infestation

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and, as a consequence, have more resources to invest in a large ornament. Thus, recent studies have focused on whether male ornaments indicate genes for greater immune capacity.

Genes for enhanced immune system function are one form of genetic benefits that females may gain from extra-pair mating (or mating with a highly ornamented male). Immune responses are excellent indicators of disease resistance, which is crucial to survival, especially during the nestling stage of altricial birds (Lochmiller *et al.* 1993). Thus, females could benefit indirectly if the fitness of their offspring is greater due to the inheritance of an enhanced immune system from the extra-pair sire. However, to date, only one study has found evidence that extra-pair mating can improve the individual immune response (immuno-competence) of nestlings. In bluethroats (*Luscinia svecica*) extra-pair young were more immunocompetent than their within-pair half-sibs (Johnsen *et al.* 2000). Two other studies of reed buntings (*Emberiza schoeniclus*; Kleven & Lifjeld 2004) and barn swallows (*Hirundo rustica*; Kleven *et al.* 2006) have found no effect of paternity on nestling immunocompe-tence. Therefore, it is still unclear if extra-pair mating leads to offspring that are more immunocompetent and if male ornaments signal immunocompetence.

The common yellowthroat (*Geothlypis trichas*) provides a rare opportunity to determine if females use male orna-mental traits to choose extra-pair sires with superior genes for immunocompetence. It is one of a handful of species (see Table 3 in Griffith *et al.* 2002) in which extra-pair mating success is related to the size of a male plumage ornament, the black facial mask (Thusius *et al.* 2001; Abroe *et al.* 2006). In aviary experiments, females prefer males with larger masks, and males with larger masks are dominant over males with smaller masks (Tarof *et al.* 2005). Although extra-pair mating occurs regularly in common yellowthroats (20% of young and 46% of broods; Thusius *et al.* 2001), it remains unknown what genetic benefits females gain from extra-pair mating. In this study, we investigated whether extra-pair young had enhanced immune system function by comparing the T-cell-mediated immune response of within-pair and extra-pair nestlings. We also investigated whether the mask size of sires was related to the T-cell-mediated immune response of nestlings.

Methods

Field techniques

Field research was performed at the University of Wisconsin-Milwaukee (UWM) Field Station in Saukville, WI (43°23′N, 88°01′W) from May to August 2002–2004. Territories of common yellowthroats were located in 5.4 hectares of contiguous conifer bog and willow/sedge marsh. Nests were located using behavioural cues of adults and were monitored from the date of egg-laying until the young fledged (at 8 days of age). Tarsus length and mass of nestlings ($n = 284$ young in 79 broods) were measured on days 4 and 5 after hatching. Nestling growth rate was estimated by the change in mass from day 4 to day 5.

Adult birds were captured in mist nets and banded with unique combinations of three coloured plastic bands and a US Fish and Wildlife Service (USFWS) band. Small (30–50 µL) blood samples were collected via the brachial vein from all adults and nestlings for molecular analyses of paternity and sex. We recorded body mass, and wing, tail and tarsus length of adults, and photographed males to estimate the size of the mask and bib. Mask and bib size were measured using image analysis software (Thusius *et al.* 2001), and size estimates were highly repeatable between different pictures of both the mask and bib (both $r = 0.99$). Bib colour was estimated by measuring the hue, saturation and brightness using ADOBE PHOTOSHOP ELEMENTS 2.0 (Adobe Systems; see Tarof *et al.* 2005). We used a prin-cipal components analysis to combine the three bib colour variables into a single colour score (PC1) that explained 46% of variation in bib colour and loaded positively for hue (0.70) and brightness (0.06) and negatively for saturation (−0.71).

