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Stress Hormones: A Link between Maternal Condition and Sex-Biased Reproductive Investment

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ABSTRACT: In species where offspring fitness is sex-specifically influenced by maternal reproductive condition, sex allocation theory predicts that poor-quality mothers should invest in the evolutionarily less expensive sex. Despite an accumulation of evidence that mothers can sex-specifically modulate investment in offspring in relation to maternal quality, few mechanisms have been proposed as to how this is achieved. We explored a hormonal mechanism for sex-biased maternal investment by measuring and experimentally manipulating baseline levels of the stress hormone corticosterone in laying wild female European starlings (*Sturnus vulgaris*) and examining effects on sex ratio and sex-specific offspring phenotype adjustment. Here we show that baseline plasma corticosterone is negatively correlated with energetic body condition in laying starlings, and subsequent experimental elevation of maternal baseline plasma corticosterone increased yolk corticosterone without altering maternal condition or egg quality per se. Hormonal elevation resulted in the following: female-biased hatching sex ratios (caused by elevated male embryonic mortality), lighter male offspring at hatching (which subsequently grew more slowly during postnatal development), and lower cell-mediated immune (phytohemagglutinin) responses in males compared with control-born males; female offspring were unaffected by the manipulation in both years of the study. Elevated maternal corticosterone therefore resulted in a sex-biased adjustment of offspring quality favorable to female offspring via both a sex ratio bias and a modulation of male phenotype at hatching. In birds, deposition of yolk corticosterone may benefit mothers by acting as a bet-hedging strategy in stochastic environments where the correlation between environmental cues at laying (and therefore potentially maternal condition) and conditions during chick-rearing might be low and unpredictable. Together with recent studies in other vertebrate taxa, these results suggest that maternal stress hormones provide a mechanistic link between maternal quality and sex-biased maternal investment in offspring.

Keywords: corticosterone, sex ratio theory, sex allocation theory, maternal condition, maternal effects, yolk hormones.

Sex allocation theory predicts that selection should act on parents to sex-specifically vary the level of investment in offspring when the fitness returns differ for the two sexes (Charnov 1982; Frank 1990; Hardy 2002). Trivers and Willard (1973) originally proposed that maternal quality should influence the direction of this investment for species exhibiting high variance in male reproductive success (compared with variance in female reproductive success). Cameron and Linklater (2002) have recently summarized the assumptions of Trivers and Willard (1973), including predictions for both sex ratio and sex-biased investment: (1) mothers in better condition would be favored by producing more of and investing more in the evolutionarily more costly sex, and alternatively, (2) mothers in poorer condition would be favored by producing more of the less costly sex and investing more in this sex. Although originally conceived with polygynous mammals such as ungulates in mind (Sheldon and West 2004), where larger male size and/or higher adult reproductive variance may select poor-condition mothers to invest in less costly female offspring (Clutton-Brock et al. 1984; Kruuk et al. 1999; reviewed in Sheldon and West 2004), the theory can be extended to numerous systems, including but not limited to cooperatively breeding avian species in which one sex is philopatric and may help parents in poor-quality habitats raise future offspring (Komdeur et al. 2002; Ewen et al. 2003), size-dimorphic species in which one sex has greater energetic demands during postnatal growth (Wiebe and Bortolotti 1992; Velando 2002; Martins 2004), and even species that exhibit sex-specific differences in life-

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history traits such as age of sexual maturity (Krebs et al. 2002).

Mothers have two ways in which they may bias sex allocation: producing unequal numbers of male and female offspring and/or varying the quality of the sexes produced (Laaksonen et al. 2004). In birds, females are the heterogametic (ZW) sex, and mothers therefore theoretically have control over the sex of future offspring at the time of ovulation (Komdeur and Pen 2002). However, no preovulatory mechanisms by which female birds might alter primary (laying) sex ratios have been discovered (Krackow 1995; Palmer 2000), despite evidence that alterations occur in some species (Appleby et al. 1997; Sheldon et al. 1999; Komdeur et al. 2002). Furthermore, producing an optimal offspring sex ratio at laying may be difficult or even maladaptive because, for birds, the correlation between environmental cues at laying (and therefore potentially maternal condition) and conditions during chick-rearing might be low and unpredictable (Nager et al. 2000).

Alternatively, some coordination between sex ratio manipulation both before and after hatching may lead to an optimal sex brood composition aimed to maximize fitness (Heinsohn et al. 1997). For example, sex-specific embryo mortality leading to biased hatching sex ratios combined with sex-specific phenotypic effects influencing postnatal developmental sensitivity to variation in maternal resources would be one possible mechanism for adaptive sex allocation. However, despite good correlational (Wiebe and Bortolotti 1992; Appleby et al. 1997; Ewen et al. 2001, 2003; Velando 2002; Whittingham et al. 2002; Alonso-Alverez and Velando 2003) and experimental evidence (Bradbury and Blakey 1998; Kilner 1998; Nager et al. 1999; Kalmbach et al. 2001; Albrecht and Johnson 2002; Clout et al. 2002; Krüger et al. 2005) for a link between maternal energetic condition and variation in sex ratios and sex-specific investment in offspring, a physiological mechanism whereby mothers sex-specifically fine-tune their brood has not been identified (Cameron 2004; Krüger et al. 2005). Moreover, although Trivers and Willard (1973) were explicit in the predictions that the less expensive sex would be favored when mothers are in poor condition (Cameron and Linklater 2002), researchers have focused largely on the outcome in good-quality females (prediction 1), routinely ignoring the evolutionary decisions of poor-condition females (prediction 2; Cameron and Linklater 2002).

For the last decade, evolutionary physiologists have recognized that hormonal exposure during embryonic life has great potential for contributing to phenotypic plasticity (Adkins-Regan et al. 1995; Dufty et al. 2002). For example, studies of the transfer of maternally derived yolk androgens in birds have provided evolutionary physiologists with a hormonal mechanistic model by which mothers may be able to adaptively modulate offspring phenotype in relation to the social conditions the mother experiences at the time of laying (Schwabl 1993, 1996, 1997; Lipar and Ketterson 2000; Sockman and Schwabl 2000; Eising et al. 2001, 2003; Petrie et al. 2001; Veiga et al. 2004; Daisley et al. 2005; Gorman and Williams 2005; Groothuis et al. 2005). Considerably less attention has been paid to endocrine pathways related to energetic balance and condition (such as stress hormones [glucocorticoids]), despite their potential role in sex allocation theory (Pike and Petrie 2003; Cameron 2004). Glucocorticoids serve to mediate adaptive physiological and behavioral responses to stressful events in nonmammalian tetrapods via corticosterone and in mammals and fish via cortisol (reviewed in Sapolsky et al. 2000) and are therefore intimately tied to physiological/physical condition through their prominent role in homeostatic energy balance (Harvey et al. 1984; Dallman et al. 1993). Because of this, a decline in body condition is strongly associated with increased plasma baseline corticosterone levels in many species of birds (Holberton et al. 1996; Heath and Dufty 1998; Kitaysky et al. 1999*a*, 1999*b*, 2001, 2003), an effect that can be enhanced by local ecological conditions (Wingfield 1994; Marra and Holberton 1998).

