

Maternal corticosteroids influence primary offspring sex ratio in a free-ranging passerine bird

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When fitness benefits of investment in sons and daughters differ, animals are predicted to manipulate the sex ratio of their offspring. Sex ratio manipulation occurs in many taxa, but the mechanisms underlying the phenomenon in vertebrates remain largely unknown. Factors favoring skewed sex ratios, such as reduced maternal condition or food availability, also induce elevated corticosteroids. Recent experimental studies support a causal relationship between corticosteroids and sex ratio. Evidence of a natural correlation between maternal corticosteroids and offspring sex ratio has been lacking, however. Without such evidence, the importance of corticosteroids in influencing sex ratios in natural populations was unknown. We measured baseline corticosteroids in 19 free-ranging female white-crowned sparrows (*Zonotrichia leucophrys*) and the sex ratios of their offspring. Females with high corticosteroids produced more daughters than females with low hormone levels. We then conducted a controlled, field-based experiment investigating the effects of moderately increased maternal corticosteroids on offspring sex ratios to determine if the observed correlation reflects a causal relationship between maternal corticosteroids and offspring sex ratio. Hormone-implanted females produced more female embryos than control females. These findings provide the first evidence of a natural correlation between maternal corticosteroids and offspring sex ratios in free-ranging birds, and the first experimental evidence of a causal link between moderate increases in corticosteroids and biased primary sex ratios. *Key words*: avian, corticosterone, sex ratio, Trivers-Willard model, *Zonotrichia leucophrys*. [*Behav Ecol* 18:1045–1050 (2007)]

When net fitness benefits conferred by investment in sons and daughters differ, animals are predicted to manipulate the sex ratio of their offspring (Trivers and Willard 1973; Oddie 1998). In many systems, daughters, regardless of their quality, are more likely than sons to reproduce (Rose et al. 1998; Kruuk et al. 1999; Bercovitch et al. 2000; Widdig et al. 2004). High-quality sons can leave many more offspring than daughters, but low-quality sons may fail to reproduce at all, making sons a more risky investment (Cameron and Linklater 2002). In systems where the fitness consequences of offspring quality are sex specific, a female that can produce high-quality offspring should produce more sons, whereas a female constrained to produce low-quality offspring should produce more daughters. This theory is supported by empirical evidence of biased offspring sex ratios in many taxa (reviewed in Pike and Petrie 2003; Rosenfeld and Roberts 2004). Further, experimental and empirical studies have demonstrated that skewed sex ratios are associated with variation in numerous factors, including maternal hormone levels (von Engelhardt et al. 2004; Veiga et al. 2004; Correa et al. 2005; Love et al. 2005; Pike and Petrie 2006), food abundance (Austad and Sunquist 1986), mate quality (Svensson and Nilsson 1996; Pike and Petrie 2005), habitat quality (Wiebe and Bortolotti 1992; Komdeur et al. 1997, 2002), rainfall (Burley et al. 1989), dominance status (Leonard and Weatherhead 1996), and maternal condition (Parker 2002; Pike 2005). Although the mechanisms that underlie sex ratio manipulation in vertebrates with genetically determined sex remain unknown, re-

cent experimental studies provide some evidence of a causal relationship between maternal corticosteroids and offspring sex ratio (Love et al. 2005; Pike and Petrie 2006).