Immunoassay

The T-cell-mediated immune response of nestlings was tested in 2003 and 2004 with a subcutaneous injection of a nonpathogenic antigen, phytohaemagglutinin (PHA). In other species of birds, the PHA skin test is a sensitive indicator of immunocompetence (Cheng & Lamont 1988) and is also correlated with nestling survival (Christe *et al.* 1998) and longevity (Birkhead *et al.* 1999). At 4 days of age, each nestling ($n = 159$ in 39 broods) was tested with a subcutaneous injection of 0.05 mg PHA dissolved in 10 µL of saline into the right wing web. A control of 10 µL of saline was injected into the left wing web. Wing thickness was measured to the nearest 0.01 mm before injection on day 4 posthatch, and 24 (± 1) hours later using a digital thickness gauge (Mitutoyo model 700–118). Several measurements were taken of each wing web until a consistent value was reached and recorded. T-cell-mediated immune response was measured as the increase in wing thickness of the PHA-injected wing minus the change in the control wing (Lochmiller *et al.* 1993). All measurements of immune response were performed by J.G. In our analyses of T-cell-mediated immune response we controlled for air temperature (during the 24-h period after injection) and nestling body mass. The T-cell-mediated immune response is lower during cold, wet weather that reduces food abundance (Christe *et al.* 2001; Lifjeld *et al.* 2002) and greater among nestlings with greater body mass (Brinkhof *et al.* 1999; Christe *et al.* 2001; Kleven & Lifjeld 2004). Air temperature was measured at an automated weather station located on the study site.

Paternity analysis

Paternity was examined in broods produced from 2002 to 2004. Parentage of each nestling was determined using four highly variable microsatellite loci developed for closely related warblers [*Dpu*01 and *Dpu*16, Dawson *et al.* 1997; *Dca*24 (2002 & 2003 only), *Dca*28, and *Dca*32 (2004 only), Webster *et al.* 2001]. Details of DNA extraction, polymerase chain reaction (PCR) conditions and thermal cycling programs for common yellowthroats at *Dpu*01, *Dpu*16, *Dca*24 and *Dca*28 are given in Abroe *et al.* (in press). For *Dca*32, we used 50–100 ng of genomic DNA, 0.3 pmol of both the fluorescently labelled forward primer and reverse primer, 10 mM Tris-HCl (pH 8.3), 3.0 mM $MgCl_2$, 0.8 mM dNTPs, and 0.1 U *Taq* in a 10-μL reaction (see Webster *et al.* 2001 for thermal cycling conditions). PCR products and a GeneScan-500 fluorescently labelled size standard were run on an automated sequencer (ABI 373; 6% polyacrylamide gel). Alleles sizes were determined with GENOTYPER 2.0 software (Applied Biosystems Inc.).

The number of alleles for each locus ranged between 16 and 32. The combined probability of exclusion (P_{et}; Jamieson 1994) was greater than 0.999 for both combinations of four loci (see above). All nestlings matched their mothers at all four loci. Nestlings were considered within-pair young if they matched the putative sire at all four loci ($n = 228$). Nestlings were considered extra-pair young if they mismatched the putative sire at one or more loci ($n = 56$). For offspring that mismatched at just one locus ($n = 18$), we calculated the probability of chance inclusion (Jeffreys *et al.* 1992) for the three matching loci. In all 18 of these cases, offspring had a high probability of chance inclusion (> 0.05; mean = 0.36 ± 0.03; range = 0.08–0.69) and, thus, were considered extra-pair young (Johnsen *et al.* 2000). Sires of extra-pair young were assigned by comparing the paternal genotype of a nestling to the genotypes of all males in the population until a match at all four loci was found. Using this criterion, we were able to assign parentage to 49 of 56 (88%) extra-pair young (including 16 of the 18 young with a single mismatching locus). Two of the remaining seven extra-pair young (both in the same brood) matched two males at all four loci. One of these matching males resided five territories away and the other was a neighbour observed intruding onto the territory. Over 90% of extraterritorial forays by radio-tracked males and females were to immediate neighbours (Pedersen *et al.* 2006), so in this case we assigned these two nestlings to the neighbouring male. We were unable to find an extra-pair sire in our population for the remaining five extra-pair young (includes two young with a single mismatching locus). The probability that a randomly chosen male would match the paternal genotype of a specific nestling at all four loci (combined probability of chance inclusion) was low, both for within-pair young (mean = 0.0034 ± 0.0003; range = 0.00006–0.024) and extra-

pair young (mean = 0.0037 ± 0.0004; range = 0.0003–0.013; see Jeffreys *et al.* 1992).

