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Manipulating rearing conditions reveals developmental sensitivity in the smaller sex of a passerine bird, the European starling *Sturnus vulgaris*

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Traditionally, studies of sexually size-dimorphic birds and mammals report that the larger sex is more sensitive to adverse environmental conditions during ontogeny. However, recent studies in avian species that exhibit moderate size-dimorphism indicate that the smaller sex may be more sensitive to poor rearing conditions. To better understand sex-specific sensitivity in a passerine exhibiting moderate size-dimorphism, we examined growth, cell-mediated immunity (CMI) and survival of European starling *Sturnus vulgaris* nestlings following an experimental reduction of maternal rearing ability (via a feather-clipping manipulation). Contrary to conventional theory, daughters showed reduced growth in both body mass and measures of structural size in response to the maternal treatment. In contrast, sons showed no reductions in any of these traits in relation to the treatment. No sex-specific differences in nestling CMI were found for either group, although CMI of nestlings raised by manipulated mothers were higher than those of control nestlings. Finally, fledging sex ratios did not change from those at hatching indicating that neither sex appeared differentially sensitive to the maternal treatment in terms of mortality. These results reveal that variation in the quality of the rearing environment can have significant effects on the smaller sex of a passerine exhibiting moderate dimorphism and as such support recent studies of species with small-moderate sexual size-dimorphism. Combined results suggest that sex-specific effects of environmental variation on nestling development may be both context- (i.e., brood size, resource level, hatching order) and temporally- (when during development they occur) specific. Furthermore, more studies are needed that examine multiple traits at several developmental stages and then follow the sexes over the longer-term to examine potential effects on fitness.

Significant research effort has been invested into trying to understand how and why parents sex-specifically invest in offspring in species where sons and daughters differ in both their proximate (e.g., energetic) and ultimate (i.e., evolutionary) costs to parents (Trivers and Willard 1973, Clutton-Brock et al. 1985, Bensch 1999, Magrath et al. 2004, Love et al. 2005). To this end, species exhibiting large degrees of sexual size-dimorphism (SSD) have been useful models to examine the potential costs and benefits of the sexes under varying environmental conditions, since male and female nestlings can differ in energetic requirements during rearing (Teather and Weatherhead 1988, Krijgsveld et al. 1998, Riedstra et al. 1998, Badyaev 2002, Vedder et al. 2005), as well as differ in their reproductive success and survival as adults (Trivers and Willard 1973, Clutton-Brock et al. 1982). These studies have revealed that it is the larger sex that is more sensitive to a reduction in the quality of environmental conditions (Richter 1983, Clutton-Brock et al. 1985, Weatherhead and Teather 1991, Anderson et al. 1993, Torres and Drummond 1997, 1999, Velando 2002, Kalmbach et al. 2005, although see Fargallo et al. 2002). For example, in mammals, patterns of juvenile mortality in the highly size-dimorphic red deer *Cervus elaphus* indicate that the larger males show significant sensitivity to declining resource availability (Clutton-Brock et al. 1985). In birds, great-tailed grackle *Quiscalus mexicanus* males are 91% heavier than females and have significantly increased nestling mortality rates when resources decline during postnatal development (Teather and Weatherhead 1989). However, studies examining species with small-moderate degrees of SSD find that the smaller sex may be more sensitive to poor environmental conditions (Bortolotti 1986, Oddie 2000, Hipkiss et al. 2002, Råberg et al. 2005, Dubiec et al. 2006). Why these differences between species exhibiting differential degrees of SSD occur is therefore a question of considerable interest.

The purpose of the current study was to determine whether experimentally decreasing maternal provisioning rates reveals sex-specific sensitivity in nestling phenotype of a passerine with moderate SSD, the European starling *Sturnus vulgaris*. In this passerine species, SSD begins after hatching, where males have higher growth rates than females during early development (Love et al. 2005) resulting in males being larger and heavier than females by 7–8% as both fledglings (Chin et al. 2005, Love et al. 2005, this study) and adults (Cabe 1993, this study). These combined traits make starlings good candidates for testing sex-specific sensitivity in a species exhibiting moderate SSD. We chose to examine three distinct indicators of nestling sensitivity in starlings faced with a reduction in maternal rearing ability. Firstly, we examined morphological traits expected to be sensitive to the quality of rearing conditions, namely body mass, body size and flight feather growth (Oddie 2000, Hipkiss et al. 2002, Råberg et al. 2005). We predicted that under stressful rearing conditions (being raised by a feather-clipped mother), male nestlings would be able to maintain investment in body mass and structural size because the larger sons may be able to effectively compete against their smaller sisters for reduced parental resources.