Endocrine signals provide organisms with information about individual and local environmental conditions. Measures of baseline corticosteroids, a class of stress hormones found in all vertebrates, correlate with indices of individual condition (Marra and Holberton 1998; Kitaysky, Piatt, et al. 1999; Kitaysky, Wingfield, et al. 1999; Duckworth et al. 2001; Romero and Wikelski 2001), dominance status (Creel 2001), food abundance and quality (Clinchy et al. 2004; Schoech et al. 2004), weather conditions (Wingfield 1984; Romero et al. 2000), and habitat quality (Marra and Holberton 1998). Given the coincidence between factors that affect sex ratio and factors that affect baseline corticosteroid (cort) levels, maternal cort could provide an appropriate signal that results in sex ratio manipulation (Pike and Petrie 2003; Cameron 2004; Love et al. 2005). Alternatively, cort levels may simply be correlated with other indicators of condition and quality that are directly involved in adaptive manipulation of offspring sex ratios. In either case, one would predict that increasing maternal cort levels would correlate with increases in the proportion of offspring that are female. While this idea has been suggested previously (Pike and Petrie 2003) and maternal fecal cort levels have been found to predict offspring sex ratios in captive quail (Pike and Petrie 2006), no evidence of such a relationship in a free-ranging population has been reported. Experimental studies have documented an effect of elevated cort on offspring sex ratio (Love et al. 2005; Pike and Petrie 2006). The significance of these findings for natural populations has been difficult to interpret, however, because these studies employed traditional silastic implants to increase cort levels. These implants typically result in a rapid and significant increase in circulating hormone levels well beyond the normal range of variation in baseline hormone levels (Gray et al. 1990; Criscuolo et al. 2005). Although these studies

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provide strong evidence that dramatically increased cort can cause sex ratio biases, the effect of cort at more moderate levels, within the range of normal baseline levels, is unknown. Linking cort to documented sex ratio biases requires evidence that a similar effect is seen with moderate increases in cort, within the range that is likely to be associated with slight declines in body condition, habitat quality, or other factors that have been shown to influence offspring sex ratios.

We tested the prediction that increasing maternal cort levels would correlate with female-biased offspring sex ratios in free-ranging white-crowned sparrows (*Zonotrichia leucophrys*). White-crowned sparrows are socially monogamous, but extrapair paternity is common (Chilton et al. 1995; Bonier et al. 2007), so males can produce a disproportionate number of offspring with minimal investment through extrapair copulations. In contrast, females only produce offspring with significant parental investment, as conspecific brood parasitism has never been documented in this species (Chilton et al. 1995).

After documenting a natural relationship between maternal cort levels and offspring sex ratio, we conducted a controlled, field-based experiment in the same population of birds. We moderately increased maternal cort levels within natural baseline levels and documented the effects on the primary sex ratio (proportion of female embryos at the time of laying).

METHODS

Empirical study

We tracked 19 free-ranging breeding pairs of white-crowned sparrow in Seattle, Washington, through regular observations and location of nests. Birds were monitored from the time when they began arriving on the breeding grounds in late March 2005 through the time when they abandoned territories in early August 2005. Once pairs were established, we monitored birds regularly for evidence of breeding activity and located all the focal pairs' nests throughout the season. We monitored nests regularly from the time when they were located to record incidence of laying, hatching, predation, brood parasitism, and fledging.

During incubation of the first clutch, we captured females using mist nets placed less than 3 m from the active nest and flushing them directly from the nest into nets just prior to dawn. We took a small blood sample within 3 min of initial disturbance using alar vein puncture and then fitted all birds with unique colored leg band combinations to allow subsequent identification. We used the blood sample for measurement of the birds' circulating baseline cort levels. In 4 cases, first nests were depredated before we could capture both adults, so birds were netted during the incubation period of subsequent nesting attempts. Blood samples were separated through centrifugation within 8 h of collection, and plasma was removed and stored at -20°C until assay.

We were unable to measure cort levels during all stages of breeding. Sex ratio manipulations could occur prior to or after laying, yet we only quantified cort levels during incubation. Further, we quantified a cumulative sex ratio across multiple nesting attempts in one breeding season but only have one measure of cort levels for each female. Despite this, we expect that cort levels from one sampling period should be representative of relative cort levels among females within our population. Evidence from other populations of this species demonstrate that cort levels tend to be relatively stable within individuals across early breeding stages, that is, individuals with high cort levels during laying tend also to have high cort levels during incubation and nestling-provisioning periods (Wingfield and Farner 1976, 1978).

We took a small sample of blood from nestlings between days 5 and 7 of the nestling period, which lasts between 8 and 12 days in white-crowned sparrows (Chilton et al. 1995). Whole-blood samples were stored at -20°C until DNA extraction and analysis (see below). For each focal female, data across repeated nests were combined to represent seasonal sex ratios (as these birds are multiply brooded). Sex ratios did not vary with nest number or progression of the breeding season.