Like androgens, corticosterone can be transferred from mother to egg via the yolk (McCormick 1998; Hayward and Wingfield 2004). Maternally derived corticosteroids can have significant phenotypic effects on developing offspring, including reduced body masses of developing embryos, reduced masses of offspring at parturition, reduced postnatal growth rates, and even long-term "fetal programming" (sensu Seckl 2001) effects on behavior and physiology in a wide range of vertebrate taxa (fish: McCormick 1998, 1999; amphibians: Hayes et al. 1993; Hayes 1995; Glennemeier and Denver 2002; reptiles: Sinervo and DeNardo 1996; Cree et al. 2003; Meylan and Clobert 2005; birds: Jelinek et al. 1983; Mashaly 1991; Heiblum et al. 2001; Hayward and Wingfield 2004; Rubolini et al 2005; and mammals: Quinlivan et al. 1998; Hansen et al. 1999; Lesage et al. 2001, 2004; Seckl 2001; Walker et al. 2001). These effects are largely thought to reveal a prenatal plasticity of the embryo and neonate to the developmental effects of glucocorticoids (Hales and Barker 1992; Gluckman 2001; Gluckman et al. 2005), given that glucocorticoids may play significant roles in growth and developmental pathways in both pre- and postnatal young (De Jesus et al. 1990; Redding et al. 1991; Woodall et al. 1999; Ghosh et al. 2000; Nolan et al. 2001). Embryonic glucocorticoid exposure may therefore mechanistically link maternal environmental factors with fetal growth and developmental programming (Seckl 2001; Dufty et al. 2002; Lesage et al. 2004), a physiological result that may benefit

mothers evolutionarily via a fine-tuning of offspring quality to match maternal quality. Therefore, given that elevated maternal glucocorticoids are related to decreased maternal condition, are transferred to young via the placenta and yolk, and have direct observable phenotypic effects on developing embryos, hatchlings, and postnatal offspring, glucocorticoids are excellent candidates to play a role in sex-biased investment in vertebrates.

We examined the effects of experimentally elevated maternal corticosterone on sex ratio and sex-specific embryo and nestling development as a potential mechanism for sex-biased investment in birds by using a wild nest box breeding population of European starlings (*Sturnus vulgaris*). European starlings are good candidates for testing evolutionary sex-biased investment hypotheses because they fulfill assumptions set out by Trivers and Willard (1973). Briefly, males exhibit a high variance in reproductive success, and this success depends largely on their ability to defend both nest sites and potential mates against competing males (Cabe 1993). In addition, starlings are sexually size-dimorphic at fledging (Cabe 1993; Chin et al. 2005) because of faster male growth rates during postnatal development (Cramp and Perrins 1994; data presented in this article).

We first examined intraspecific variation in baseline and stress-induced corticosterone in laying female starlings in 2001 to confirm a relationship between energetic condition and baseline corticosterone levels and to determine the range of baseline corticosterone within which to experimentally manipulate laying females. We then experimentally elevated baseline corticosterone levels of wild laying female starlings in 2002 and 2003 within the physiological range exhibited by poor-condition female starlings in 2001, thereby rendering the results as biologically meaningful. Because the manipulation was acute and lasted for the egg production period only, we were able to directly examine the effects of elevated yolk corticosterone available to developing embryos without altering overall maternal body condition, the quality of the eggs (egg size), or maternal behavior during incubation or chick rearing. We measured multiple indicators of nestling quality including (at hatching) body masses and size and (throughout development) growth rates and cell-mediated immune response at fledging. We predicted that male offspring of corticosterone-implanted females would be more developmentally sensitive to elevated corticosterone during embryonic development, given their higher growth rates and growth requirements following hatching. Furthermore, we predicted that elevated maternal corticosterone would result in female-biased sex ratios at hatching via male-specific embryonic mortality (caused by increased sensitivity to elevated yolk corticosterone), given that embryos have been shown to be developmentally sensitive to elevated maternal corticosterone. The result would be an indirect investment in female offspring by mothers with experimentally elevated corticosterone.

Methods

Fieldwork and Reproductive Output Measures

Research in 2001 was carried out at the Pacific Agri-Food Research Center in Agassiz, British Columbia (49°14′N, 121°46′W), a site consisting of approximately 175 nest boxes on farm buildings and telephone poles. Research in 2002/2003 was carried out at a nearby site located at the Davistead Dairy Farm in Langley, British Columbia (49°10′N, 122°50′W), a site consisting of 215 nest boxes mounted on farm buildings and on posts in large fields, used annually by breeding starlings.

All work was conducted between April and August in each year under a Simon Fraser University Animal Care permit (657B-96), following guidelines of the Canadian Council on Animal Care. Nest boxes were checked daily to determine clutch initiation and clutch completion dates as well as the laying sequence of eggs; boxes were checked hourly during hatching to record individual nestling identity.

Starlings at the two field sites lay 5.9 ± 0.2 (mean ± SE) eggs per clutch within the main peak of laying, incubate for 10.3 ± 0.1 days, and fledge nestlings 22 ± 0.9 days following hatching (O. P. Love and T. D. Williams, unpublished data; Love et al. 2004). Moreover, traits such as egg size, clutch size, female body size, and baseline corticosterone levels of laying females in the two populations do not differ significantly (O. P. Love and T. D. Williams, unpublished data). Some pairs are able to raise two broods per season, and therefore the successful reproductive season (within which pairs successfully fledge at least one nestling) runs from the beginning of April to the beginning of July.

Blood Sampling and Measures of Maternal Condition

To examine individual variation in plasma corticosterone levels of egg-laying females, we captured 35 individual females in 2001 at the middle laying (three-egg) stage while they roosted in their nest boxes at night (between 2000 and 2400 hours), when baseline corticosterone levels in starlings are at their daily mean with respect to daily variation (Romero and Remage-Healey 2000). Because corticosterone increases rapidly in birds after capture (Wingfield 1994; Romero and Romero 2002), we blood sampled (~100 μL) all birds from the wing vein within 2 min of capture to ensure that we measured baseline levels; birds were subsequently weighed and metal banded (permit

10646), and their exposed culmen and metatarsus were measured to the nearest millimeter. We detected no effect of time after capture (within a 0–2 min interval) on total and free baseline corticosterone levels in initial blood samples ($r^2 = 0.09$, $P = .56$). Thus, initial blood samples were considered to reflect baseline corticosterone levels. Each bird was then placed in a cloth bag and one additional blood sample was taken 30 min after capture (referred to as stress-induced corticosterone levels), as in earlier work on starlings (Romero and Remage-Healey 2000). Stress-induced corticosterone was measured as part of a different study and is therefore not reported here. We returned birds to their nest boxes following sampling; blood samples were centrifuged within 2 h, and plasma was frozen and stored at −20°C until further analysis.