Sex determination of nestlings

There is evidence of sex-based differences in nestling immune response (Tschirren *et al.* 2003; Chin *et al.* 2005), so we examined the T-cell-mediated immune response while controlling for nestling sex. Common yellowthroat nestlings are monomorphic, so we used a PCR-based molecular technique (P2 and P8 primers in Griffiths *et al.* 1998) to determine the sex of all 284 nestlings. Details are given in Abroe *et al.* (in press).

Statistical analysis

We examined the prediction that extra-pair young will have a greater T-cell-mediated immune response than within-pair young with a mixed-model analysis of variance (ANOVA) of all nestlings in the population. In this analysis, paternity, air temperature during the 24-h period after injections, nestling body mass, and sex were included as fixed factors, and territory was included as a random effect to control for the nonindependence of young from the same territory (two pairs were double-brooded in the same season, and, thus, the first and second broods were pooled for analysis). We also examined the immune response of within-pair young and their extra-pair half-siblings in the same nest (maternal half-siblings) with a similar mixed-model ANOVA using territory as a random effect that controls for maternal genotype and nestling environment. This analysis used the same fixed factors as above except for air temperature, as all young in a particular brood experienced the same temperatures during the immunoassay. An additional mixed-model ANOVA was performed comparing the immune response of young sired by the same male, but from different nests (paternal half-siblings) to investigate the effects of temperature, nestling body mass, maternal genotype and any maternal–paternal genotype interactions (compatibility effects). In this model, paternity, air temperature, body mass, and sex were fixed factors, while sire was a random effect. Statistical analyses were performed with JMP (version 5.0.1, SAS Institute Inc.). Least-squares means are reported with their standard error from mixed models. All tests were two-tailed. Sample sizes for some analyses differ because all ornament measurements were not available for all males.

Results

Over all 3 years, 56 of 284 (20%) offspring were sired by extra-pair males in 35 of 79 broods (44%). The proportion of extra-pair young (16/82 in 2002, 22/101 in 2003, and 18/101 in 2004; $\chi^2_2 = 0.50$, $P = 0.78$) and the proportion of

Table 1 Mixed-model analyses of the T-cell-mediated immune response of nestlings. The first analysis compares the immune response of all nestlings in the population. The analysis of maternal half-sibs only includes broods with both extra-pair and within-pair young (*n* = 15). The analysis of paternal half-sibs only includes males who sired both within-pair young and young in other nests (extra-pair; *n* = 11 males). Territory was included as a random effect in the first two analyses, while identity of the sire was included as a random effect in the paternal half-sib analysis. Paternity was coded as extra-pair or within-pair, and a positive estimate indicates extra-pair young had a greater immune response

Response	Fixed effect	Estimate	F	P
Immune response of all nestlings (d.f. = 1,114)				
	Paternity	0.144	6.42	**0.013**
	Air temperature	0.019	5.23	**0.024**
	Body mass	0.060	5.59	**0.020**
	Nestling sex	0.001	0.01	0.954
	Year * paternity	−0.178	6.94	**0.010**
	Year	0.085	0.57	0.213
Maternal half-sib immune response (d.f. = 1,48)				
	Paternity	0.146	4.40	**0.041**
	Body mass	0.045	0.99	0.325
	Nestling sex	0.009	0.059	0.809
	Year * paternity	−0.182	4.64	**0.036**
	Year	0.095	0.90	0.347
Paternal half-sib immune response (d.f. = 1,45)				
	Paternity	0.287	9.82	**0.003**
	Air temperature	0.039	5.45	**0.024**
	Body mass	0.054	1.32	0.257
	Nestling sex	0.010	0.067	0.798
	Year * paternity	−0.318	9.85	**0.003**
	Year	<−0.001	< 0.001	0.998

broods with at least one extra-pair young (12/26 in 2002, 12/26 in 2003, 11/27 in 2004; $\chi^2_2 = 0.21$, $P = 0.90$) did not differ between years.