Second, we examined a physiological indicator of nestling sensitivity to decreased quality of the rearing conditions by examining the immune response to phytohaemagglutinin (PHA) injection given that research supports the role of SSD in sex-specific trade-offs in resource allocation to cell-mediated immunity (CMI; Müller et al. 2003, Tschirren et al. 2003, Chin et al. 2005, Dubiec et al. 2006, although see Fargallo et al. 2002). We predicted that females should maintain investment in this trait whereas males would exhibit reduced CMI in relation to the maternal treatment based on previous results in this species (Chin et al. 2005). Finally, we examined sex-specific mortality as an indicator of sensitivity to poor rearing conditions. Although traditional theory in highly SSD species predicts that the larger sex should experience higher mortality in the nest because of higher energetic demands during growth (reviewed in Råberg et al. 2005), since SSD is moderate in starlings (males are only 7–8% larger than females both at fledging and as adults; Cabe 1993, this study), we predicted that males may be able to out-compete females under poor rearing conditions and therefore expected female-biased mortality.

Methods

Field site

This study was conducted from April to May, 2005 at the Davistead dairy farm in Langley, British Columbia, Canada (49°10′N, 122°50′W). The site consists of approx. 250 nest boxes mounted on posts around pastures and on farm buildings throughout the site. The site is used yearly by a wild colony of breeding European starlings which lay 5.9 ± 0.2 (mean $\pm$ SE) eggs per clutch, incubate for 10.3 ± 0.1 d, and fledge chicks 21 ± 0.7 d following hatching (Love et al. 2005). Nest boxes were checked daily to determine clutch initiation, laying sequence, and clutch completion dates. At day ten of incubation (within 0.5 d of hatching), clutches were removed and placed in an incubator for approximately 12–15 h until hatching; dummy eggs were replaced to maintain maternal incubation behavior. At hatching, nestling body mass and structural size measures were taken, a small blood sample was collected for PCR analysis and nestlings were returned to their nest.

Manipulation of maternal quality

Females were caught at day eight of incubation and were split pair-wise by laying date whereby half underwent a feather clipping treatment ($n = 15$ females) designed simply to reduce maternal provisioning rates (Winkler and Allen 1995, Hill 2003); the other half remained un-manipulated ($n = 12$ females). The treatment consisted of clipping the ninth, sixth, and third primary feathers, the sixth and third secondary feathers, and the sixth and third rectrices (tail feathers) near the base of each feather; control birds were only captured, measured and released. All females were measured (beak length, tarsus length, wing chord, and mass), banded with metal and color bands and were released to return to normal activities. Females were re-caught at day 7–8 of chick rearing (control: 11 of 12; clipped: 10 of 15) to determine any post-treatment change in body mass; the remaining 6 birds not re-caught had abandoned their nests.

Growth, survival and immune responses of nestlings

All nestlings were weighed and measured (exposed culmen, meta-tarsus) at hatching and at 5, 10, 15 and 17 d of age in order to assess growth rates, 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 d of age) and during the asymptotic period (10 d to fledge; Ricklefs and Peters 1979). We started measuring flattened wing cord at 10 d of age when primary feathers began to appear and wing cord at fledging (17 d of age) was used for analysis. 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 bands (permit #10646) so that individual nestlings could be identified. All nestlings underwent a phytohaemagglutinin (PHA) test at 17–18 days of age as a means of evaluating the CMI in 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 (patagia) of each bird. Patagium thickness was measured three times to 0.01 mm prior to and again 24 hours after injection using a gauge micrometer (The Dyer Company, model number 304–196). Cell-mediated immune response to PHA was calculated as the change in thickness of the wing-web prior to and following injections as outlined in Smits et al. (1999). Repeatability of both initial ($r = 0.92$, $P < 0.001$), and final ($r = 0.88$, $P < 0.001$) measurements was high, and we used mean values of the three measurements. Finally, to assess parental provisioning rates, we performed a 30 minute behavioral observation of each nest box, per day, over three consecutive days using spotting scopes when nestlings were aged 6–10 days. Provisioning rates were calculated as the number of feeds per chick, per hour of each parent based on the mean brood size of the nest for the three-day observation period (Chin et al. 2005).

