FREE-LIVING MALE MOUNTAIN WHITE-CROWNED SPARROWS EXHIBIT TERRITORIAL AGGRESSION WITHOUT MODULATING TOTAL OR FREE PLASMA TESTOSTERONE

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Abstract.   In some species, expression of territorial aggression is accompanied by a rise in testosterone secretion, but in others aggressive behavior is expressed while testosterone levels remain unchanged. Corticosteroid binding globulin (CBG) binds both corticosterone and testosterone in avian plasma. Thus, increasing corticosterone may result in fluctuations in unbound ("free") testosterone; this could result in greater biological activity of testosterone without an increase in testosterone secretion. We investigated whether such plasma interactions of testosterone, corticosterone, and CBG might result in alterations of free testosterone in male Mountain White-crowned Sparrows (*Zonotrichia leucophrys oriantha*). We conducted simulated territorial intrusions during incubation and compared total and free testosterone of males captured immediately following a simulated territorial intrusion with that of males captured passively. All experimental males showed aggressive behavior, but apparently did not modulate total or free testosterone relative to controls.

Key words: aggression, corticosteroid binding globulin, free hormone, simulated territorial intrusion, testosterone, Zonotrichia leucophrys oriantha.

Los Machos Silvestres de *Zonotrichia leucophrys oriantha* Exhiben Agresión Territorial sin Modular la Testosterona Plasmática Total o Libre

Resumen.   En algunas especies, la expresión de las agresiones territoriales es acompañada por un incremento en la secreción de testosterona, pero en otras especies que expresan agresividad territorial los niveles de testosterona se mantienen constantes. La globulina ligadora de corticosteroides (CBG) se liga tanto a la corticosterona como a la testosterona en el plasma de las aves. Así, el aumento de corticosterona puede resultar en testosterona no ligada ("libre"). Esto puede resultar en una mayor actividad biológica de la testosterona sin un aumento en la secreción de la testosterona. Investigamos si estas interacciones plasmáticas de la testosterona, corticosterona y la CBG pueden resultar en alteraciones de la testosterona libre en machos de *Zonotrichia leucophrys oriantha*. Realizamos intrusiones territoriales simuladas durante la incubación y comparamos la testosterona total y libre de machos capturados inmediatamente después de la intrusión territorial simulada con la de machos capturados pasivamente. Todos los machos experimentales mostraron comportamiento agresivo, pero aparentemente no modularon la testosterona total o libre con relación a los machos control.

Environmental and social cues are well known to elicit changes in circulating levels of steroid hormones in birds (Wingfield 1985, 1990, Beletsky et al. 1992, Wingfield and Farner 1993). Total levels of plasma steroids are often assumed to represent changes in physiological activity at the level of hormone receptors; however, growing evidence indicates that the fraction of steroid in plasma that is reversibly bound to a steroid-specific binding globulin may have different physiological activity than the fraction that is unbound, or free. The "free hormone hypothesis" suggests that only unbound hormone is available to enter target tissues and bind to receptors, or be cleared by the liver (Mendel 1989, Ekins 1990). Although additional evidence indicates potential alternative roles for steroid-specific binding globulins (Nakhla et al. 1988, Strel'chyonok and Avvakumov 1991; reviewed by Breuner and Orchinik 2002), support for the free hormone hypothesis is widespread (Siiteri et al. 1982, Rosner 1990, Bright 1995, Hammond 1995, Breuner and Orchinik 2002, Gozansky et al. 2005), suggesting that the free fraction of hormone in the plasma is likely the primary mediator of hormone action. Consequently, in this study we focus on the physiological relevance of the free fraction of hormone based on predictions of the free hormone hypothesis.

Two main steroid binding globulins have been identified in vertebrates: sex-hormone binding glob-

ulins (SHBG), which bind both androgens and estrogens, and corticosteroid binding globulin (CBG), which binds glucocorticoids (Hammond 1990). Surprisingly, a sex-steroid-specific binding globulin has not been identified in birds and, until recently, biologists assumed that testosterone and estradiol circulated unbound in the plasma (Wingfield et al. 1984). However, Deviche et al. (2001) recently identified avian CBG as a biologically relevant testosterone binding globulin. They demonstrated that CBG has a relatively high affinity for testosterone and may bind 90%–95% of circulating testosterone under unstressed conditions. Thus, in birds, CBG may function as a regulator of both free corticosterone and free testosterone.