We tested the hypothesis that females with elevated cort would have a higher proportion of daughters than females with low cort using general linear model analysis of sex ratio of all nestlings that reach sampling edge with a binomial error structure and logit link function, weighted by number of nestlings sampled, such that the proportion of daughters in larger broods had a greater effect on the analysis than data from smaller broods (Crawley 2005). All statistical analyses were conducted using R version 2.2.1 (The R Foundation for Statistical Computing).

Experimental study

In the spring and summer of 2006, we again monitored the arrival and establishment of territories of white-crowned sparrows in the same population. Once females completed laying first clutches and began incubation, we captured them, using methods described above. We collected a blood sample within 3 min of disturbance and also fitted females with unique color bands if they were not already marked from previous years of study.

Following sampling, banding, and collection of basic morphometric measurements, we subcutaneously implanted females with either a placebo or a 0.5-mg corticosterone (the primary cort found in birds) pellet. These pellets are designed to release their contents in a relatively stable manner over a 60-day period, in contrast to more frequently used silastic tubing implants, which release a large bolus followed by a long tapering dose of hormone (Gray et al. 1990; Criscuolo et al. 2005). According to the manufacturer, the pellets are comprised of cholesterol, cellulose, lactose, phosphates, and stearates. Placebo pellets only differ from hormonally active pellets in the absence of hormone (information from manufacturer, Innovative Research, Sarasota, FL, USA). We selected the hormone dose using a captive trial of 3 doses (0.1, 0.5, and 2.5 mg cort) implanted into 12 white-crowned sparrows. In weekly sampling, we found hormone levels in birds with the 0.5-mg dose to be increased on average by 2.2 ± 1.9 ng/ml over preimplant levels beginning 1 day after implantation through 6 weeks after implantation (Figure 1). This extremely moderate increase in hormone level is equivalent to 0.26 of 1 standard deviation of the observed variation in natural circulating baseline hormone levels found in our previous field study (see Figure 2). Because the implants increase hormone levels consistently for a period of several weeks, even this moderate increase could have significant, biologically relevant effects. In our captive study, we observed a transient peak followed by a decrease in hormone levels before levels stabilized, which is likely attributable to the balancing of negative feedback mechanisms and the exogenous hormone being delivered by the time-release pellets (Westerhof et al. 1994).

After birds were captured and implanted, we collected their clutch of eggs, measured egg mass (± 0.001 g with an Acculab portable scale), and placed the eggs in an incubator (at $37 \pm 0.2^{\circ}\text{C}$) until they reached at least 6 days of incubation to ensure sufficient tissue availability for embryo sexing. We moved clutches into the incubator, rather than leaving them with the female for incubation to prevent cowbird brood parasitism and predation from affecting the clutches. Despite

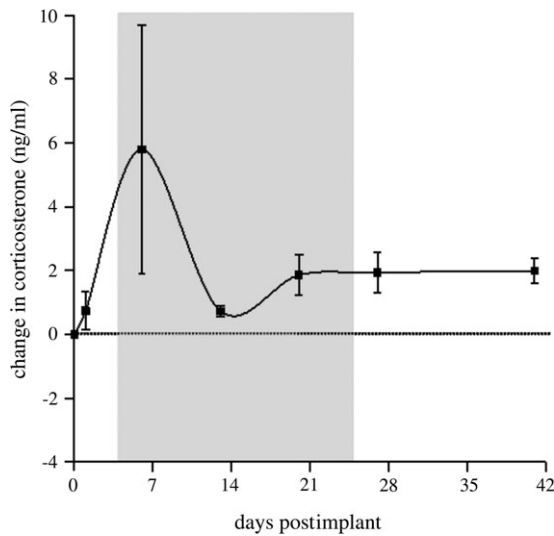


Figure 1
Effect of 0.5-mg corticosterone implants on circulating hormone levels in 4 captive white-crowned sparrows. Data shown are differences in mean cort levels before and after implant (postimplant – preimplant hormone level). Gray shaded area depicts range of days when free-ranging, hormone-implanted birds were ovulating.

taking this precaution, 5 of 26 broods were parasitized by cowbirds (Table 1) and one placebo-implanted female’s second brood was depredated prior to sampling. This female’s third clutch was used for postimplantation data collection.