Molecular sexing

A small blood sample was collected on a piece of filter paper from the nestlings at hatching, which was then stored in a labeled tube and frozen at −20 C. Based on techniques reported by Love et al. (2005), nestling sex was determined using polymerase chain reaction (PCR) amplification. DNA was isolated from the blood samples using Insta-gene matrix (Bio-Rad Laboratories, Hercules, California, Cat. No. 732-6030) following the manufacturer's protocol. PCR amplification was carried out in a total volume of 10 μl and run using the P2 (5′-TCTGCATCGCTAAATCCTTT) and CW (5′-AGAAATCATTCCAGAAGTTCA) primers followed by digestion with HAE III Enzyme.

Statistical data analysis

We used Analysis of Covariance (ANCOVA) to analyze maternal treatment effects on maternal characteristics (body mass, brood size, provisioning rates); maternal treatment was included as a fixed factor and relevant covariates (all $P < 0.05$ when included in the analysis) were included where necessary (i.e., original capture date was included in the analysis of pre-treatment maternal body mass, clutch size was included in the analysis of brood size and original mass was included in the analysis of post-treatment body mass change). We used GLMM to analyze sex-specific effects of the maternal treatment on nestling traits (growth, fledgling body mass, fledgling tarsus, fledgling wing cord and fledgling CMI) by including nestling sex and maternal treatment as fixed factors and relevant covariates (all $P < 0.05$ when included in the analysis) where necessary (i.e., brood size and fledging body mass were included in the analysis of fledgling CMI); maternal identity was included as a random factor to control for non-independence due to the inclusion of siblings in the analysis. Post-hoc comparisons for significant interaction terms were carried out using the adjusted Bonferroni post-hoc procedure, with the P-value corrected for the number of pair-wise comparisons made depending on the type of analysis (Rice 1989). Sex ratio of offspring (measure of sex-specific mortality), as a function of maternal treatment, was analyzed using GLMM with a binomial error structure (Love et al. 2005). For sex ratio analysis, maternal identity was included as a random factor. The significance of the explanatory variables was determined by their Wald statistic using the χ^2-distribution with alpha set to 0.05 in all analyses (Crawley 1992).

Results

There was no significant difference between clutch size ($F_{1,24} = 0.19$, $P = 0.66$; Table 1), hatch brood size ($F_{1,23} = 1.23$, $P = 0.27$; Table 1), or pre-treatment body mass ($F_{1,23} = 0.07$, $P = 0.80$; Table 1) of the birds assigned to either treatment, indicating that we had successfully obtained a random sample of birds. The feather-clipping manipulation resulted in a significant reduction in maternal body mass by the mid-chick-rearing stage compared with control mothers ($F_{1,23} = 0.07$, $P = 0.80$; Table 1). Furthermore, both the maternal and parental provisioning rates (number of feeds/nestling/h) in nests of treatment mothers were significantly lower than nests attended by control

Table 1. Maternal characteristics in relation to initial feather clipping treatment (control or clipped feathers; see Methods); least squares mean ± SEM are given.

Trait	Control	Clipped	F	df	P
Clutch size	5.41 ± 0.22	5.29 ± 0.20	0.19	1,24	0.66
Hatching brood size	4.70 ± 0.24	4.33 ± 0.22	1.27	1,23	0.27
Pre-treatment body mass (g)	85.68 ± 4.15	86.13 ± 3.80	0.07	1,23	0.80
Change in body mass (g)	7.25 ± 2.09	8.86 ± 1.89	3.18	1,20	0.05
Maternal provisioning rate (feeds/nestling/h)	4.81 ± 1.27	2.40 ± 1.32	7.26	1,19	0.02
Parental provisioning rate (feeds/nestling/h)	8.43 ± 0.98	6.09 ± 1.12	4.58	1,19	0.05

mothers (maternal: $F_{1,19} = 7.26$, $P = 0.02$; parental: $F_{1,19} = 4.58$, $P = 0.05$; Table 1).