Because avian CBG binds corticosterone and progesterone with higher affinity than it binds testosterone (Deviche et al. 2001), several scenarios may result in a dramatic release of testosterone from CBG. For example, an increase in corticosterone secretion, an increase in progesterone secretion, or a decrease in CBG levels might result in a rapid increase in free testosterone. Resulting increases in free testosterone might have important behavioral consequences for free-living birds. For example, a surge in circulating free testosterone might significantly increase testosterone availability to receptors in the brain, and thus either initiate rapid changes in behavior (e.g., as a result of membrane receptor binding; Shakil et al. 2002, Remage-Healey and Bass 2005a, 2005b), or influence existing neural circuits and thus enhance future behavioral responses in appropriate contexts (Wingfield et al. 1987). Alternatively, a surge in free testosterone might speed the metabolic clearance of testosterone from the plasma, thus quickly reducing circulating testosterone levels under certain circumstances.

Such plasma interactions of sex steroids, corticosterone, and CBG might be especially relevant to a free-living bird during aggressive encounters. Testosterone plays an important role in the initiation and maintenance of reproductive behavior (Balthazart 1983, Harding 1983) and is generally associated with the expression of aggressive behavior in temperate-zone birds (Balthazart 1983, Wingfield et al. 1990, Wingfield and Farner 1993). However, patterns of plasma testosterone and expression of aggressive behavior vary among species. In males of some species, expression of aggressive behavior is accompanied by a rise in testosterone secretion (Wingfield 1985, Wingfield and Wada 1989, Wingfield et al. 1990, Wingfield and Hahn 1994), but in others, aggressive behavior is expressed while testosterone levels remain unchanged (Romero et al. 1998, Meddle et al. 2002; see also Wingfield and Hunt 2002).

Several hypotheses exist to explain the uncoupling of aggressive behavior and increased testosterone secretion, suggesting this may reflect differing ecological pressures, such as a short breeding season or the need for a smooth transition to caring for young (Wingfield and Hunt 2002). However, these studies assumed that the lack of increase in testosterone secretion reflected no overall increase in testosterone action. That is, these studies assessed only total circulating testosterone levels, and did not account for fractions that were free or bound to CBG. Thus, the possibility remains that testosterone *availability to receptors* may be modulated by additional mechanisms, e.g., at the level of binding globulins. We suggest that the interaction of steroid hormones and binding globulins in circulation may result in modulation of *free* testosterone, potentially eliciting changes in behavior independent of changes in testosterone secretion.

Because avian CBG binds corticosterone and progesterone with similarly high affinity (Deviche et al. 2001), changes in plasma progesterone or corticosterone could potentially elicit changes in free testosterone. Progesterone has been implicated in the activation of aggressive behavior in reptiles (Weiss and Moore 2004) and mammals (Davis and Marler 2003), but evidence for fluctuations of plasma progesterone following a territorial challenge in birds is scant. One study of female Song Sparrows (*Melospiza melodia*) found no change in plasma progesterone following simulated territorial intrusion during pre-breeding, breeding, or molt (Elekonich and Wingfield 2000). We are unaware of similar published studies of male birds. In addition, a study of breeding European Starlings (*Sturnus vulgaris*) demonstrated that circulating progesterone levels were not high enough to significantly alter estimations of circulating free corticosterone (Love et al. 2004). In contrast, a variety of environmental stimuli are well known to elicit dramatic and rapid increases in corticosterone in birds (Sapolsky et al. 2000). Deviche et al. (2001) estimated that in Dark-eyed Juncos (*Junco hyemalis*), a rapid increase in corticosterone would dramatically increase free testosterone. Thus, although the role of progesterone in aggressive encounters in male birds is an interesting avenue for future study, we focused the present study on interactions of corticosterone, testosterone, and CBG in regulating free testosterone levels.

