

Mediation of a corticosterone-induced reproductive conflict

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Received 4 August 2003; revised 16 September 2003; accepted 2 February 2004

Available online 22 April 2004

Abstract

Current research in birds suggests that a conflict should exist during reproduction for the role of the glucocorticoid corticosterone (CORT). While elevated levels have been correlated with the increased energetic demand of raising offspring, elevated CORT levels have traditionally been implicated in reproductive abandonment. We examined the relationship between CORT and nest desertion in breeding wild female European starlings (*Sturnus vulgaris*) incorporating analyses of both total circulating levels and ‘free’, unbound CORT through analysis of corticosteroid-binding globulin (CBG). Free baseline CORT levels of nest-abandoning birds were significantly higher than nonabandoning birds within each stage, with chick-rearing birds exhibiting the highest free baseline CORT levels, while concurrently remaining the most resistant stage to nest desertion. Elevated free baseline CORT levels in chick-rearing birds were not due to increased total CORT secretion, but rather to a decrease in CBG levels. Overall, our results suggest that CORT and CBG interact to play a role in mediating the increased energetic demand of offspring, while minimizing the chances of nest desertion, thereby alleviating any potential behavioral conflict for CORT during reproduction. Furthermore, these results demonstrate that the traditional view of the role of CORT during reproduction is much more complex than previously appreciated. Together with mounting evidence, we suggest that elevated corticosteroid levels are an inherent and necessary part of reproduction in nonmammalian tetrapods.

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Keywords: Corticosterone; Corticosteroid-binding globulin; Reproduction; Nest desertion; European starling (*Sturnus vulgaris*)

Introduction

Corticosterone (CORT) serves to mediate adaptive physiological and behavioral responses to stressful events (reviewed in Sapolsky et al., 2000). One of the more prominent roles of glucocorticoids is their involvement in homeostatic energy balance (Dallman et al., 1993; Harvey et al., 1984): in birds, elevated CORT levels increase foraging activity (Wingfield et al., 1990), locomotory activity (Breuner et al., 1998), food intake rate (Astheimer et al., 1992; Wingfield and Silverin, 1986), plasma glucose levels via gluconeogenesis (Holmes and Phillips, 1976; Remage-Healey and Romero, 2001) and lipogenesis (Gray et al., 1990), and fat deposition (Holberton, 1999). Thus, modulation of CORT levels plays an adaptive role in energy balance and acquisition, allowing birds to respond to food shortages and environmental perturbation

(Wingfield et al., 1997). Paradoxically, elevated CORT levels are traditionally viewed to have a negative impact on reproduction in general (Greenberg and Wingfield, 1987); for example, in birds, elevated CORT levels have been associated with abandonment of reproduction (Silverin, 1986; Wingfield and Silverin, 1986). It has therefore been suggested that birds should adaptively down-regulate CORT secretion to reduce the chance of nest abandonment when investment in offspring is substantial (chick rearing) or when the probability of reneating is low (Breuner et al., 2003; Pereyra and Wingfield, 2003; Silverin, 1986; Wingfield et al., 1995). However, the issue of how parents physiologically modulate the effects of elevated CORT (which may be beneficial during offspring rearing, but may increase the chances of offspring desertion) to avoid a behavioral-mediated reproductive conflict has never been addressed.

There are many downstream factors such as the level of corticosteroid-binding globulins, receptor number and location, and second messenger signaling which affect the behavioral and physiological outcome of an increase in CORT levels (Breuner and Orchinik, 2002; Breuner and

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Wingfield, 2000). Corticosteroid-binding globulin (CBG) binds CORT with high affinity in the plasma of birds (Breuner and Orchinik, 2001; Breuner et al., 2003; Lynn et al., 2003) and mammals (Westphal, 1971) and regulates the bioavailability of 'free', unbound steroids to tissue (Siiteri et al., 1982; reviewed in Breuner and Orchinik, 2002). Since it is potentially the unbound proportion of the hormone titer which is therefore the biologically relevant fraction, analysis of free (incorporating levels of CBG), rather than total CORT, may provide a better understanding of the role of CORT during reproduction.