Hormonal Manipulations

For hormonal manipulation in 2002/2003, female starlings were captured 1–3 days after completing a first clutch, while roosting in their nest box at night (usually between 2000 and 2400 hours). Birds were first anesthetized with a 0.3-mL injection of 50/50 ketamine/rompun mixture into the pectoral muscle and were then given two subcutaneous silastic implants (i.d. 1.46 mm, o.d. 1.97 mm, length 20 mm) containing either crystalline corticosterone (2002, $n = 48$; 2003, $n = 46$) or no hormone (empty; 2002, $n = 48$; 2003, $n = 46$) placed subcutaneously along the flank beneath each wing; all females were randomly assigned to one or the other treatment. Implants were designed to elevate baseline corticosterone within the physiological range measured in our laying population during 2001 (see "Results"). Birds were measured (body mass, exposed culmen, and tarsus) and banded (metal and color; permit 10646) for individual identification. Birds were allowed to recover from anesthesia and returned to their nest boxes. We found no significant differences in body mass ($F = 1.40$, df $= 1, 184$, $P > .20$), mean egg mass ($F = 1.73$, df $= 1, 184$, $P > .20$), clutch size ($F = 0.64$, df $= 1, 185$, $P > .40$), or laying date ($F = 1.74$, df $= 1, 185$, $P > .20$) of first clutches for birds assigned to control or corticosterone-implanted groups in 2002/2003 (controlling for laying date where necessary). Similarly, within each treatment, we found no significant differences in preimplant body mass, mean egg mass, or clutch size when comparing birds that did or did not lay a second clutch (all $P > .40$); that is, renesting birds represented a random sample of all birds initially assigned to the treatment groups.

Females were induced to lay a second clutch by removing the first clutch at the time of treatment; females were then left alone to raise the second clutch. A subsample of 14 birds (equally split between both treatment groups) were blood sampled (as above) at the first-egg stage in 2002 to confirm that baseline corticosterone had been elevated; first eggs laid by these females were collected to determine whether elevated maternal corticosterone had been transferred to yolks (all egg yolks were weighed, mixed with distilled water, and immediately frozen).

Offspring Quality

To assess nestling growth, we determined the mass of each nestling within 4 h after hatching, as well as at 5, 10, 15, and 17 days after hatching, and measured beak, tarsus, and antibrachium at each of these stages; nestling growth rate was calculated as the gain in body mass per day per nestling (based on average brood size that day). As soon as primary feathers began to appear (10 days after hatching), we also measured flattened wing chord. Nestling age was tracked using nontoxic food coloring and nestling-specific feather clipping, and at 10 days of age, each nestling was metal and color banded. Blood samples were collected from nestlings near fledging in heparinized collecting tubes by means of brachial vein puncture (80–100 μL), a portion of which was immediately transferred to a piece of filter paper and then frozen at −20°C for sex analysis.

We assessed the ability of nestlings to mount a T-cell-mediated immune response, one of the three main components of immunocompetence in vertebrates (Norris and Evans 2000), using a phytohemagglutinin (PHA) test at 17 days of age (Tella et al. 2002). Briefly, we injected 50 μg of PHA (PHA-p; Sigma) in 50 μL of sterile phosphate-buffered saline (PBS) subcutaneously with a 27-gauge needle into the right wing web of each bird (patagium); the left patagium was injected with 50 μL of PBS only. Wing web thickness in each wing was measured three times to 0.01 mm using a gauge micrometer (Dyer model 304-196) before and 24 h 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 ($r = 0.96$, $P < .0001$) and final ($r = 0.94$, $P < .001$) measurements was high, and we used mean values of the three measurements.

Parental Behavior

Based on evidence from previous studies, we were confident that the size of the implants used in starlings would elevate plasma corticosterone for only a short period of time (L. M. Romero, personal communication, unpublished data). However, to be certain that behavior in successive reproductive stages was not affected by the im-

plants, we measured incubation and parental provisioning behavior of all implanted birds. First, we used StowAway TidbiT temperature data loggers (Onset Computer, Pocasset, MA) equipped with thermocouples that were placed between eggs within the nest cup to examine possible effects of hormone-treatment on incubation behavior (given that elevated baseline corticosterone can affect activity and restlessness [Breuner et al. 1998; Breuner and Wingfield 2000; Lynn et al. 2003] and hence may affect incubation behavior and therefore embryo development). Data loggers recorded incubation temperatures every 22 s for three consecutive days during midincubation and allowed us to determine the periods and duration that any parent was present on the nest (see Reid et al. 1999). We calculated total daily incubation duration, the number of foraging bouts, the number of interruptions in incubation behavior, and the total incubation period in days for each implanted female that renested. Using general linear mixed models (GLMM), we examined treatment differences in these variables with clutch size and maternal body mass included as covariates; year was included as a random factor. We could not detect any treatment effects for any of these measured variables relating to incubation behavior (all $P > .80$).

To examine any possible effects of the implants on maternal feeding rates (with the knowledge that elevated baseline corticosterone may decrease parental provisioning behavior (Silverin 1986; Wingfield and Silverin 1986) as well as increase foraging behaviors in adult birds (Wingfield et al. 1990; Astheimer et al. 1992), we performed 30-min behavioral observations using spotting scopes on all nest boxes over three consecutive days when nestlings were aged 6–10 days. Provisioning rates were calculated per nestling per hour and based on the mean brood size of the nest for the 3-day observation period. Again using GLMM, we examined treatment differences in provisioning rates, with brood size included as a covariate and year and maternal identity included as random factors. We also found no treatment differences in provisioning rates by females ($P > .75$).