Across the population, extra-pair young had greater immune responses than within-pair young in a mixed model using all 39 immunochallenged broods from 2003 and 2004 ($F_{1,114} = 6.42$, $P = 0.013$; Table 1). There was an interaction between year and paternity ($F_{1,114} = 6.94$, $P = 0.010$; Table 1; Fig. 1), because extra-pair young had a much stronger PHA response than within-pair young in 2003 (least-squares means: 0.23 ± 0.11 and -0.05 ± 0.04 mm, respectively), while there was no difference between extra-pair and within-pair young in 2004 (0.14 ± 0.07 and 0.21 ± 0.03 mm, respectively). Immune response was also related positively to air temperature during the 24-h period after injections ($F_{1,114} = 5.23$, $P = 0.024$; Table 1). Temperature during the 24-h period after injections was lower in 2003 (17.3 ± 0.35 °C) than in 2004 (18.4 ± 0.26 °C) when we compared corresponding dates (19 June to 14 July; mixed model with nest as a random factor $F_{1,99} = 1.82$, $P = 0.014$). Thus, the interaction between years may be explained, at least in part, by how extra-pair and
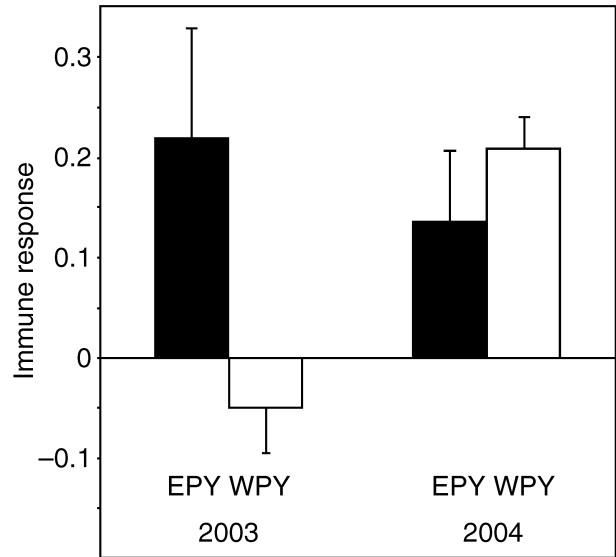

Fig. 1 T-cell-mediated immune response (mm) of extra-pair (EPY) and within-pair (WPY) young in 2003 and 2004. Bars indicate least squares means (and SE) from the mixed model in Table 1.

Table 2 Mixed-model analyses of nestling growth rate (g/day). Territory was included as a random effect in the analysis. Paternity was coded as extra-pair or within-pair, and a positive estimate indicates extra-pair young had a greater response

Response	Fixed effect	Estimate	F	P
Nestling growth rate (d.f. = 1,115)				
	Paternity	0.147	4.05	**0.047**
	Paternity * year	−0.147	2.88	0.092
	Nestling sex	−0.057	4.11	**0.045**
	Year	0.113	0.80	0.374
	Immune response	−0.261	3.33	0.071
	Immune response * year	0.262	1.79	0.183

within-pair young respond to differences in air temperature. Immune response was also related positively to nestling body mass ($F_{1,114} = 5.59$, $P = 0.020$; Table 1). There was no qualitative change to the results when we added date of the immunoassay to the model or removed nestling sex.