The maternal feather-clipping treatment affected male and female nestling body mass and structural size differently. Body mass, tarsus and wing cord of male fledglings were unaffected by the maternal treatment; however, female nestlings raised by wing-clipped mothers showed a significant reduction in all three traits compared to daughters raised by control mothers-body mass (Repeated-measures ANCOVA, maternal treatment × sex; $F_{4,67} = 2.69$, $P < 0.05$; Bonferroni post-hoc analysis – all male comparisons $P > 0.6$, all female comparisons $P < 0.03$; Table 2), fledging tarsus (ANOVA, maternal treatment × sex; $F_{1,73} = 4.51$, $P = 0.03$; Bonferroni post-hoc analysis-male: $P = 0.28$, female: $P = 0.001$; Table 3), and fledging wing cord (ANCOVA, maternal treatment × sex; $F_{1,73} = 3.98$, $P < 0.05$; Bonferroni post-hoc analysis-male: $P = 0.41$, female: $P = 0.013$; Table 3). As the result of decreased growth rates in females during the linear phase of growth (hatch to 10 d of age in starlings; ANOVA, maternal treatment × sex; $F_{1,73} = 4.13$, $P < 0.05$; Bonferroni post-hoc analysis-male: $P = 0.65$, female: $P = 0.02$), only daughters showed catch-up growth following this linear phase in relation to maternal treatment (ANOVA, maternal treatment × sex; $F_{1,73} = 4.87$, $P = 0.03$; Bonferroni post-hoc analysis-male: $P = 0.52$, female: $P = 0.014$). Interestingly, nestlings of both sexes raised by treatment mothers showed similarly higher immune responses than nestlings raised by control mothers (ANOVA, maternal treatment; $F_{1,73} = 5.97$, $P = 0.017$; Table 3). Sex ratio at hatching was not significantly different than 1:1 ($\chi^2 = 1.76$, $P = 0.18$) and despite sex-specific differences in growth parameters, there was no treatment effect on offspring sex ratio at fledging ($\chi^2 = 0.44$, $P = 0.62$), indicating that the maternal treatment did not result in sex-specific mortality during post-natal development.

Discussion

Although previous work in highly size-dimorphic species indicate that the larger sex is more sensitive to a stressful rearing environmental (Richter 1983, Clutton-Brock et al. 1985, Weatherhead and Teather 1991, Anderson et al. 1993, Torres and Drummond 1997, 1999, Velando 2002, Kalmbach et al. 2005), recent results in species with only small, or moderate, degrees of SSD indicate the opposite pattern (Oddie 2000, Hipkiss et al. 2002, Råberg et al. 2005). As such, we expected in the moderately dimorphic starling that smaller daughters would show greater sensitivity to a poor environment post-hatching compared with larger sons. This is exactly what we saw, with sons showing no effects of the maternal treatment on any of the morphological traits measured, while daughters showed significant reductions in all traits. These results are consistent with other recent studies examining sensitivity in species exhibiting moderate SSD. Råberg et al. (2005) found that the morphology of the smaller sex (females) in blue tits *Cyanistes caeruleus* (6% SSD) was more negatively affected by poor rearing conditions than in males in the more severe year of their two year

Table 2. Sex-specific changes in body mass through post-natal development in relation to maternal treatment (control or clipped flight feathers-see Methods); least squares mean ± SEM are given.

Age	Male nestlings		Female nestlings	
	Control	Clipped	Control	Clipped
Hatch	5.18 ± 0.05	5.11 ± 0.04	5.03 ± 0.05	5.07 ± 0.05
5 d	34.66 ± 1.40	32.00 ± 1.34	33.52 ± 01.09	29.51 ± 1.37*
10 d	69.45 ± 1.75	65.69 ± 1.63	66.89 ± 1.69	58.51 ± 2.04*
15 d	77.54 ± 1.32	75.60 ± 1.23	73.64 ± 0.94	70.57 ± 1.16*
17 d	76.52 ± 1.29	75.42 ± 1.20	73.91 ± 1.26	69.54 ± 1.53*

*Indicates a significant difference between treatments within sex, cf. Methods.

Table 3. Morphological traits and cell-mediated immune responses (CMI) of fledgling European starlings in relation to maternal treatment (control or clipped flight feathers; see Methods); least squares mean ± SEM are given.

Trait	Male nestlings		Female nestlings	
	Control	Clipped	Control	Clipped
Tarsus (mm)	35.20 ± 0.48	34.12 ± 0.45	34.93 ± 0.22	33.80 ± 0.26*
Wing cord (mm)	94.24 ± 1.2	93.87 ± 1.2	92.15 ± 0.8	88.6 ± 0.5*
CMI (mm × 10)	15.6 ± 1.39	20.0 ± 1.29*	15.1 ± 1.59	20.9 ± 1.90*

*Indicates a significant difference between treatments within sex, see Methods.

field study. Likewise, a study by Oddie (2000) manipulating the degree of hatching asynchrony in great tit broods *Parus major* (7% SSD), revealed that late-hatched females suffered more in terms of growth (mass, tarsus, wing) than late-hatched males. Finally, Dubiec et al. (2006) recently reported that tarsus, but not body mass, was more retarded only in female blue tits in response to a brood enlargement manipulation.