Hormone Determination

The concentration of total corticosterone (see Breuner and Orchinik 2002; Love et al. 2004) in yolk and plasma was determined using a corticosterone enzyme immunoassay kit (EIA; 0.31% cross-reactivity with testosterone; Assay Designs, Ann Arbor, MI) with a four-parameter logistic fit. Assay sensitivity was 32–20,000 pg/well, and all determinations fell within this range. Briefly, plasma was assayed at a volume of 100 μL (diluted 1 : 80 in assay buffer) in triplicate on two assay plates yielding an intra- and interassay variation of 7.1% and 8.3%, respectively. Yolk was extracted before assay by the methods outlined in an article by Williams et al. (2005). Following equilibration with distilled/deionized (dd) water, dilution with distilled-in-glass (DIG) methanol, and centrifugation at 1,500 *g* for 7 min under refrigeration (2° ± 1.5°C), supernatant was extracted on C18 columns (IS2200050C Isolute SPE columns, Chromatographic Specialties, Brockville, Ontario) under vacuum filtration. Columns were primed with DIG methanol, followed by dd water, followed by the entire 10-mL sample volume, and then washed with dd water. Corticosterone was eluted with 5 mL of 90% methanol into 7-mL borosilicate vials (03-337-26; Fisher). Each sample was evaporated to dryness under a stream of nitrogen gas and reconstituted in 1.2 mL of assay buffer (5% ethanol) before being quantified in triplicate on a single plate of the corticosterone EIA.

As internal controls, four additional yolks were used to quantify extraction efficiency. The four yolks were mixed by hand in a small borosilicate beaker and four fractions were weighed from the combined yolk and thereafter treated as independent yolk samples. After dilution into a total volume of 10 mL, two of the four samples were spiked with an 83.3-ng bolus of corticosterone in a 200-μL volume drawn from commercial standards from a corticosterone I^{125} radioimmunoassay kit (ICN, Orangeburg, NY), and the remaining two samples were spiked with 200 μL of assay diluent from the same kit. The resulting samples settled overnight and were handled exactly the same way as the unknown yolk determinations so that the controls contained raw yolk or raw yolk plus 8.33 ng of corticosterone. Corticosterone concentration in the raw yolk was 11.06 ± 0.78 ng/mL (mean ± SEM), with an intra-assay variation of 14.3%. Recovery of the 8.3 ng spike in the two replicates was 83.5% and 84.8%, suggesting that this method (see also Williams et al. 2005) is two to three times more efficient than other yolk steroid extraction methods in routine use (Schwabl 1993; Eising et al. 2003; Verboven et al. 2003). Values are reported as quantified and are not corrected for the recovery efficiency.

Molecular Sexing

We were able to collect all unhatched eggs and deceased nestlings from nests and these were frozen at −20°C until analysis. Starling nestlings were sexed using a polymerase chain reaction (PCR) amplification process based on techniques used by Griffiths et al. (1998). DNA was isolated from the red blood cell samples using Insta-Gene Matrix (Bio-Rad, Hercules, CA) and from tissue samples using DNeasy kits (Qiagen) following manufacturers' protocols. PCR amplification was run using primers P2 (5′-TCTGCATCGCTAAATCCTTT) and CW (5′-AGAAATCATTCCAGAAGTTCA), as proposed by Vanderkist et al. (1999).

Statistical Analyses

Correlation coefficients were calculated between baseline corticosterone levels and body mass corrected for body size for birds comprising the intraspecific sample in 2001. To calculate an estimate of body mass corrected for body size (energetic body condition) for these laying females, we regressed body mass against the first principal component score calculated from a principal component analysis for body size based on exposed culmen, tarsus, and flattened wing chord. For the hormonal manipulation data collected in 2002/2003, GLMM was used to analyze treatment and sex differences in morphological traits, growth rates, and immune response. For body mass and growth analyses, sex and treatment were included as factors, and egg mass, hatch mass, and maternal body mass were included as covariates (where required); maternal identity and year were used as random factors. For immune response analysis, sex and treatment were included as factors, nestling fledging mass was included as a covariate, and maternal identity and year were used as random factors. Nonsignificant interactions were backward eliminated, and all tests were two-tailed with the significance level set at $P = .05$. For post hoc comparisons of nestling masses, growth rates, and PHA immune responses for the sex × treatment analyses, we used the Bonferroni procedure (Rice 1989), with the P value being corrected to 0.0083 for the six possible pairwise comparisons.

We analyzed renesting probability of implanted females, sex ratio of the offspring, and sex-specific posthatching mortality as a function of treatment using GLMM with a binomial error structure (Kruuk et al. 1999). We included treatment, year, nestling hatch mass, and maternal body mass in the analysis where required, and the significance of the explanatory variables was determined by their Wald statistic using the χ^2 distribution. Preliminary analysis revealed no statistically significant relationship between laying order and sex within either treatment and no differences between sex and laying order between the treatments. However, this may be caused by imperfect knowledge of hatching order for all broods rather than a true lack of a relationship in this species. Least squares means ± SEM are reported throughout.

Results

Relationship between Maternal Condition and Plasma Corticosterone

Mean baseline corticosterone level for the 35 laying birds sampled in 2001 was 18.3 ± 3.5 ng/mL (means ± SEM;

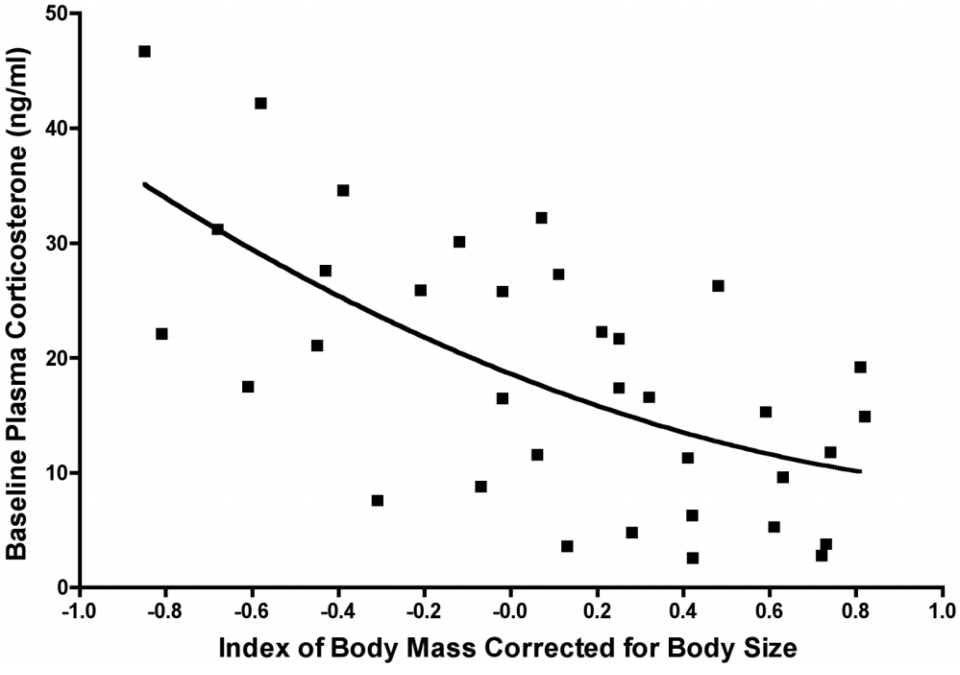

Figure 1: Relationship between baseline plasma corticosterone and body mass corrected for body size in laying female European starlings (based on the residuals from a regression between body mass and scores from the first axis of a principle component analysis involving tarsus, exposed culmen, and flattened wing chord).

range 3.8–46.7 ng/mL). Mean stress-induced plasma levels 30 min after capture were 80.5 ± 11.6 ng/mL (range 42.9–129.3 ng/mL). Body condition of laying female starlings was significantly negatively correlated with baseline corticosterone levels (second-order polynomial regression analysis: $r^2 = 0.40$, $P < .01$, $n = 35$; fig. 1).