We also investigated the effect of extra-pair paternity on nestling growth rate (from day 4 to day 5) using all 39 broods. Growth in body mass was greater for extra-pair (1.4 ± 0.15 g/day) than within-pair (1.1 ± 0.09 g/day; $F_{1,115} = 4.05$, $P = 0.047$) young in a mixed-model ANOVA with paternity, immune response, nestling sex, the interaction between immune response and year and the interaction between paternity and year as fixed effects (Table 2). Immune response and its interaction with year were included in the model because previous studies have found evidence for a trade-off between growth in mass and immune response (e.g. Soler *et al.* 2003; Brommer 2004). Our analysis supported

this hypothesis as growth rate tended to be slower when nestlings had a stronger immune response ($F_{1,115}$ = 3.33, P = 0.07). In this analysis male nestlings (1.32 ± 0.10 g/day) also grew faster than female nestlings (1.21 ± 0.11 g/day; $F_{1,115}$ = 4.11, P = 0.045; Table 2).

A comparison of within-pair and extra-pair young in the same brood (maternal half-siblings) allowed us to examine the effects of paternity on immune response while controlling for rearing environment and maternal genetic effects. The sample of broods with both maternal half-siblings and data on immune response was small (n = 15 broods), but we found a similar pattern of immune response as in the mixed-model analysis of all nests. The immune response of extra-pair young (0.26 ± 0.12 mm) was greater than that of their within-pair half-siblings (−0.04 ± 0.09 mm; $F_{1,48}$ = 4.4, P = 0.041; Table 1), and there was also an interaction between years ($F_{1,48}$ = 4.6, P = 0.036; Table 1), which occurred because the immune response of extra-pair young was greater than that of within-pair young in 2003 (0.26 ± 0.12 mm and −0.04 ± 0.09 mm, respectively), but not in 2004 (0.17 ± 0.08 mm and 0.24 ± 0.06 mm, respectively). In this analysis, immune response was not affected by nestling body mass ($F_{1,48}$ = 0.99, P = 0.32; Table 1) or sex ($F_{1,48}$ = 0.059, P = 0.81; Table 1).

We also compared the immune response of nestlings sired by the same male, but raised in different nests (paternal half-siblings); that is, as within-pair young in the male's own nest and as extra-pair young in other nests (n = 11 sires that sired both extra-pair and within-pair young). This type of analysis can potentially reveal whether there are interactions between male and female genotypes (genetic compatibility effects). In a mixed-model analysis with sire as a random effect, these paternal half-siblings had a greater immune response (0.52 ± 0.18 mm) when they were raised in other nests (as extra-pair young) than when they were raised as within-pair young in the male's own nest (−0.05 ± 0.11 mm; $F_{1,45}$ = 9.8, P = 0.003; Table 1). As found above, there was also an interaction between years ($F_{1,45}$ = 9.8, P = 0.003; Table 1), because the immune response of extra-pair young was greater than that of within-pair young in 2003 (0.52 ± 0.18 mm and −0.05 ± 0.11 mm, respectively), but not in 2004 (0.21 ± 0.09 mm and 0.27 ± 0.06 mm, respectively). In this analysis, immune response was related positively to air temperature ($F_{1,45}$ = 5.4, P = 0.024; Table 1), but was not affected by nestling body mass ($F_{1,45}$ = 1.3, P = 0.26; Table 1), or sex ($F_{1,45}$ = 0.067, P = 0.80; Table 1).

Consistent with previous studies of this population (Thusius *et al.* 2001), extra-pair males had larger masks (335 ± 6.5 mm²) than the males they cuckolded (316 ± 8.5 mm²; paired t_{29} = 2.26, P = 0.03; data from all 3 years). However, mask size was not related directly to immune response when we replaced paternity with mask size as a predictor in the mixed models above (all P > 0.36). Bib size (paired t_{28} = 1.67, P = 0.11), bib colour (PC1; paired

t_{28} = 0.35, P = 0.73) and body mass (paired t_{27} = 0.56, P = 0.58) did not differ between extra-pair and within-pair males. Mask size of extra-pair males averaged 11% larger in 2003 than in 2004 (364.0 ± 7.5 and 323.2 ± 7.9 mm², respectively; t_{21} = 3.7, P = 0.001), and it was also less variable in 2003 (SD = 13.7, n = 12) than in 2004 (SD = 35.0, n = 11; Bartlett's F = 7.9, P = 0.005). Among all males, there was no difference in the average size of masks in 2003 and 2004 (315.5 ± 7.3 and 317.3 ± 8.7 mm², respectively; t_{68} = 0.2, P = 0.87), but mask size was more variable among all males in 2003 (SD = 54.2, n = 41) than in 2004 (SD = 34.1, n = 29; Bartlett's F = 6.4, P = 0.01).