Although current research in birds suggests that a CORT-mediated behavioral conflict should exist during reproduction, it is clear that birds have a mechanism(s) to modulate their response to CORT to minimize the chances of this occurring. To investigate this, we examined CORT and CBG levels in female European starlings (*Sturnus vulgaris*) during reproduction at a wild nest-box-breeding colony. Here, we show that free CORT levels in breeding female starlings are dramatically different from the total CORT pattern commonly reported for birds in previous studies, with chick-rearing starlings exhibiting the highest free baseline CORT levels. Furthermore, we show that this rise in free baseline CORT levels is not due to increased total CORT secretion, but rather to a decrease in CBG levels. Importantly, we show that although free baseline CORT levels are higher in abandoning vs. nonabandoning birds within all reproductive stages, chick-rearing birds have the lowest rates of abandonment. Overall, these results suggest that CORT and CBG interact to modulate the potentially conflicting behaviors of raising and desertion of offspring during reproduction. Together with mounting evidence in reptiles and amphibians (reviewed in Moore and Jessop, 2003), we propose that elevated CORT levels may be an inherent and necessary component of reproduction in non-mammalian tetrapods.

Materials and methods

Study site and species

This research was carried out at the Pacific Agri-Food Research Center (PARC) in Agassiz, British Columbia, Canada (49°14'N, 121°46'W), under a Simon Fraser University animal care permit (499B), following guidelines of the Canadian Council on Animal Care. The site consists of approximately 175 nest boxes on farm buildings and telephone poles that were used each year by breeding starlings. All boxes were checked daily to determine dates of clutch initiation and clutch completion and the laying sequence of eggs and growth of chicks. Starlings at our field site generally lay four to six eggs per clutch, incubate for 10–11 days and fledge chicks 20–24 days following hatching (F. Vézina, unpublished data).

Sampling and assessment of abandonment

We caught female starlings while they roosted in their nest box at night (generally between 2000 and 2300 h). Birds were blood sampled at the three-egg stage ($n = 35$), mid-incubation ($n = 14$) or early/mid chick rearing ($n = 8$) for analysis of CORT and CBG. All birds were blood sampled (ca. 200 μ l) from the wing vein within 2 min of capture, massed, metal- and color-banded, and their beak and tarsus measured to the nearest millimeter. There was no effect of time after capture (within 0- to 2-min intervals) on total and free baseline CORT levels in initial blood samples ($r^2 = 0.09$, $P = 0.56$ and $r^2 = 0.03$, $P < 0.68$, respectively). Thus, initial blood samples were considered to reflect baseline levels of corticosterone. Birds were then placed in a cloth bag and one additional blood sample was taken 30 min following capture (called stress-induced CORT levels) based on previous work on starlings (Romero and Remage-Healey, 2000). We returned birds to their nest boxes following sampling. We collected blood in heparinized capillary tubes that were immediately stored on ice, centrifuged within 2 h, with plasma frozen and stored at -20°C until further analysis. We monitored nest boxes for 3 days following blood sampling to assess nest desertion rates based on data collected over 7 years in our colony, indicating that laying birds have indeed deserted nests if they do not return after 2 days (Williams' lab, unpublished data). Furthermore, birds that do not return to incubated eggs or chicks within 1/2 a day lose embryos due to exposure to cold spring temperatures experienced at our colony (Williams' lab, unpublished data).

Measurement of CBG, total and free CORT levels

We determined total plasma levels of CORT with a direct radioimmunoassay (Wingfield et al., 1992) after extraction of 20- μ l samples in 4 ml of redistilled dichloromethane. Recoveries after extraction were 85–95% (measured for each sample independently and adjustments to the final assayed concentration made accordingly). Inter- and intra-assay variation was less than 7% and 9%, respectively.