Following release, we monitored implanted females ($N = 7$ cort, 6 placebo) for evidence of renesting. All 13 females in this study renested after implantation. As soon as females had completed their second clutches, we again netted them, collected an immediate blood sample for hormone assay, and collected their clutches of eggs, which were again relocated to an incubator for a minimum of 6 days of completed in-

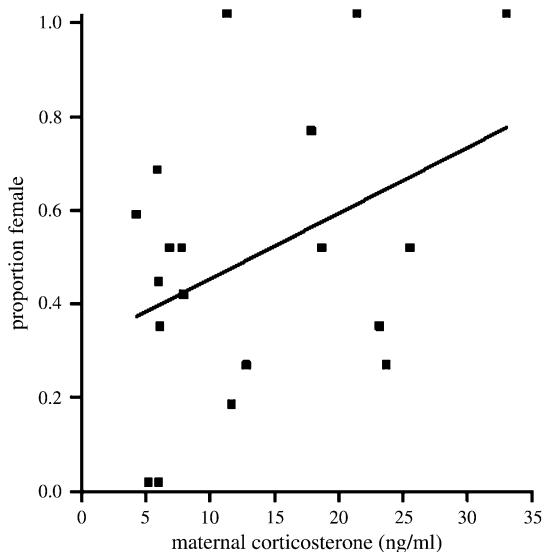


Figure 2
Regression of offspring sex ratio (proportion of female offspring) on maternal baseline corticosterone levels. Female white-crowned sparrows with high baseline corticosterone levels had more daughters than females with low baseline corticosterone levels ($N = 19$, weighted binomial general linear model, $z = -2.62$, one-tailed $P = 0.01$).

Table 1
Summary statistics for placebo-implanted ($N = 6$, above) and cort-implanted ($N = 7$, below) female white-crowned sparrows and their broods

Parameter	Preimplant	Postimplant
Cort level (ng/ml)		
Placebo	10.6 ± 1.7	13.8 ± 2.5
Cort	13.8 ± 2.8	14.2 ± 3.2
Sex ratio (proportion of female)		
Placebo	0.5 ± 0.1	0.4 ± 0.1
Cort	0.5 ± 0.2	0.6 ± 0.1
Clutch size (number of eggs)		
Placebo	4.1 ± 0.3	3.9 ± 0.3
Cort	4.0 ± 0.2	4.1 ± 0.3
Number of sterile eggs		
Placebo	0.3 ± 0.2	1.2 ± 0.4
Cort	0.8 ± 0.3	0.4 ± 0.2
Egg mass (g)		
Placebo	2.8 ± 0.1	2.8 ± 0.1
Cort	2.8 ± 0.1	2.9 ± 0.1
Number of parasitized nests		
Placebo	2	1
Cort	1	1
Interegg interval (days)		
Placebo	NA	8.4 ± 1.0
Cort		10.0 ± 2.3

Only postimplant offspring sex ratio (in bold) differed between groups. All data are presented as means ± 1 standard error, except for number of nests parasitized by brown-headed cowbirds, which is total count per group. NA, not applicable.

cubation. Two females could not be recaptured, but their clutches were collected for inclusion in the analysis.

We compared postimplant sex ratio (proportion of female embryos) between placebo and cort implanted birds using a general linear model with a binomial distribution and logit link function, weighted by the number of fertile eggs in each postimplantation clutch. Overdispersion was not a problem in our model (i.e., residual deviance was less than degrees of freedom) (Crawley 2005). To investigate the within-female effect of implants, we compared the change in number of female embryos produced between pre- and postimplant clutches using a t -test, weighted by total number of fertile eggs produced. All tests were one tailed as we had made a priori, directional predictions. All hormone and genetic samples were randomly assigned a number, such that the researcher conducting analyses was unaware of the sample’s origin or the implant status (placebo or cort) of the female.