Elevation of Maternal/Yolk Corticosterone

Silastic implants successfully elevated baseline plasma corticosterone levels in female starlings at the first-egg stage ($F = 5.40$, df = 1, 12, $P < .04$; fig. 2*A*) within the natural range of baseline levels for our population (see "Methods"). Elevation of maternal corticosterone also resulted in a significant elevation of yolk corticosterone ($F = 6.73$, df = 1, 12, $P = .02$; fig. 2*B*). We detected a significant positive relationship between maternal plasma corticosterone and yolk corticosterone in control (CTL) and corticosterone-implanted (CORT) females (second-order polynomial regression analysis: $r^2 = 0.39$, $P < .05$, $n = 14$; fig. 3).

There was no significant difference between the two treatments in either renesting probability ($\chi^2 = 2.14$, $P > .1$; CORT: 40.4%, CTL: 37.2%), renesting interval (number of days between removal of first clutch and initiation of second clutches; $F = 0.52$, df = 1, 56, $P > .4$; CORT: 9.73 ± 0.28 days, CTL: 9.57 ± 0.23 days, mean ± SEM), mean egg mass ($F = 0.06$, df = 1, 56, $P > .8$; CORT: 7.12 ± 0.05 g, CTL: 7.08 ± 0.04 g, mean ± SEM), clutch size ($F = 2.64$, df = 1, 56, $P > .1$; CORT: 5.8 ± 0.3 eggs, CTL: 5.6 ± 0.2 eggs, mean ± SEM), or female body mass ($F = 0.24$, df = 1, 56, $P > .6$; CORT: 87.5 ± 3.8 g, CTL: 89.6 ± 2.6 g, mean ± SEM). Twenty-two control females (out of 35) and 24 corticosterone-implanted females (out of 38) successfully reared nestlings to fledging ($F = 0.94$, df = 1, 44, $P > .75$).

Effects of Hormonal Manipulation on Reproductive Output and Offspring Quality

Although we did not detect a treatment effect on primary (laying) sex ratio ($\chi^2 = 1.21$, $P = .45$), females in the corticosterone-implanted group produced significantly more daughters at hatching than females in the control group over the 2-year study ($\chi^2 = 4.96$, $P = .026$; fig. 4*A*). This was a direct result of a significant difference in male-biased embryo mortality in the corticosterone-implanted group (treatment × sex interaction: $\chi^2 = 4.91$, $P < .03$), although we could not detect treatment differences in brood size at hatching ($F = 0.45$, df = 1, 56, $P > .5$). Moreover, males of corticosterone-implanted mothers that hatched were significantly lighter at hatching than males from control mothers, while female nestlings hatched at similar masses in both treatments (sex × treatment interaction: $F = 4.54$, df = 1, 201, $P = .034$; fig. 4*B*). We did not detect any sex-specific treatment effects on the length of the tarsus, ex-

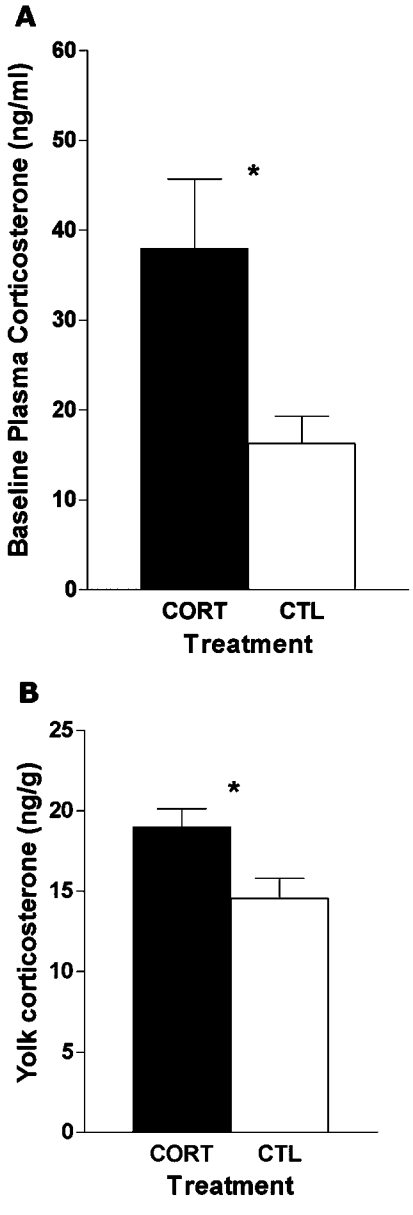

Figure 2: *A*, Baseline plasma levels of corticosterone (CORT) in CORT-implanted (*solid bars*) and sham-implanted (*open bars*) female European starlings at the first-egg stage (least squares means [LSM] ± SEM − LSM ± SEM). Asterisk indicates $P < .05$. *B*, Yolk corticosterone levels of first-laid eggs laid by CORT-implanted (*solid bars*) and sham-implanted (*open bars*) female European starlings (LSM ± SEM). Asterisk indicates significant difference of $P < .05$.

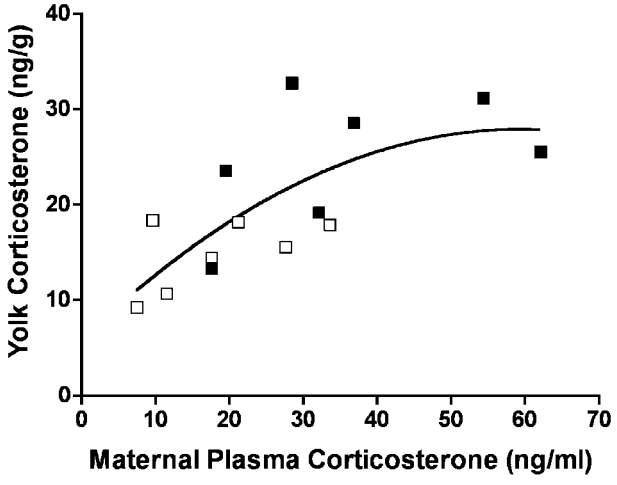

Figure 3: Relationship between maternal plasma corticosterone (CORT) at the first-egg stage and yolk corticosterone of first-laid eggs for laying female European starlings, CORT-implanted (*solid*) and sham-implanted (*open*).

posed culmen, or wing measurements at hatching (all $P > .25$); that is, male nestlings were lighter but were not different in structural size.

