

Brood size and environmental conditions sex-specifically affect nestling immune response in the European starling *Sturnus vulgaris*

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In sexually size-dimorphic species, the larger sex can be more sensitive to stressful environmental conditions, often resulting in reduced growth and elevated mortality rates. Development of the immune system is regarded as highly resource dependent, and recent data suggest that nestling passerines experience a possible resource-based trade-off between growth and immunity. Given the hypothesized importance of maximizing growth for the larger sex, the corresponding immune system may also exhibit similar sensitivity to limited resources. To better understand how natural variation in brood size and resources might differentially affect growth and immune function in nestlings of a sexually size-dimorphic species, we examined the relationship between brood size and inter-sexual differences in cell-mediated immunity (CMI) and survival in European starling *Sturnus vulgaris* nestlings where males are larger in both mass and structural size. We hypothesized that male CMI response should be negatively impacted by increasing sibling competition (brood size), especially during periods of low resource availability. In a year of reduced parental provisioning rates and reduced chick growth rates, male offspring exhibited the predicted negative relationship, whereas female CMI response was unaffected. However, in a year of improved provisioning rates and chick growth, neither sex exhibited a negative relationship between immune response and brood size. Thus, natural variation in brood size can affect sex-specific immunity differently in offspring of a sexually size-dimorphic passerine. However, this relationship appears resource-dependent, suggesting that the hypothesized resource-based trade-off may be compensated for in years of adequate resource abundance.

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Within sexually size-dimorphic birds, the larger sex can exhibit higher sensitivity to stressful conditions such as lower resource availability compared with the smaller sex (Weatherhead and Teather 1991, Torres and Drummond 1997, Hipkiss et al. 2002). This sensitivity is largely considered to be due to greater energetic demands and/or food consumption (Wiebe and Bortolotti 1992, Anderson et al. 1993, Krijgsveld et al. 1998, Riedstra et al. 1998, Love et al. 2005), or differential resource allocation required during development of the larger sex (Newton 1978, Richter 1983, Torres and

Drummond 1999). Greater resource requirements of the larger sex, coupled with a greater sensitivity to stressful environmental conditions such as food shortage and/or competition from siblings, can result in reduced growth rates and even increased mortality (Clutton-Brock et al. 1985, Weatherhead and Teather 1991, Torres and Drummond 1997, Hipkiss et al. 2002). Furthermore, this sex-specific sensitivity to resource availability during growth may have far-reaching consequences upon fitness-related traits as adults (Metcalfe and Monaghan 2001).

Recent studies have shown that the development of the immune system appears to be energetically costly and resource-limited (Klasing and Leshchinsky 1999, Lochmiller and Deerenberg 2000, Norris and Evans 2000, Alonso-Alvarez and Tella 2001, Martin et al. 2003, Bonneaud et al. 2003). During energetically stressful periods therefore, resources may be reallocated towards other costly functions (Sheldon and Verhulst 1996) such as growth (Birkhead et al. 1999, Hōrak et al. 1999, Soler et al. 2003, Love et al. 2005), given that it may be very costly to compensate for a poor start in terms of growth (Metcalf and Monaghan 2001). Consistent with this idea, studies under both wild (Saino et al. 1997a, Hōrak et al. 1999) and captive conditions (Naguib et al. 2004) indicate that increasing sibling competition via increases in brood size negatively impacts immune function of individual nestlings, suggesting a possible trade-off between immune function and growth (Saino et al. 1997a, Soler et al. 2003). In a sexually size-dimorphic species, any resource allocation trade-off between growth and immune function should also be dependent on nestling sex (Fargallo et al. 2002) since the larger sex may have to allocate more resources and energy towards factors such as growth or sexual ornamentation rather than immune function (Møller et al. 1998, Tschirren et al. 2003, Love et al. 2005). Although numerous studies have examined potential energetic costs of the larger sex in sexually size-dimorphic birds (Wiebe and Bortolotti 1992, Anderson et al. 1993, Torres and Drummond 1997, Krijgsveld et al. 1998, Riedstra et al. 1998, Torres and Drummond 1999), few have examined resource-based effects on inter-sexual differences in immune function (but see Fargallo et al. 2002). Furthermore, no studies have yet documented the combined effects of brood size variation and changes in resources across years on the potential sensitivity of immune function in a sexually size-dimorphic bird.