We investigated the expression of aggressive behavior and modulation of free and total testosterone in male Mountain White-crowned Sparrows (*Zonotrichia leucophrys oriantha*) following simulated territorial intrusion (hereafter also referred to as "territorial challenge"). Mountain White-crowned Sparrows breed at mid-latitudes but are constrained to a relatively short breeding season due to their high altitude breeding grounds (Morton 2002). Hence, current hypotheses would predict no increase in testosterone secretion during aggressive encounters (Wingfield and Hunt 2002). We subjected male Mountain White-crowned Sparrows to territorial challenges early in the breeding season and recorded aggressive behavior. We also measured plasma testosterone, corticosterone, and CBG levels following territorial challenge and compared these to levels in free-living males captured without territorial challenges (controls). With these data, we can assess if aggressive behavior during a territorial challenge is accompanied by alterations in biological activity of testosterone through: (1) modulation of testosterone secretion, or (2) elevations in corticosterone secretion, leading to rapid changes in circulating free testosterone.

METHODS

STUDY POPULATION AND STUDY AREA

Our study population of White-crowned Sparrows breeds near Tioga Pass, Mono County, California ($\sim$37°30′N, $\sim$3000 m elevation). We captured males ($n = 19$) along Lee Vining Creek and the neighboring Tioga Pass Meadow between 15 and 24 June 2003, when the majority of females in the population were incubating eggs. All males not banded previously were banded with an aluminum U.S. Fish and Wildlife Service band and colored plastic leg bands for identification.

SIMULATED TERRITORIAL INTRUSIONS AND BLOOD SAMPLING

Nests of all eight experimental males were located at least one day prior to testing. Experimental males were subjected to a territorial challenge in which a caged conspecific male (decoy) and a loop recording of male Mountain White-crowned Sparrow song were placed within 5 m of the nest. This protocol (described by Wingfield and Ramenofsky [1985]) has been widely used as a standardized method for assessing aggressive behavior in birds. To avoid pseudoreplication we used two decoys and songs recorded from three different males for territorial challenges. Assignment of a decoy and song to each challenge was randomized. During the first 5 min of the challenge, we recorded the time it took the resident male to approach the decoy (latency), the closest approach the resident male made to the decoy, and the frequency of three behaviors: number of low flights over the decoy (within 1–2 m vertical distance), number of songs, and number of trills. Songs and flights toward an intruder have been widely recognized as aggressive behaviors among passerines, and trills made by White-crowned Sparrows during male–male encounters are also assumed to relate to aggression (Petrinovich and Patterson 1981).

After 5 min, we stopped recording the male's behavior, and quickly set up a mist net to capture the responding male. We continued the territorial challenge during this time. At capture we collected blood samples (approximately 250 µl) in heparinized microhematocrit capillary tubes following puncture of the alar vein with a 26-gauge needle. The first 50 µl (obtained within 3 min of capture) were collected for the corticosterone assay to ensure that plasma corticosterone levels were not elevated due to capture stress (Wingfield et al. 1982). The remaining volume (obtained within 10 min) was collected for the CBG and testosterone assays. Plasma was separated by centrifugation, and stored at $-20°$C until assay.

BLOOD SAMPLING FROM CONTROL MALES

Eleven male Mountain White-crowned Sparrows, designated as controls, were captured using Potter traps baited with seed. Thus, males were captured when they were foraging, and not while engaged in a territorial interaction. Blood samples were collected in the manner described above. The initial 50 µl of blood (obtained within 3 min of our approach to the trap) was collected for corticosterone assay. The remainder of the blood sample (collected within 10 min of our approach) was designated for testosterone and CBG analysis. For two of the control males, corticosterone samples were not obtained within 3 min, and consequently did not represent baseline corticosterone levels (Wingfield et al. 1982). Thus, we only report total testosterone levels for these two birds.

HORMONE ASSAYS

Plasma hormone levels were evaluated using enzyme immunoassay kits from Assay Designs (Ann Arbor, Michigan). Corticosterone and testosterone assays were optimized for Mountain White-crowned Sparrow plasma. Briefly, pooled plasma from breeding Mountain White-crowned Sparrows was stripped of endogenous steroid using dextran-coated charcoal. Stripped plasma was spiked with hormone (to 200 pg ml^{-1} corticosterone or 125 pg ml^{-1} testosterone) and serially diluted (1:10, 1:20, 1:40, and 1:80) with assay buffer of the same hormone concentration. Two dilution series were prepared: one with steroid displacement buffer (Assay Designs; designed to eliminate interference of binding globulins with antibody binding in the assay), and one without. Thus, all dilutions contained the same concentration of hormone, but other plasma components were diluted in a regular way. All eight plasma dilutions (1:10, 1:20, 1:40 and 1:80, with and without steroid displacement buffer) and an external standard were then assayed in triplicate. This allowed us to determine which plasma dilutions allowed for accurate measurement of steroid without extraction. This plasma dilution was subsequently used for samples in the assay. For a more detailed description of the optimization process, see Wada et al. (in press).