Discussion

We found significant annual variation in the effect of extra-pair paternity on the immune response of nestling common yellowthroats. Extra-pair young had greater T-cell-mediated immune responses than within-pair young, both across the population and between half-sibs, in 2003 but not in 2004. Furthermore, extra-pair young grew faster than within-pair young in 2003, but not in 2004. While our results from 2003 support the hypothesis that females gain more immuno-competent offspring from extra-pair mating, our results from 2004 do not. We discuss some possible explanations for this difference between years, including environmental variation that affects the relative benefits to females of extra-pair mating.

Effects of extra-pair mating

In 2003, extra-pair young had a greater immune response than within-pair young when we examined all nests in our population. When the analysis was restricted to broods with mixed paternity, we also found that extra-pair young had a greater immune response than their maternal half-sibs raised in the same nest; this suggests a paternal genetic effect (Johnsen *et al.* 2000), but may also indicate differential investment by the female (see below). In addition, extra-pair young had a greater immune response than their paternal half-siblings raised as within-pair young in the nest of their genetic father. The stronger immune response of extra-pair young compared with both their paternal and maternal half-sibs may suggest an interaction between the genotypes of females and their extra-pair mates. A similar effect of both paternal and maternal genotypes on nestling immune response was found in bluethroats by Johnsen *et al.* (2000). These results suggest that extra-pair matings result in more immunocompetent young through genetic compatibility, rather than specific alleles for enhanced immune response. There is no evidence in bluethroats that females choose extra-pair males based on their phenotypic traits; thus, Johnsen *et al.* (2000) suggested that a post-copulatory mechanism within the female's reproductive tract

may play a role in selecting genetically compatible sperm.

In contrast to bluethroats, there is evidence from aviary (Tarof *et al*. 2005) and field studies (Thusius *et al*. 2001; Pedersen *et al*. 2006; this study) that a male ornament (mask size) is related positively to extra-pair mating success. This directional selection for a larger ornament is expected when females gain a good genes benefit, as all females mating with highly ornamented males should gain offspring with greater fitness (Neff & Pitcher 2005). We did not find a direct relationship between mask size and immune response in this study, which is not consistent with the traditional view of good genes benefits, but the relationship between ornaments and immunity is likely to be confounded by tradeoffs with other components of fitness (Westneat & Birkhead 1998). Furthermore, if ornament expression of males is correlated positively with individual heterozygosity, then females could receive a compatible genes benefit from mating with more ornamented males, because their offspring are more likely to be heterozygous and, as a consequence, have greater fitness (e.g. see Foerster *et al*. 2003; Reid *et al*. 2005). On the other hand, it is possible that mating with a male with a larger mask provides good genes for some benefit other than immune response, or that males with larger masks are better competitors for extra-pair matings, and, thus, these patterns are not due simply to female choice. Indeed, males that make extra-territorial forays tend to have larger masks, and they visit the territories of neighbours with smaller masks (Pedersen *et al*. 2006), where they are likely to be dominant (Tarof *et al*. 2005). It seems likely that both female choice and male-male competition contribute to extra-pair mating in common yellowthroats, and, consequently, the greater immune response of extra-pair young. It is also difficult to ascribe these genetic benefits to additive ('good genes') or nonadditive (compatibility) genes, although the analysis of halfsibs supports the latter. These two types of genetic benefits are not mutually exclusive and may be best considered opposite ends of a spectrum of genetic benefits (Neff & Pitcher 2005).