Growth rates from hatching to 5 days of age were also significantly different in a sex-specific manner in regard to treatment, with male nestlings from corticosterone-implanted mothers growing more slowly than males from control mothers (sex × treatment: $F = 3.92$, df $= 1, 200$, $P < .05$; fig. 5*A*). In addition, we therefore detected a sex-specific difference in growth rates in control-treated nestlings but not corticosterone-treated chicks ($F = 4.12$, df $= 1, 200$, $P < .05$; fig. 5*A*). This difference in growth rates resulted in a significant difference between the treatments in the masses of only male nestlings at 5 days of age (sex × treatment: $F = 6.14$, df $= 1, 201$, $P = .014$; fig. 5*B*). However, sex-specific effects of treatment on body masses disappeared by 10 days of age and remained nonsignificant at 15 and 17 days of age ($P = .08$, $P > .65$, $P > .71$, respectively), although males in both groups were heavier than females (both $P < .05$; male CORT: 74.64 ± 1.21 g, male CTL: 75.11 ± 0.98 g; female CORT: 70.47 ± 0.87 g, female CTL: 68.71 ± 0.72 g; mean ± SEM). We did not detect any significant sex-specific effects of treatment on either tarsus or wing chord at 17 days of age (all $P > .4$). However, while we did detect a sex difference in PHA immune response (sex: $F = 4.85$, df $= 1, 186$, $P < .03$; fig. 6), only corticosterone-born males exhibited significantly lower PHA responses than their control counterparts (sex × treatment: $F = 4.5$, df $= 1, 186$, $P = .035$; fig. 6). Finally, we detected no treatment ($\chi^2 = 0.91$, $P > .8$) or sex-specific treatment ($\chi^2 = 6.01$, $P > .1$) effects on nestling survival during postnatal development, and therefore fledging sex ratios of corticosterone-implanted mothers remained female biased ($\chi^2 = 4.7$, $P < .05$).

Discussion

Although evolutionary biologists have produced abundant evidence in support of the Trivers and Willard (1973) maternal investment model in numerous taxa (see the introduction to this article), evolutionary physiologists have not determined the key mechanistic link(s) between maternal quality and the sex-specific phenotypic adjustments observed in offspring. Experimental elevation of maternal baseline corticosterone in laying European starlings (thereby resulting in a decreased energetic condition phenotype) produced a concomitant increase in yolk corticosterone resulting in female-biased hatching sex ratios, lighter male offspring at hatching, and slower growing of males during early postnatal development. This hormonal manipulation resulted in reduced investment in male offspring, consistent with the prediction that mothers in poorer condition would be favored by producing more daughters than sons and investing more in daughters than sons (Trivers and Willard 1973; Cameron and Linklater 2002). Although this evolutionary investment in daughters occurred indirectly via a reduction in son quality (because a poor-condition mother would not be expected to be able to directly increase investment in the less expensive sex), it is probable that the relative investment in a daughter in relation to a son is important for a mother's fitness rather than the absolute investment the daughter receives. Previous studies that used egg removal techniques in indeterminate (continuous) laying birds and aimed at decreasing maternal laying condition (Nager et al. 1999; Kalmbach et al. 2001) did not separate the transfer of smaller-scale maternal physiological components of maternal condition (such as the transfer of hormones to eggs) from the large-scale changes in the transfer of resources to eggs (as seen in Nager et al. 1999). We were able to manipulate a hormonal signal of maternal condition without affecting condition itself, suggesting that the transfer of corticosterone to eggs can affect offspring phenotype directly without large-scale changes in egg or maternal quality.

Our results are consistent with previous studies reporting a negative relationship between baseline plasma corticosterone and energetic condition in birds. That is, individuals with low body mass given their body size (i.e., in poor energetic condition) had higher baseline corticosterone levels, whereas individuals that were heavier or at a sufficient mass, given their body size, had lower baseline corticosterone levels. Marra and Holberton (1998)

reported that American redstarts (*Setophaga ruticilla*) wintering in poor-quality habitats exhibited poor energetic condition and high baseline corticosterone levels. Furthermore, birds also exhibited a negative relationship between body mass changes (from arrival in autumn to departure in spring) and baseline corticosterone levels. Experimental food restriction studies in American kestrels (*Falco sparverius*; Heath and Dufty 1998) and various seabird species (Kitaysky et al. 1999*a*, 1999*b*, 2001, 2003) have also shown that decreasing the dietary energetic quality subsequently decreases energetic body condition, which results in elevated baseline corticosterone levels. Our data combined with those of previous studies therefore provide good evidence that baseline corticosterone can be a reliable hormonal indicator of physical and physiological condition in various avian species, which confirms our first prediction.

To our knowledge, this is the first study to measure yolk corticosterone in a wild avian species, and as predicted, maternal plasma corticosterone levels were positively related to yolk corticosterone of eggs laid by these females. Importantly though, acute experimental elevation of maternal plasma corticosterone occurred without altering maternal condition, egg quality (size and number), or the time between hormonal manipulation and re-laying of the second clutch. Although there is good experimental evidence indicating that even large-scale elevation of maternal corticosterone during laying does not affect yolk protein, total protein, or yolk lipid of eggs (Salvante and Williams 2003), there still exists the possibility that acute elevation of maternal corticosterone may have altered other components of egg quality (including such things as nutrients, carotenoids, antibodies, or even other hormones) that we were not able to measure but which should be examined in future studies. The transfer of experimentally elevated maternal corticosterone to avian yolks was recently demonstrated in captive Japanese quail (*Coturnix coturnix japonica*) by Hayward and Wingfield (2004), and maternal transfer to offspring has also been documented in other studies involving several egg-laying taxa (Sinervo and DeNardo 1996; McCormick 1998; Cree et al. 2003). It is interesting to note that variation in maternal plasma corticosterone is much higher than that of yolk corticosterone and that elevated maternal plasma levels do not directly translate to correspondingly high yolk concentrations, potentially because very high concentrations are fatal to all offspring (Mashaly 1991; Heiblum et al. 2001). Maternal buffering of embryonic glucocorticoid exposure has been examined in reptiles (Painter et al. 2002) and extensively examined in mammals (Burton and Waddell 1999; Seckl 2001; McMullen et al. 2004; Speirs et al. 2004). In birds, maternal buffering may take the form of transport proteins