Corticosterone assay. Prior to the assay, plasma was incubated for 10 min with steroid displacement buffer (1% of raw plasma volume, diluted 1:100 before addition to plasma). Treated plasma was then diluted 1:20 with assay buffer for a final dilution of 1:40 in the assay. 100 µl of diluted, treated plasma was assayed in triplicate. Sensitivity was 0.6 ng ml^{-1}, and the intra-assay coefficient of variation was 7%. Cross-reactivity of the corticosterone antibody was as follows: testosterone $<$1%, progesterone $<$1%, and estradiol $<$1% (Assay Designs).

Testosterone assay. Plasma was run at a 1:20 dilution. Steroid displacement buffer was not used in this assay, as optimization studies determined binding globulins did not interfere with binding at a 1:20 dilution (M. Swett and CWB, unpubl. data). Sensitivity was 0.4 ng ml^{-1}, and the intra-assay coefficient of variation was 5%. Cross-reactivity of the testosterone antibody was as follows: androstenedione 7.2%, estradiol $<$1%, dehydroepiandrosterone $<$1%, dihidrotestosterone $<$1%, and progesterone $<$1% (Assay Designs).

CORTICOSTEROID BINDING GLOBULIN ASSAY

For both competition and individual point sample analysis, assays were performed as described in Breuner and Orchinik (2001). Briefly, plasma samples

were charcoal stripped, and incubated with [³H]-corticosterone for 2 hr at 4°C. Final plasma dilution was 1:900. Bound and free fractions were separated by vacuum filtration over glass-fiber filters soaked in a polycationic solution. To determine K_i (the inhibition constant, an estimate of affinity), competition assays were completed using pooled White-crowned Sparrow plasma incubated with 1.5 nM [³H]-corticosterone and 0.1 nM − 1 μM unlabelled steroid (either corticosterone or testosterone). To estimate individual CBG capacity (point sample analysis), stripped plasma samples were incubated with 20 nM [³H]-corticosterone with and without 1 μM corticosterone to determine nonspecific binding. To avoid interassay variation, all samples were run at once. Based on affinity estimates for Mountain White-crowned Sparrows (Breuner et al. 2003; K_d [dissociation constant] = 3.7 ± 0.3 nM), 20 nM [³H]-corticosterone should occupy ~91% of total binding sites.

FREE HORMONE CALCULATIONS

We estimated free hormone levels using the equation of Barsano and Baumann (1989), rather than measuring free hormone directly. Recent studies in mammals have demonstrated a high correlation between direct free corticosterone measures and estimations of free corticosterone (Taymans et al. 1997, Adcock et al. 2006). Whereas direct measures of free corticosterone (e.g., by ultrafiltration) account for albumin-bound hormone, albumin's role in binding corticosterone is not incorporated into Barsano and Bauman's (1989) model for estimating free hormone. However, because the affinity of albumin for corticosterone is quite low, corticosterone that is bound to albumin is considered biologically active (Taymans et al. 1997). Thus, estimations of free hormone made using Barsano and Bauman's (1989) equation may provide a more biologically relevant measure of free hormone than direct measurement by ultrafiltration.

Free corticosterone titers were estimated using total hormone, CBG capacity, and the dissociation constant (K_d) of corticosterone for CBG (Barsano and Baumann 1989). Individual CBG capacity estimations for corticosterone represent approximately 91% of B_{max} (i.e., the total binding capacity of CBG), so capacity values were increased to 100% for free corticosterone calculations.