In this study, we examined the effects of natural variation in brood size, parental provisioning rates and chick growth rates on inter-sexual differences in T-cell-mediated immune response in nestlings of a sexually size-dimorphic species, the European starling *Sturnus vulgaris*. Males are larger in both mass and structural size, which is important in competition for nest sites and mates as adults (reviewed in Cabe 1993). Our goal in this study was to extend the intra-year observations made by Saino et al. (1997a) of the negative relationship between natural brood sizes and nestling immune responsiveness in a sexually size-dimorphic species. Furthermore, we examined this relationship across years that differed in the resources available to nestlings for allocation towards immune function and growth. Based on the findings of Saino et al. (1997a), we predicted that in a lower resource year male immune response should be negatively impacted in larger brood sizes, whereas smaller females would exhibit no relationship. Moreover, in a

year of improved resource availability, if the development of the immune system is related to resources (Alonso-Alvarez and Tella 2001, Soler et al. 2003), neither sex may exhibit a relationship.

Materials and methods

Study site and species

Research was carried out between April and July of 2002 and 2003 at the Davistead Dairy Farm in Langley, British Columbia, Canada, under a Simon Fraser University Animal Care permit (499B), following guidelines of the Canadian Council on Animal Care. A wild colony of European starlings is maintained at this site with approximately 215 nest boxes mounted on buildings and on posts in large fields at the edge of forest openings. Boxes were checked daily to determine clutch initiation and clutch completion dates as well as the laying sequence of eggs and chick growth. Starlings at our field site generally lay 4–6 eggs per clutch, incubate for 10–11 days and fledge chicks 18–20 days following hatching (Love unpubl. data).

Growth and immune response

All chicks during 2002 and 2003 were weighed and measured (exposed culmen, meta-tarsus) at hatching and 5, 10, 15 and 17 days of age in order to assess growth rate, calculated as the change in body mass in grams, per day, per nestling within the linear growth phase of post-natal development (hatch to 10 days of age, Ricklefs and Peters 1979). We began measuring flattened wing chord at 10 days of age when primary feathers began to appear. Nestling identity and subsequent age were tracked using non-toxic food colouring and chick-specific feather clipping until 10 days of age, at which time all chicks were banded with metal and colour bands (permit #10646) so that individual chicks in a nest could be identified. All nestlings underwent a phytohaemagglutinin (PHA) test at 17–18 days of age as a means of evaluating the cell-mediated immune response of individuals. We injected 50 µg of PHA (PHA-p, Sigma) in 50 µL of sterile phosphate buffered saline (PBS) subcutaneously with a 27 G needle into the right wing-web of each bird (patagia); the left patagia was injected with 50 µL of PBS only. Patagium thickness in each wing was measured three times to 0.01 mm using a gauge micrometer (The Dyer Company, model number 304-196) prior to and again 24 hours after injection. The difference between the responses to PHA and PBS were calculated (i.e., each wing independently) and the immune response was then calculated as the change in thickness of the PBS wing from the change in thickness of the PHA wing. Repeatability of both initial ($r = 0.89$,

$P < 0.0001$) and final ($r = 0.90$, $P < 0.0001$) measurements was high, and we used mean values of the three measurements. Finally, in both years we performed 30 minute behavioral observations using spotting scopes of all nest boxes over three consecutive days when nestlings were aged 6–10 days, for assessment of parental provisioning rates. Provisioning rates were calculated per chick, per hour based on the mean brood size of the nest for the three day observation period.

Molecular sexing

Blood samples were collected from chicks near fledging (17 days of age) in heparinized collecting tubes by means of brachial vein puncture (80–100 μL), and a portion of this sample was transferred to a piece of filter paper and frozen at -20°C for sex analysis. Collected carcasses of deceased nestlings and blood samples were analyzed using a polymerase chain reaction (PCR) amplification process based on techniques used by Griffiths et al. (1996). DNA was isolated from the red blood cell samples using Insta-gene matrix (Bio-Rad Laboratories, Hercules, California, Cat. No. 732-6030), and from the tissue samples using the Qiagen DNAeasy kit, both following manufacturers' protocols. PCR amplification was run using the P2 (5'-TCTGCATCGCTAAATCC-TTT) and C2 (5'-AGAAATCATTCCAGAAGTTCA) primer set, followed by digestion with HAE III (Vanderkist et al. 1998).

Statistical analyses

General linear mixed models (GLMM) were used to analyze sex and brood size effects on nestling immune response. Briefly, sex and brood size were included as factors, and nestling body mass was included as a covariate (where required); maternal identity and year were used as random factors. Non-significant interactions were backward eliminated and all tests were two-tailed with the significance level set at $P = 0.05$. Mortality analysis was performed with a Generalized Linear Model with binomial error distribution and a logit link function (Crawley 1993). Brood size and offspring sex were included in the model as categorical factors, and hatch mass as a covariate. The fit of this model is measured as a χ^2 statistic.