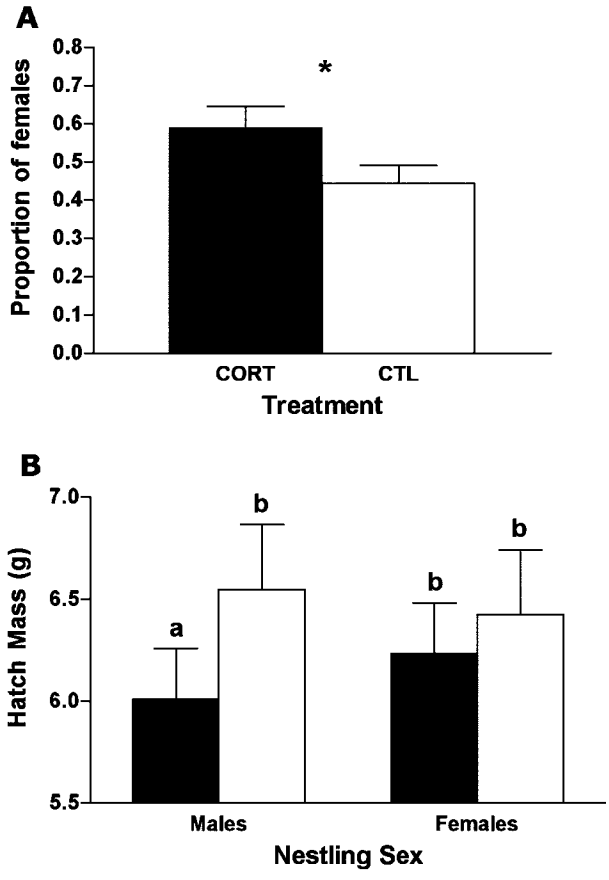

Figure 4: *A*, Proportion of daughters produced at hatching by female European starlings receiving either corticosterone (CORT; *solid bars*) or sham (*open bars*) implants during egg formation (least squares means [LSM] ± SEM). Asterisk indicates $P < .05$. *B*, Sex-specific effects of maternal hormonal manipulation of female European starling (CORT-implanted [*solid bars*] and sham-implanted [*open bars*]) on nestling hatch masses (LSM ± SEM). Different letters represent significant difference between stages ($P < .0083$, as calculated for six pairwise Bonferroni comparisons).

such as corticosterone binding globulin, which regulates the bioavailability of "free" unbound steroids to tissue (Siiteri et al. 1982; reviewed in Breuner and Orchinik 2002), or P-glycoprotein, a member of the ATP-binding cassette superfamily that performs unidirectional transport of steroid hormones in various vertebrate tissues (Meijer et al. 1988; Ueda et al. 1992; Golden and Partridge 2000; Barnes 2001; Green et al. 2005).

A large body of work in vertebrates has indisputably shown that prenatal exposure to elevated maternal glucocorticoids results in significant effects on offspring phenotype (see the introduction for references). To our knowledge however, few studies have examined these effects in

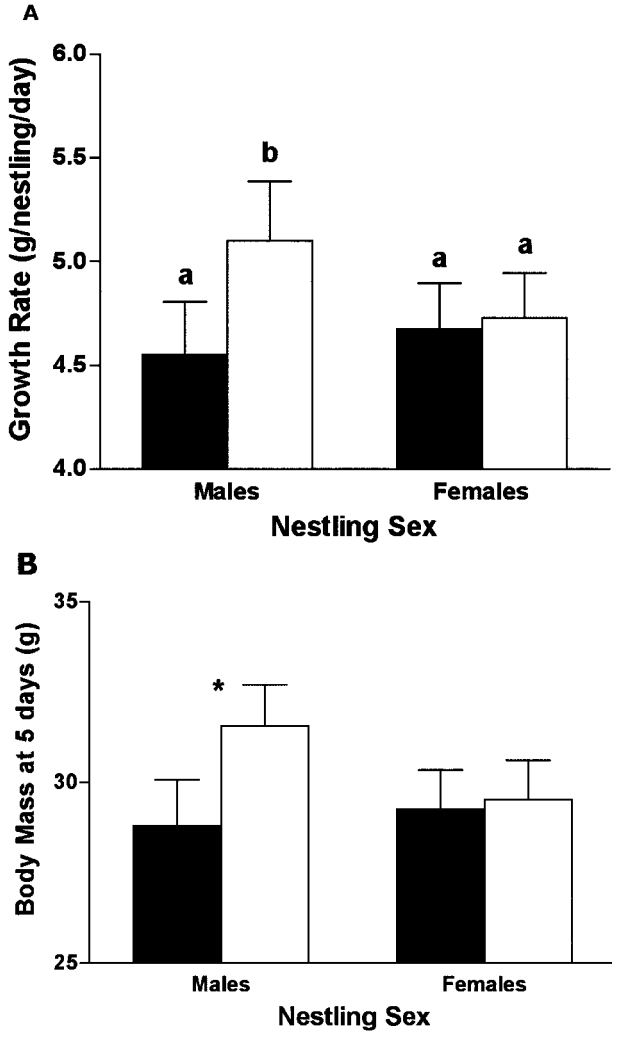

Figure 5: Sex-specific effects of maternal hormonal manipulation of female European starling (corticosterone-implanted [*solid bars*] and sham-implanted [*open bars*]) on (*A*) nestling growth rates from hatch to 5 days of age (least squares means [LSM] ± SEM) and (*B*) body mass at 5 days of age (LSM ± SEM). Different letters represent significant difference between stages ($P < .0083$, as calculated for six pairwise Bonferroni comparisons).

a sex-specific or evolutionary framework, with two notable exceptions. Sinervo and DeNardo (1996) reported that an elevation of maternal corticosterone in side-blotched lizards (*Uta stansburiana*) resulted in sex-specific effects on hatchling masses. Exogenous corticosterone delivered via silastic implants (within the physiological range for the population) increased clutch mass in laying females, resulting in larger female offspring at hatch compared with sham-implanted females; this effect was not observed in male offspring. Meylan and Clobert (2005) reported sex-specific effects of maternal corticosterone treatment in the common lizard *Lacerta vivipara* (however, levels should be considered pharmacological rather than within the natural physiological range), with male offspring showing apparent negative growth effects of the hormonal treatment while at the same time exhibiting positive effects on their survival. A possible mechanism for the paralleled sex differences observed in both these and this study is sex-specific differences in developmental sensitivity of embryos to maternal glucocorticoids during prenatal development.