Radioligand binding methods for measuring plasma CBG were based on those described in Deviche et al. (2001), and although CBG has not yet been cloned, radioligand binding is a well-documented and well-characterized method for identifying CBG levels (Breuner and Orchinik, 2002). Briefly, incubation time (2 h), temperature (4°C), rinse volume (9 ml ice-cold buffer) and plasma concentration (1:720) were optimized for starlings to maximum specific binding. All samples were run in triplicate, and bound and free radioligand were separated using rapid vacuum filtration (Brandel Harvester) over glass fiber filters (Whatman GF/B, soaked in 25 mM Tris with 0.3% PEI for 1 h before filtering). Following filtration, radioactivity bound to the filters was measured by standard liquid scintillation spectroscopy. The equilibrium binding parameters for the specific binding of ^3H -CORT were determined through equilibrium saturation

binding analysis of pooled female starling plasma which was incubated in the presence of ^3H -CORT at concentrations varying between 0.23 and 14 nM (Fig. 1). Affinity (Kd) estimates for European starlings were 3.46 ± 0.09 nM. Individual variation in CBG capacity was determined by incubating samples with 15.5 nM ^3H -CORT. A plasma standard was included in all CBG capacity assays, which yielded inter-assay coefficients of variation of less than 4%. Based on affinity estimates derived from equilibrium saturation analysis, this ligand concentration should occupy approximately 86% of total binding sites (B_{max}), so capacity values were increased to 100% for free CORT calculations. Free CORT titers were estimated using the equation of Barsano and Baumann (1989) outlined in Deviche et al. (2001).

Statistical analyses

Baseline and stress-induced CORT data were analyzed using analysis of variance (ANOVA) models. In addition, to examine possible interactions between breeding stage and nest desertion on CORT levels (free baseline, free stress-induced, total baseline and total stress-induced), separate ANOVAs were performed for each breeding stage. Where appropriate, we used unpaired students *t* test to examine differences in CORT levels between stages in relation to nest desertion. Finally, we used regression analysis to examine the potential relationship between CORT levels and Julian date and we used linear correlation analyses to examine the potential relationship between CORT/CBG levels and brood size. During statistical analyses, initial data were tested for homoscedastic assumptions required by a parametric statistical test according to Sokal and Rohlf (1995). At no time were any of these assumptions violated, and therefore data transformation was not necessary. Preliminary analysis indicated that CORT or CBG levels did not vary with Julian date and we therefore excluded this variable in subsequent analysis.

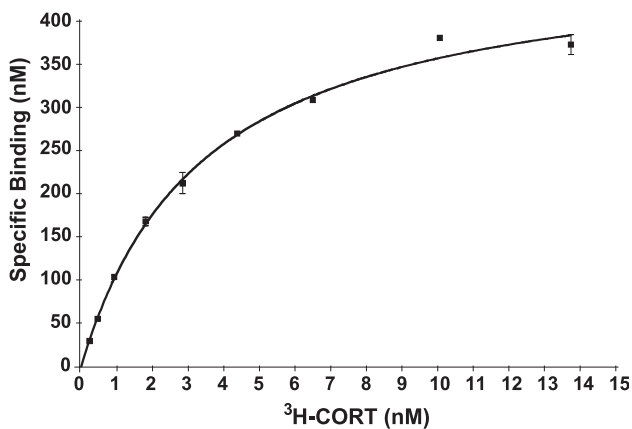


Fig. 1. Equilibrium saturation binding curve demonstrating specific binding of ^3H -CORT to European starling plasma (4°C) as a function of increasing concentrations of radiolabeled CORT. Points represent means \pm SEM.