Results

Provisioning and growth rates

Behavioral observations revealed that parental feeding rates were higher in 2003 than in 2002 (2002: 3.09 ± 0.36 feeds/nestling/hour, 2003: 3.96 ± 0.23 feeds/nestling/hour,

$F_{1,52} = 4.09$, $P < 0.05$). Growth rates within the linear growth phase of starlings (hatch to 10 days of age; Ricklefs and Peters 1979) were also higher in 2003 than 2002 (2002: 5.68 ± 0.09 g/day, 2003: 5.98 ± 0.07 g/day, $F_{1,102} = 9.21$, $P < 0.005$). We found no significant effect of year and sex in relation to brood size on growth rates (sex \times year \times brood size interaction; $F_{1,194} = 0.68$, $P = 0.41$).

Nestling survival

Controlling for hatch mass, there was no difference in mortality between male and female offspring ($\chi^2 = 0.197$, $P = 0.657$). There was a significant difference between years in offspring mortality, that is, 2002 had a higher mortality rate than 2003 ($\chi^2 = 11.77$, $P < 0.05$), and thus the years were analyzed separately. However, within both years we again found no difference in mortality between the sexes ($\chi^2 = 0.2955$, $P = 0.5867$; $\chi^2 = 0.012$, $P = 0.90$, respectively).

Immune response and body mass

We detected both a significant interaction between year and nestling sex ($F_{4,271} = 2.49$, $P < 0.05$, Table 1), and year, nestling sex and brood size on immune response ($F_{4,271} = 2.61$, $P < 0.05$, Table 1). Given these results and the inter-year differences in parental feeding rates and hence growth rates, we analysed inter-sexual differences in immune response separately for both years. In 2002, males exhibited significantly lower immune response in larger broods while females showed no relationship between brood size and immune response (sex \times brood size: $F_{4,108} = 2.62$, $P < 0.05$, Fig. 1a). However in 2003, we detected neither a sex difference nor an interaction between nestling sex and brood size (both $P > 0.45$, Fig. 1b). Furthermore, although we detected the predicted effect of sex on body mass at fledging given that starlings are sexually-size dimorphic ($F_{1,249} = 8.78$,

Table 1. Inter-sexual differences in immune response in European starling nestlings at the end of the nestling period as assessed by the PHA assay. General linear mixed models (GLMM) with a normal distribution were used with sex and brood size as fixed factors and maternal identity and year as random factors. Non-significant interactions were backward eliminated.

Source of variation	df	F	P
PHA response			
Maternal identity	1,271	10.4	<0.001
Year	1,271	1.20	0.27
Sex	1,271	0.19	0.66
Brood size (BS)	4,271	1.1	0.36
Year \times Sex	4,271	2.49	<0.05
Year \times Sex \times BS	4,271	2.61	<0.05
Error	271		

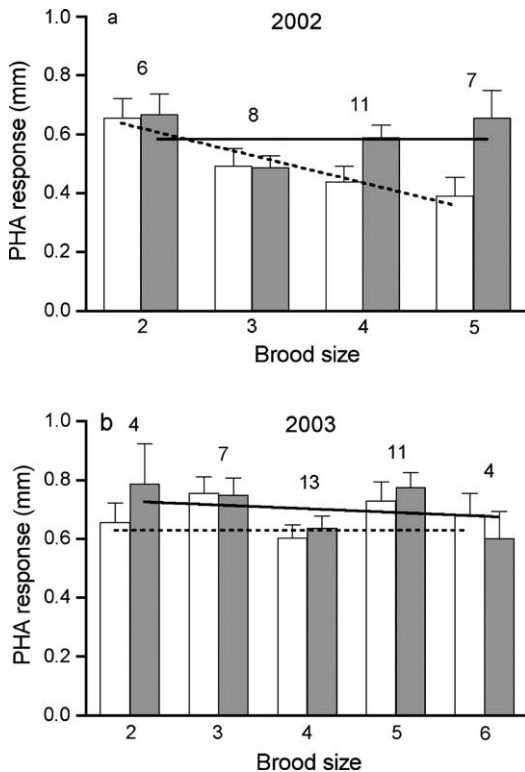


Fig. 1. Mean intra-brood inter-sexual variation in cell-mediated immune response of European starling nestlings as assessed by the PHA assay in relation to brood size in two study years (a) 2002 and (b) 2003 (male: open bars and dashed line, female: gray bars and line). Bars indicate the least-square mean \pm SEM; numbers above bars represent numbers of nests sampled for a given brood size. Lines were fitted to the data points using regression analysis.