We found no support for sex-specific sensitivity in cell-mediated immunity (CMI) to a reduction in the quality of the rearing environment. In the present study, CMI in both sexes appeared similar and was in fact more robust in fledglings raised under apparently stressful conditions. In nestling birds, predictions of how individuals should face trade-offs between the immune system and growth have been based on the hypothesized energetic costs of the development of the immune system (Birkhead et al. 1999, Hõrak et al. 1999, Soler et al. 2003). During energetically stressful periods, resources should theoretically be allocated away from the immune system and towards other functions, such as growth (Hõrak et al. 1999, Soler et al. 2003, Chin et al. 2005, Dubiec et al. 2006). It has generally been assumed that in sexually size-dimorphic species, any resource allocation trade-off between growth and the immune system should also be dependent on nestling sex (Fargallo et al. 2002, Chin et al. 2005) 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). However, studies thus far have produced mixed results, some indicating that female fledglings exhibit higher cell-mediated immune responses than males regardless of whether they are the smaller (great tit; Tschirren et al. 2003 and European starlings; Chin et al. 2005), or larger sex (Eurasian kestrels *Falco tinnunculus*; Fargallo et al. 2002), and some indicating that the larger males have higher CMI (blue tit; Dubiec et al. 2006), although male CMI decreased more than in females with a brood size manipulation. Many factors other than just resource availability can impact inter-sexual differences in immune responses, for example, sex-specific differences in nutritional requirements (Klasing 2002), exposure to pathogens (Christe et al. 1998) and sexually-selected differences (Folstad and Karter 1992, Westneat and Birkhead 1998, Saino et al. 1997, Tschirren et al. 2003). The only way to understand why nestlings of different species respond differently immunologically to reductions in the quality of the rearing environment is to measure numerous traits as well as understand the life-history of the species in question.

Although we predicted that females would be more vulnerable to mortality in relation to the maternal treatment, we found no effects of reduced maternal rearing quality on survival in either sex in the nest. These results are actually consistent with results in species exhibiting moderate degrees of SSD (Sheldon et al. 1998, Oddie 2000, Råberg et al. 2005). It is generally accepted that size differences significantly impact the survival of the larger sex of species exhibiting large degrees of SSD (Clutton-Brock et al. 1985, Teather and Weatherhead 1989). However, species exhibiting large degrees of SSD may tell us little about how mortality, an extreme consequence of sensitivity to stressful rearing conditions, will be affected in a species with moderate SSD. Nestlings of different species may trade-off different physiological traits when faced with decreased resources, potentially in a sex-specific manner (Clutton-Brock et al. 1985, Chin et al. 2005). Therefore, nestlings of species with moderate SSD may be able to employ reallocation strategies selected to minimize the chance of mortality when reared under environmentally stressful conditions that highly dimorphic species cannot.

Along with data from recent studies, our results suggest that researchers should be cautious in applying broad hypotheses about SSD and sex-specific sensitivity to environmental conditions to all species exhibiting SSD. This is because the degree of SSD can vary greatly between species and the life-histories of these species can be dramatically different, potentially leading to different selection pressures on the sexes in relation to SSD (Bortolotti 1986). Furthermore, which sex is sensitive appears to be dependent on when during development a stressor is applied. For example, Love et al. (2005) recently reported that male embryos exposed to the maternal stress hormone corticosterone in the egg were more sensitive than females, the result being higher male embryonic mortality and lower hatch masses and growth in males but not females. Whereas the present study examines how males and females respond to an unexpected change in maternal quality post-hatching, the results of Love et al. (2005) potentially test how developmental trajectory is programmed as an embryo responds to a signal of environmental or maternal quality. Sex-specific effects of environmental variation on nestling development therefore appear to be both context (i.e., brood size, resource level, hatching order) and temporally (i.e., when during development they occur) specific. Future studies are therefore needed that examine the effects of a reduction in environmental quality on multiple offspring traits and at several developmental stages. Following the sexes once they fledge will allow researchers to examine the long-term effects of sex-specific sensitivity on fitness.

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