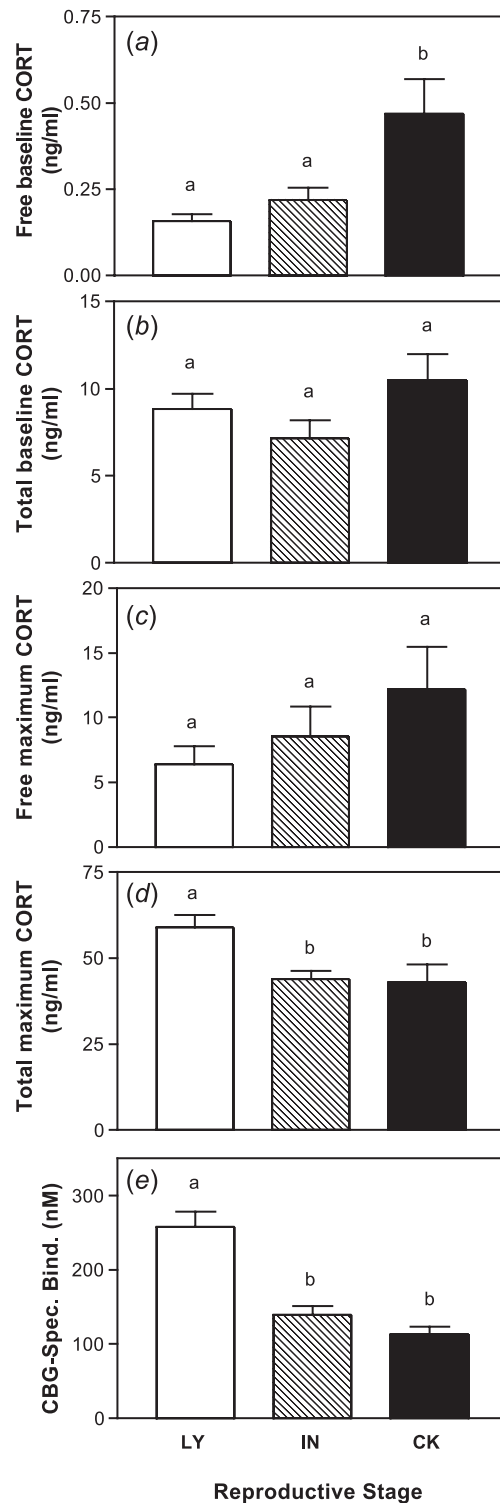


Fig. 2. Plasma levels of corticosterone (CORT) and corticosterone-binding globulin (CBG) during three reproductive stages in breeding female European starlings: laying (LY, $n = 35$), incubation (IN, $n = 14$) and chick rearing (CK, $n = 8$). (a) Free baseline CORT; (b) total baseline CORT; (c) free maximum CORT; (d) total maximum CORT; (e) CBG; mean \pm SEM [different letters represent significant ($P < 0.05$) difference between stages].

Results

Free baseline CORT levels were significantly higher in chick-rearing birds compared with laying and incubating birds [$F_{(2,48)} = 9.09$, $P < 0.001$, Bonferroni multiple comparison: $P < 0.001$; Fig. 2a], but total baseline CORT levels did not differ between stages ($P = 0.20$; Fig. 2b). Free stress-induced CORT levels did not differ significantly between stages ($P = 0.54$; Fig. 2c), whereas total levels were significantly higher in laying compared with incubating and chick-rearing birds [$F_{(2,48)} = 5.44$, $P < 0.01$, Bonferroni multiple comparison: $P < 0.001$; Fig. 2d]. The significant increase in free baseline levels was therefore not due to an increase in total baseline CORT, but rather to a significant drop in plasma CBG levels during reproduction [$F_{(2,48)} = 9.72$, $P < 0.0003$; Fig. 2e].

Chick-rearing birds had significantly lower rates of nest abandonment (14.2%) compared with laying (67.7%) and incubating birds (28.6%) [$F_{(2,48)} = 4.04$, $P < 0.05$, Bonferroni multiple comparison: $P < 0.05$]. We additionally analyzed CORT levels in relation to reproductive stage (laying/incubation/chick rearing) and nest abandonment (yes/no) using a two-factor nested ANOVA. Free baseline CORT was significantly higher in birds which abandoned nests following blood sampling within each reproductive stage [laying: $F_{(1,28)} = 4.60$, $P < 0.05$; incubation: $F_{(1,28)} =$