$P=0.003$; males: 73.11 ± 0.67 g, females: 69.41 ± 0.32 g), we did not detect either a year ($P=0.28$) or brood size ($P=0.13$) effect or any possible interactions of these variables on fledging body mass. That is, body mass did not significantly decline within either sex in either year in relation to increasing brood size.

Discussion

We investigated the effect of inter-year variation in resource availability on a possible growth-immune function trade-off in sexually-size dimorphic European starling nestlings. Assessing inter-year variation in parental provisioning rates and nestling growth revealed that 2002 exhibited lower parental provisioning rates, lower growth rates and higher chick mortality compared to 2003. Concurrently in 2002, immune responses of the larger male nestlings appeared to be negatively impacted in larger brood sizes; no such relationship was detected for males in 2003 or for female nestlings in either year. Furthermore, fledging masses

appeared consistent between years, regardless of the differences in the quantity resources delivered to nestlings. Although results support both previous work on brood size effects on immune response in passerines (Saino et al. 1997a, H \ddot{o} rak et al. 1999, Naguib et al. 2004) and the idea that the larger sex should exhibit a negative relationship between immune response and brood size (Saino et al. 1997a, H \ddot{o} rak et al. 1999), the data further emphasize that immune response of growing nestlings appears highly resource-dependent (Birkhead et al. 1999, Alonso-Alvarez and Tella 2001).

Interestingly, we did not detect in either sex any relationship between brood size and year differences in resource availability on fledging body masses. While this is not necessarily surprising given the importance of maintaining growth rates during development to obtain an adult body size that ensures future reproductive success (Starck and Ricklefs 1998, Metcalfe and Monaghan 2001), it appears that male starlings may face a possible resource-based trade-off between growth and immune response. Evidence for the competing demands of somatic growth and development of the immune system comes from 1) brood manipulation studies where increasing brood size negatively influences immune function in individual nestlings (Saino et al. 1997a, H \ddot{o} rak et al. 1999), and 2) experimental food restriction studies indicating that food intake directly influences immune response in nestlings (Birkhead et al. 1999, Alonso-Alvarez and Tella 2001). Female starlings do not appear to be as significantly impacted by lowered feeding rates combined with a large brood size, perhaps because they have lower energetic growth costs and therefore may be able to allocate more resources towards immune function. Whether female offspring require a higher immune response upon fledging than males is as yet unknown, however this trend has recently been reported in both great tits *Parus major* (Tschirren et al. 2003) and Eurasian kestrels nestlings *Falco tinnunculus* (Fargallo et al. 2002), species that exhibit conventional and reversed sexual size dimorphism, respectively. Sex-specific strategies in the allocation of resources towards immune function have been suggested (M \ddot{o} ller et al. 1998, Tschirren et al. 2003, Love et al. 2005), however, further study is needed to firmly establish the potentially different immune requirements of the sexes. This is especially important given the fact that nestling immunocompetence may be more plastic than that of adults (Sz \acute{e} p and M \ddot{o} ller 1999), suggesting that males in 2002 in the present study may have been able to develop immunity further following fledging, thus compensating for any resource-linked delays suffered in the nest. It should be noted that many factors other than resources alone may also impact inter-sexual differences in immune responses, such as sex-specific differences in nutritional requirements (Klasing 2002) and exposure to pathogens (Christe et al. 1998) as well as

sexually-selected differences (Folstad and Karter 1992, Westneat and Birkhead 1998, Saino et al. 1997b).

We did not observe a consistent relationship between nestling immune response and brood size which contrasts with results of recent studies, all of which report a consistent negative relationship between brood size and immune response in nestlings (Saino et al. 1997a, Hōrak et al. 1999, Naguib et al. 2004). The present study was undertaken across years rather than within one year or under controlled laboratory conditions as in previous studies. Since post-natal growth in avian nestlings requires substantial resources (Starck and Ricklefs 1998), inter-annual variation in resource variability should affect nestling immune response more than growth in relation to brood size. Ecological variation across years can have significant effects on a number of physiological processes (Piersma and Lindström 1997, Vézina and Williams 2003), and our results reinforce the need for multi-year studies in the examination of immunocompetence in wild birds. Future studies examining inter-sexual differences in immune response should also separate maternal quality at laying (i.e., clutch size) from maternal rearing potential via brood manipulations (as in Hōrak et al. 1999, Naguib et al. 2004), which was not possible in the present study. Finally, previous multi-year studies have focused on immunocompetence in adults (Saino et al. 1997b), however, little is known about the development of immunity in juvenile passerines, especially post-fledging. Since immune function has been shown to be correlated with survival (Saino et al. 1997b, Tella et al. 2000), it is important that future studies follow immunocompetence through various life-history stages to further examine the development of the immune system outside the nest, as well as other possible subsequent costs of immunity.

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