Molecular sexing

We used a published polymerase chain reaction (PCR)-based method to sex all sampled offspring and embryos (for details, see Griffiths et al. 1998). Briefly, we extracted DNA from whole-blood and embryonic tissue samples using the Qiagen DNeasy Tissue Kit following manufacturer’s instructions for extraction from nucleated blood cells or tissue. Primers P2 and P8 were used to amplify a portion of the *CHD-W* and its homolog, the *CHD-Z* gene, found on the avian sex chromosomes (Griffiths et al., 1998). Because of size differences in the target fragments, males and females can readily be identified with agarose separation and visualization of PCR products. We sexed each of the nestlings that reached sampling

age and all the embryonic tissue samples in duplicate, with 100% agreement in results from duplicate samples. Eggs without evidence of embryonic tissue development were treated similarly, but all failed to yield amplifiable DNA and were thus assumed to be sterile.

Hormone assay

We centrifuged blood samples within 8 h of collection to separate plasma, which was then stored at -20°C until assay. We quantified plasma levels of total cort in each sample in duplicate through direct radioimmunoassay, following extraction with redistilled dichloromethane (see Wingfield et al. 1992 for details). All samples from each season were assayed simultaneously (2 assays total) and within-assay variation between replicate known concentration control samples was 2.4% and 0.83% in 2005 and 2006, respectively.

RESULTS

Empirical study

As predicted, maternal baseline cort levels were positively correlated with offspring sex ratio in our empirical study (Figure 2, $N = 19$, $z = -2.62$, one-tailed $P = 0.01$); females with higher cort had more daughters than females with low cort. Across the focal population, the sex ratio of nestlings in 2005 was 49 male: 44 female, which does not differ from a 1:1 ratio ($\chi^2 = 0.27$, $P = 0.60$). Summed across the season, the focal females produced between 3 and 7 offspring (mean 4) that survived to the age of sampling. We observed no gaps in the laying sequences in 60 interegg intervals observed for 21 nests that were located during building or at onset of laying, as would be expected with selective resorption of fertilized ova. We also found no relationship between partial brood reduction (via hatching or fledging success) and cort levels, as would be expected if sex ratio manipulations occurred after laying.

Experimental study

Experimental manipulation of maternal cort levels had a significant effect on the primary sex ratio, regardless of the analysis we employed. In postimplant clutches, cort-implanted females produced a significantly higher proportion of female embryos than placebo-implanted birds (Figure 3, $N = 7$ cort, 6 placebo, $z = 2.32$, one-tailed $P = 0.01$). The difference in number of female embryos produced between pre- and postimplant clutches was significantly higher for cort-implanted females than for placebo-implanted females ($t = -1.67$, one-tailed $P = 0.03$).

We sampled females on average 15 days following implantation (range 10–26 days). By that time, baseline cort levels did not differ between groups (Table 1). Mean clutch size, clutch mass, egg mass, number of sterile eggs, and number of days between initial clutch collection and time of first laid egg of the subsequent clutch, in the pre- and postimplantation clutches also did not differ for cort- or placebo-implanted females (Table 1). We also found no relationship between clutch size and sex ratio. The mean interval between initial hormone or placebo implantation and ovulation of the first laid egg of the subsequent clutch was 8 days (range 4–21 days). Mean interval between implantation and initiation of postimplantation clutches of cort-implanted females was 10 days (range 6–21 days), and these clutches were completed on average 14 days after implantation (range 10–26 days).

In relation to data from our captive implant validation trial, the period of time when free-ranging, cort-implanted females began ovulating coincides with maximum and significant in-