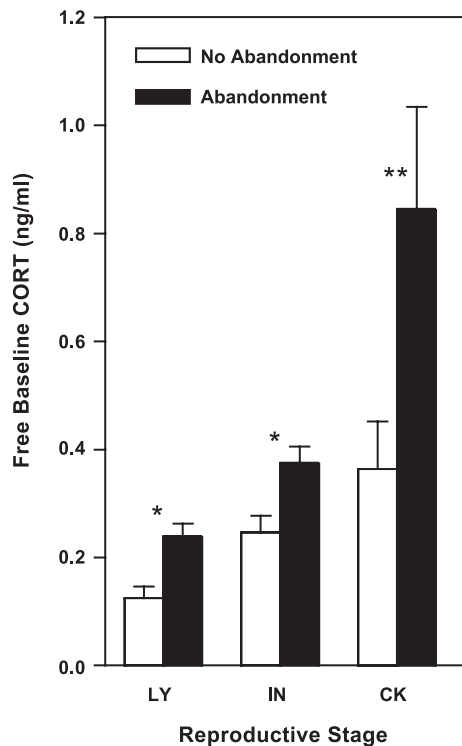


Fig. 3. Plasma levels of free baseline corticosterone (CORT) during three reproductive stages in breeding female European starlings in relation to nest abandonment following blood sampling [laying (LY, $n = 35$), incubation (IN, $n = 14$) and chick rearing (CK, $n = 8$); mean \pm SEM; * $P < 0.05$; ** $P < 0.01$ level of significance.

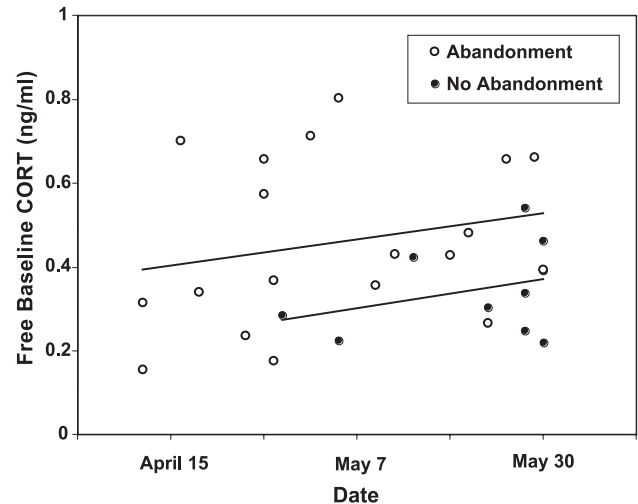


Fig. 4. Plasma levels of free baseline corticosterone (CORT) in laying female European starlings in relation to nest abandonment and Julian date ($n = 35$); mean \pm SEM.

5.96, $P < 0.04$; chick rearing: $F_{(1,28)} = 15.3$, $P < 0.01$; Figs. 3, 4]. Total baseline and total and free stress-induced CORT levels showed no such relationship ($P = 0.09$; $P = 0.2$ and $P = 0.3$, respectively) nor did CBG levels ($P = 0.96$). Most interestingly, free baseline CORT levels of chick-rearing birds which did not abandon nests were equivalent to levels of laying birds which did abandon (unpaired Student's t test: $P = 0.62$).

A total of 35 laying birds were sampled across 45 days (April 16th to May 29th) which encompasses the successful breeding season in our population. Within these birds (i.e., controlling for breeding stage), neither total or free baseline, nor total or free stress-induced CORT were related to Julian date (linear regression analyses: all $P > 0.05$; Fig. 4) and this trend was mirrored for CBG levels ($P = 0.18$). We are therefore confident that any patterns we observed in CORT