The superior immune response of extra-pair young could also have been influenced by a variety of environmental effects, including differences in maternal investment in particular eggs or rearing environment (i.e. maternal nongenetic effects). For example, females can alter their investment in eggs, by altering levels of testosterone (Gil *et al*. 1999), antibodies (Saino *et al*. 2002) or egg size (Cunningham & Russell 2000; Parker 2003) in relation to the attractiveness of their social mate. However, to our knowledge, there is no evidence that females make an adaptive decision to direct these resources to the individual eggs sired by extra-pair males (rather than to the entire clutch). If these nongenetic maternal effects increased the body mass of extra-pair nestlings, then it would tend to enhance the T-cell-mediated immune response, because it is correlated positively with body mass (Brinkhof *et al*. 1999; this study). However, in our mixed-model analysis of immune response (Table 1) we found an effect of paternity even after controlling for nestling body mass. Although males grew faster and were heavier than females, there was no effect of nestling sex on immune response in the mixed model, and there is no evidence that brood sex ratio is related to extra-pair paternity (Abroe *et al*. in press).

Extra-pair young also grew faster than within-pair young in 2003. A similar effect of extra-pair paternity on nestling growth rate was reported in a meta-analysis of 40 bird species (Royle *et al*. 1999). Life-history theory predicts that there should be a trade-off between costly activities such as immune response and growth (reviewed in Lochmiller & Deerenberg 2000) and this relationship is supported in other studies of growth and immune function in nestling birds (Soler *et al*. 2003; Brommer 2004). There was some evidence for this type of trade-off between growth and immune function ($P = 0.07$, Table 2) in common yellowthroats; however, after controlling for this effect, we found that extra-pair young were still able to grow faster and mount a stronger immune response. Thus, both immunity and growth may be independently associated with extra-pair paternity, at least in one of 2 years.

Variation between years

In the cooler year (2003) we found that extra-pair young had a greater immune response and faster growth rate than within-pair young, while there were no such differences in the warmer year (2004). It is possible that the warmer temperatures in 2004 allowed all nestlings to mount a relatively high immune response, which may have obscured the effect of paternity. These results are not entirely unexpected as the development of immune responses may be influenced by environmental conditions (Westneat & Birkhead 1998), particularly temperature. The average difference between years in temperature was small (1.1 °C), and temperature explained just 17% of the variation in immune response (mixed model with only temperature as a predictor), so it seems likely that other environmental factors associated with temperature (e.g. food abundance) contributed to the difference between years. For example, temperature and food abundance had statistically independent effects on immune response in tree swallows (*Tachycineta bicolor*; Lifjeld *et al*. 2002). Further study is needed of how immune response varies with environmental conditions, because it is not clear if limitation of energy or nutrients is a sufficient explanation for changes in immune response (see Svensson *et al*. 1998). Overall, these results are consistent with a growing body of evidence that genetic benefits are dependent on environmental context and may only appear under relatively poor conditions (Schmoll *et al*. 2005).

It is also possible that differences between years occurred as a result of variation in female choice. Overall, females prefer males with larger masks (Thusius *et al.* 2001; Tarof *et al.* 2005; Pedersen *et al.* 2006). There was less variation in mask size among extra-pair males in 2003 than in 2004, even though there was more variation in mask size among all males in the population in 2003 than in 2004. This pattern may indicate that females were more discriminating in their choice of extra-pair mates in 2003 than in 2004, or perhaps used different mate choice criteria. Thus, environmental and social factors may have led to stronger selection for genetically superior extra-pair males in 2003 than in 2004 (as indicated by mask size), and, as a consequence, a relatively stronger immune response among extra-pair young.

Our results support the prediction that extra-pair sires provide genetic benefits to females in the form of genes for increased immune response and, unexpectedly, genes for faster nestling growth. However, these benefits varied between years apparently due to differences in social and environmental factors. Such annual variation should be considered in future studies because it could obscure the genetic benefits of extra-pair mating.

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