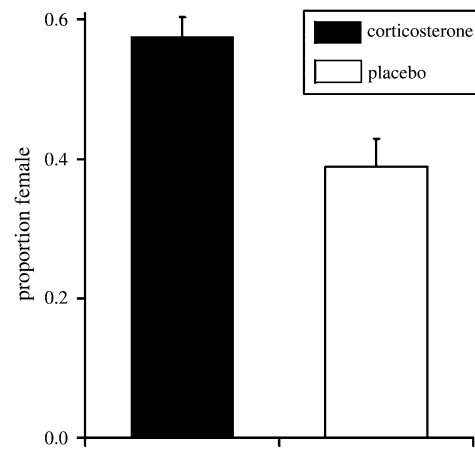


Figure 3

Primary sex ratio (proportion of female embryos) in offspring of cort-implanted (filled bars) and placebo-implanted (open bars) white-crowned sparrows (mean \pm 1 standard error). Cort-implanted birds produced significantly more female embryos than placebo-implanted birds ($N = 7$ cort, 6 placebo, weighted binomial general linear model, $z = 2.32$, one-tailed $P = 0.01$).

creases in cort levels resulting from the 0.5-mg implants in captive birds (Figure 1, paired t -test of log-transformed mean cort levels on days 6 and 13 after implantation relative to preimplant levels, $t = 3.65$, one-tailed $P = 0.02$). At day 6, the day of maximal cort increase in our captive study, cort levels were increased above preimplant levels by 5.79 ng/ml. If similar increases occurred in our field study, as is expected if the observed increase is primarily caused by the biochemical properties of the slow-release pellets we employed, then mean maximum cort levels in our free-ranging, cort-implanted birds would have been less than 20 ng/ml and well within the natural range of variation in baseline cort levels in this population (Figure 2).

DISCUSSION

This study provides the first empirical evidence of a natural correlation between maternal cort and offspring sex ratios in a free-ranging organism and the first experimental evidence of a causal link between moderate increases in maternal cort and biased offspring sex ratios. Female white-crowned sparrows with naturally elevated baseline cort levels produced more daughters than females with low cort levels (Figure 2). We did not observe delays in laying sequence or postlaying brood reduction biased by cort levels that would indicate post-ovulatory brood reduction, suggesting that sex ratio manipulation took place prior to laying. In our experimental study, we found that moderate elevation of maternal cort levels resulted in female-biased sex ratios at the time of laying (Figure 3).

Cort could affect offspring sex through a number of as yet untested mechanisms, including via inhibitory effects on estrogens or progesterone (Sapolsky et al. 2000; Murase et al. 2002), both of which have been suggested to be involved in offspring sex manipulation (Creel et al. 1998; von Engelhardt et al. 2004; Correa et al. 2005). One of the mechanisms that could account for our results is selective fertilization. If all the sterile eggs were assigned the minority sex in each postimplantation clutch, sex ratios would not have differed between clutches from placebo- and cort-implanted females. An alternative explanation is male-biased early embryonic mortality. Male embryos have been found to respond differently to elevated cort than females in previous studies (Love

et al. 2005; Hayward et al. 2006). We found no amplifiable DNA in eggs without visible evidence of development, suggesting that early embryonic mortality is unlikely to account for our results. Nonetheless, if early embryonic mortality is responsible for the sex ratio biases we report here, then cort influenced the secondary, rather than the primary, sex ratio.

Although this study provides the first reported evidence of a natural relationship between maternal cort levels and offspring sex ratio in free-ranging birds, several previous studies have provided evidence suggesting that this relationship may be found in other taxa. Geiringer (1961) found that injections of adrenocorticotrophic hormone, a pituitary hormone that triggers cort release, resulted in female-biased broods in rats, and Pratt and Lisk (1989) found that dexamethasone (a cort blocker) injections reversed stress-related sex ratio biases in golden hamsters. More recently, Pike and Petrie (2006) demonstrated that experimental elevation of cort, but not manipulation of several other hormones, resulted in female-biased sex ratios in captive Japanese quail, and Love et al. (2005) found a similar effect of cort implants in free-ranging European starlings. These studies suggest that cort may act to alter offspring sex ratios independent of female condition and other quality-related factors. Both Pike and Petrie (2006) and Love et al. (2005) employed silastic implants to elevate cort levels; thus, the biological significance of these findings was not known. In combination with the findings reported here, we now have strong evidence that cort is involved in sex ratio manipulations and that even moderate increases, such as those that would be predicted from a moderate decline in maternal condition, can have significant impacts on offspring sex ratios.

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