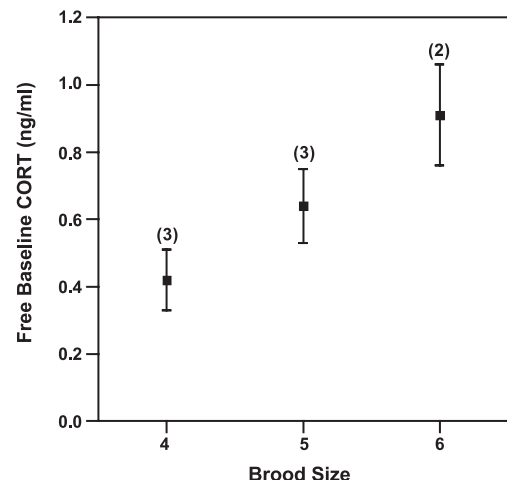


Fig. 5. Plasma levels of free baseline corticosterone (CORT) in chick-rearing adult female European starlings raising different numbers of nestlings (chicks aged 7–10 days); mean \pm SEM; sample sizes are located above each point.

levels were related to a breeding stage effect rather than to seasonal effects. Furthermore, there was a significant positive correlation between brood size of chick-rearing birds and the concentration of both total baseline ($r = 0.80$, $P = 0.034$) and free baseline CORT levels ($r = 0.73$, $P = 0.041$; Fig. 5, free CORT shown). There was no such relationship for CBG ($P = 0.28$).

Discussion

According to the traditional view of the roles of total CORT in birds, there should exist a conflict during reproduction because elevated CORT correlates with the increased demand of chick rearing but has also been linked to nest desertion. Based on this idea, chick-rearing birds should exhibit the lowest values to reduce the chance of nest abandonment when investment in offspring is substantial (chick rearing) or when the probability of re-nesting is low (Breuner et al., 2003; Pereyra and Wingfield, 2003; Silverin, 1986; Wingfield et al., 1995). However, this hypothesis fails to explain why parents would not exhibit elevated CORT to meet the energetic demands of raising offspring. We found that although chick-rearing female starlings had the highest levels of free baseline CORT during reproduction when accounting for the stage-related decrease in plasma CBG (previously reported in several passerine species: Deviche et al., 2001; Romero and Wingfield, 1998; Romero et al., 1998; Silverin, 1986), they also exhibited the lowest rates of nest abandonment through reproduction. Furthermore, our results indicate that during all breeding stages, birds that abandoned nests had higher free baseline CORT than birds that remained. Most interestingly, free baseline CORT levels of the chick-rearing female starlings that did not abandon nests were equivalent to those of laying birds that did abandon nests. We detected no breeding season decline in either free or total CORT in laying birds, reinforcing the fact that changes in CORT during reproduction were due to stage-related rather than seasonally related influences. We believe that the increase in free baseline CORT in chick-rearing birds may be adaptive in that it may increase foraging activity to meet the increased energetic demands of growing offspring. This is supported in our data by the fact that (1) free CORT levels remained low during laying and incubation, and only rose at the chick-rearing stage, and (2) there existed a positive relationship between brood size and both total and free baseline CORT levels in chick-rearing females, previously reported for total baseline CORT in Pied flycatchers (*Ficedula hypoleuca*) (Silverin, 1982).

In the passerine species examined to date, progesterone (P_4) binds CBG with equal or slightly higher affinity than CORT (Deviche et al., 2001). Although it is thought that P_4 levels are too low in birds to significantly interfere with free CORT estimations, we estimated potential interference with free CORT estimations using average P_4 levels from laying, incubating and chick-rearing starlings collected by Dawson

(1983) to calculate percentage of CBG bound by P_4 . The remaining, unbound CBG was then used in free CORT estimations, enabling an estimate of the alteration in free CORT levels resulting from P_4 competition at the globulin. We detected minimal percentage of changes in free CORT during the reproductive stages due to potential P_4 competition (laying: 12.5%, incubation: 4.5%, chick rearing: 6.4%) and this did not alter any of the relationships we originally detected with respect to free CORT and reproductive stages. We are therefore confident that P_4 levels do not significantly confound the results in this study. In addition, total androgen levels in breeding female starlings in our study population are low compared to total CORT levels (pg/ml vs. ng/ml; T.D. Williams, unpublished data) and CBG affinity for testosterone is 10 times lower than that for P_4 (Deviche et al., 2001). We are therefore also confident that testosterone levels do not significantly confound the results in this study.