In addition to regulating many other important physiological pathways, glucocorticoids also regulate the insulin-like growth factor (IGF) axis (Li et al. 1998) and as such, play essential roles in growth as well as tissue differentiation and maturation (Fisher 1992). However, at elevated levels, glucocorticoids can inhibit cell proliferation and growth (Orth et al. 1992). Studies in various vertebrate taxa have shown that elevated glucocorticoids downregulate the release and activity of pituitary growth hormones (GH) and IGFs and reduce the ability of embryos to regulate levels of glucocorticoid receptors (Tonshoff and Mehls 1997; Li et al. 1998; Woodall et al. 1999; Ghosh et al. 2000; Nolan et al. 2001) and interact with developmental hormones such as thyroid hormones (De Jesus et al. 1990; Redding et al. 1991; Porter and Dean 2001). Glucocorticoids also act as transcription factors because many genes have glucocorticoid response elements on the DNA that are activated by glucocorticoids, leading to their transcription. Thus, any potential change in glucocorticoid levels can have subtle or overt effects on the development of various organs and development overall (see Byrne 2001 for review). In a sexually size-dimorphic species, these developmental effects could be sex-specific, given that the sexes may require different levels of GHs, IGFs, or even receptors for these hormones during prenatal or postnatal development. This would explain why male starling embryos appear to be more developmentally sensitive to elevated maternal glucocorticoids than female offspring.

Large male size in starlings provides fitness benefits to both sons and mothers and is dependent on sons growing faster than daughters while in the nest. If glucocorticoids play a role in the embryonic initiation of sex-specific post-hatching growth rates, mothers could take evolutionary advantage of the greater sensitivity of male embryos to these glucocorticoid-dependent developmental pathways via the deposition of corticosterone in yolks. However, how would mothers benefit from producing fewer, smaller male offspring either during adverse conditions or in relation to poor body condition, and why do corticosterone-implanted females invest in male offspring at all, especially if these males fledge with a lower quality (i.e., decreased cell-mediated immune responses)? Starlings exhibit no na-

tal philopatry (young do not return to parental breeding grounds; Cabe 1993; O. P. Love and T. D. Williams, unpublished data), and therefore female starlings would not be expected to alter sex-specific investment to take advantage of current population sex ratios (as proposed by Fisher 1930). Rather, deposition of yolk corticosterone may benefit females by acting as a bet-hedging strategy in stochastic environments where the correlation between environmental cues at laying (and therefore potentially maternal condition) and conditions during chick-rearing might be low and unpredictable (Nager et al. 2000). If maternal condition at laying remains poor into chick-rearing, sons that weighed less at hatching would be more likely to experience postnatal mortality because low mass at hatching has a significant negative effect on survival during early postnatal development in altricial birds (reviewed in Williams 1994; O. P. Love and T. D. Williams, unpublished data for starlings), the outcome being a further relative investment in daughters via a reduction in competition with sons. Although mothers adopting this strategy would fledge fewer young than a good-condition female, it may be better for a mother in poor condition to fledge fewer young overall (so as not to impact her immediate survival or future reproductive effort). This allows her to maintain the quality of the young she does produce (in this case females), rather than attempting to raise more young at the cost of decreasing quality in both the sexes. Alternatively, if environmental conditions and maternal quality do improve by the chick-rearing stage, mothers are then able to fledge both good-quality female and male offspring of adequate body size (as exhibited in this study). However, there still appears to be a trade-off in adopting this strategy: males hatching from high-corticosterone mothers and surviving to the fledgling stage do so with decreased cell-mediated immune responses.

Although we did predict both increased male mortality and decreased fledgling body masses in the corticosterone treatment group, we detected only transitory effects of corticosterone on male body mass at hatching and early on in development; that is, these effects disappeared after 5 days of age with no significant male-biased mortality during this time and no treatment effects on mass or structural size. However, we did observe decreased PHA immune response in male corticosterone-born nestlings that could be produced via two possible pathways. Given the costs of compensatory growth (Metcalfe and Monaghan 2001) and that cell-mediated immune function can be directly resource-limited in growing nestling birds (Saino et al. 1997, 1998; Nordling 1998; González et al. 1999; Alonso-Alverez and Tella 2001), the larger-sized male starlings may have traded off PHA immune response while attempting to maintain growth. This idea has recently been presented by Chin et al. (2005) and is further supported in the present study by the fact that male PHA responses were significantly lower than those of their female counterparts in both treatment groups. Alternatively, Rubolini et al. (2005) recently reported that elevation of whole-egg corticosterone may directly affect nestling immune responses, given that chronic elevations of baseline corticosterone in adult birds have been shown to suppress PHA responses (Owen-Ashley et al. 2004; Martin et al. 2005). The transitory effects of yolk corticosterone manipulation on body mass and growth are not necessarily surprising, given that this study was not designed to alter postnatal maternal condition. Manipulating both prehatch maternal or yolk corticosterone levels in addition to manipulating posthatch maternal chick-rearing quality (energetic body condition, as in Velando 2002) will test the adaptive nature of this potential maternal hormonal strategy.

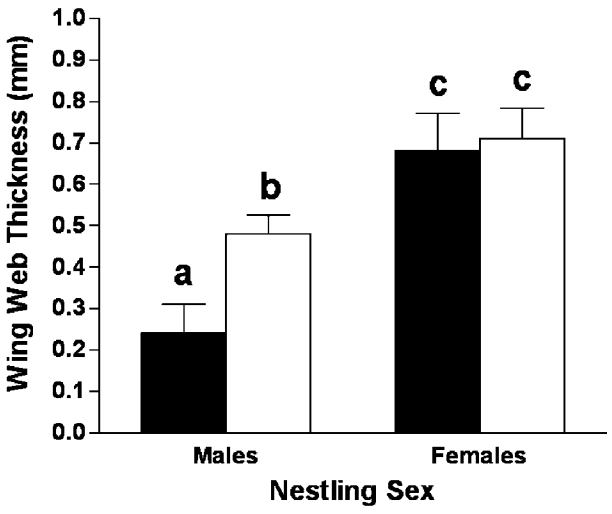

Figure 6: Sex-specific effects of maternal hormonal manipulation of female European starling (corticosterone-implanted [*solid bars*] and sham-implanted [*open bars*]) on nestling cell-mediated immune response measured using the PHA method (see text; least squares means ± SEM). Different letters represent significant difference between stages ($P < .0083$, as calculated for six pairwise Bonferroni comparisons).

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Hatchling starlings (*Sturnus vulgaris*) compete for access to feeding parents. Sons and daughters benefit mothers differentially; mothers must make decisions about which to invest in, in relation to their own condition. Photograph source: Christina Semeniuk.