We have shown that CORT and CBG interact to play a role in the modulation of a potential behaviorally mediated reproductive conflict in birds, a previously unapproached and unresolved issue in the study of corticosteroids in birds. The traditional view that elevated CORT levels are incompatible with reproduction has been based on two observations: (1) the correlation among inclement weather, highly elevated total plasma CORT and nest abandonment during laying (Astheimer et al., 1995; Wingfield et al., 1997), and (2) abandonment of offspring through exogenous pharmacological elevation of total plasma CORT (Silverin, 1982, 1986). Overall, our data indicate that chick-rearing female starlings are able to simultaneously modulate CORT-mediated energetic demands in conjunction with decreases in CBG without resulting in nest abandonment. Perhaps just as interesting is that while CORT-mediated decisions are being modulated within reproductive stages, birds are also modulating CORT during reproduction independently of Julian date (season). Within laying birds sampled across the entire breeding season, we detected no effects of date on either total or free CORT levels, indicating that the changes in CORT we found were related to breeding stage rather than season. Traditionally, researchers have reported a relationship between CORT and Julian date, with levels decreasing from breeding to molt and migration (Romero and Wingfield, 1999; reviewed in Romero, 2002); however, no studies have directly controlled for a particular breeding stage within this breeding season. The present results represent a further overall modulation of CORT, which reinforces the hypothesis that CORT is being actively modulated rather than just passively changing as the seasons progress.

The idea that the CORT sensitivity may be modulated at different life-history stages is not surprising given (1) the wide-ranging role and diverse actions of corticosteroids within biological systems (Harvey and Hall, 1990; Harvey et al., 1984; Siegel, 1980), and (2) the presence of glucocorticoid receptors in numerous avian tissue preparations (Breuner and Orchinik, 2001; Gould and Siegel, 1984; Orchinik et al., 2000; Saad and Moscona, 1985; Sabeur et al., 1993).

Moreover, the acute action of corticosteroids is not necessarily uniform within a species and the actions may be plastic, time- or context-dependent (Orchinik et al., 2000). Furthermore, modulation in the responsivity of the stress axis may influence behaviors that serve to adjust energy usage in a manner more appropriate for particular environmental or physiological states (Astheimer et al., 1995; Breuner and Hahn, 2003; Holberton, 1999; Wingfield, 1994). Our data indicate that as reproduction progresses, potentially the threshold for CORT-induced abandonment increases, possibly through increases or decreases in sensitivity to CORT via receptor modulation. This remains to be investigated in birds and will depend upon locating and quantifying glucocorticoid receptors in various tissues in combination with manipulating CORT during various reproductive stages.

Little attention has been paid to how endogenous CORT levels change through reproduction and how intraspecific variation of CORT levels relates to various adaptive behaviors. Together with mounting evidence in other taxa (reviewed in Moore and Jessop, 2003), we suggest that elevated corticosteroid levels are an inherent and necessary part of reproduction in nonmammalian tetrapods. Moreover, rather than assuming a direct relationship between total plasma corticosteroids and certain behaviors such as nest desertion, researchers should explore modulation of corticosteroids via binding globulins and receptors more closely. This will allow for the elucidation of the role of intraspecific variation in these hormones and potentially for the understanding of the evolution of endocrine-mediated reproductive conflicts.

Acknowledgments

We wish to thank John Wingfield for the generous use of his laboratory during sample analysis for corticosterone and Lynn Erkman for her assistance with sample analysis. We would also like to thank Haruka Wada and John Shyu for their extensive assistance during sample analysis of CBG and two anonymous reviewers for invaluable comments and contributions that greatly improved this work. Finally, we would like to thank Tom Scott and Lee Struthers from the Pacific Agri-Food Research Center for providing the field laboratory. This research was funded by an operating Natural Sciences and Engineering Research Council of Canada (NSERC) grant to T.D.W., a National Science Foundation (NSF) research grant (IBN-0202676) to C.W.B, a NSF research grant (OBN-9905679) to John Wingfield and postgraduate funding to F.V. from NSERC and Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR).

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