Free testosterone was estimated using the percent CBG not bound by corticosterone. There is presently no equation to determine percent hormone bound to binding globulin for two hormones concurrently. Hence, it is necessary to calculate percent CBG saturated by the higher affinity ligand, and then use the remaining capacity available for the second ligand (Deviche et al. 2001, Zysling et al. 2006). Testosterone has approximately seven-fold lower affinity for CBG than corticosterone (Fig. 1). Therefore, free corticosterone was determined first, and free testosterone estimates were calculated using total testosterone, CBG available after corticosterone had bound, and estimates of the affinity of testosterone for CBG (K_i = 9.1 nM for Mountain White-crowned Sparrow; Fig. 1).
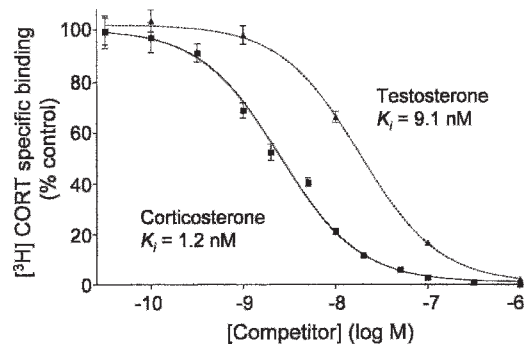

FIGURE 1. Competition curves demonstrating specific binding (as a percentage of control) of [³H]-corticosterone ([³H] CORT) as a function of increasing concentrations of unlabeled corticosterone (squares and solid line) or testosterone (triangles and dotted line). The resulting K_i (inhibition constant) for each curve is an estimate of the affinity of CBG for corticosterone or testosterone, and indicates that affinity for corticosterone is higher than affinity for testosterone. Points indicate mean ± SE of triplicate samples at each concentration.

STATISTICAL ANALYSES

Binding parameter estimates from the competition analyses were obtained by fitting untransformed data to appropriate equations using iterative, least-squares curve-fitting techniques (GraphPad Prism 2005). We log-transformed all hormone data to stabilize variances and conducted unpaired t-tests to compare data for control and experimental males. For experimental males, we conducted simple linear regressions to compare hormone levels (free and total) to the length of the territorial challenge. All statistical tests were conducted using Statview version 5.0.1 for Macintosh (SAS Institute 1999). Values reported are means ± SE, and the significance level of tests was set at $P < 0.05$.

RESULTS

BEHAVIORAL RESPONSE TO SIMULATED TERRITORIAL INTRUSION

All eight of the experimental males responded to simulated territorial intrusion with aggressive behavior. Average latency to respond was 36 ± 14 sec (range = 1–111 sec, mode = 1 sec). All but one male engaged in low flights over the decoy (mean = 3.6 ± 0.6 flights, n = 8). On average, males approached the decoy to within 4.8 ± 2.3 m during the first 5 min of playback, and resident males responded to simulated territorial intrusion with 17.3 ± 3.7 songs and 4.6 ± 2.3 trills.

COMPETITION ANALYSIS

Mountain White-crowned Sparrow plasma bound both corticosterone and testosterone with high affinity. Competition analysis gave a K_i (inhibition
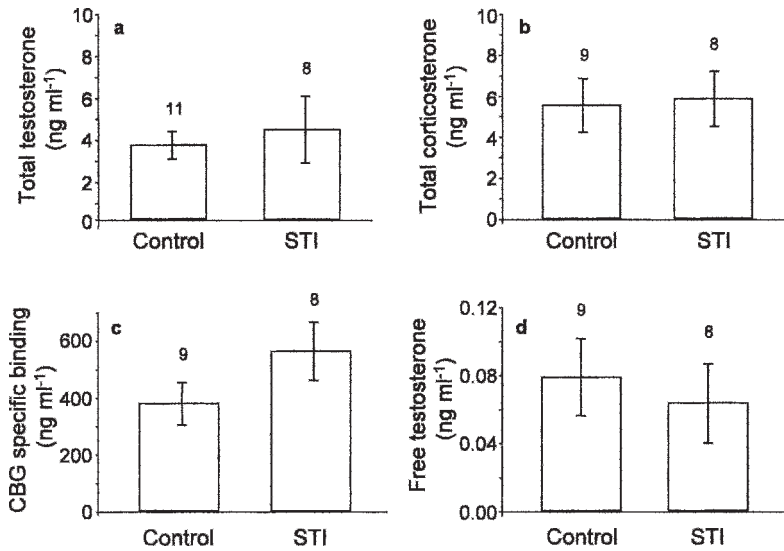
FIGURE 2. Breeding male Mountain White-crowned Sparrows captured either passively (control) or following a simulated territorial intrusion (STI) showed no differences in total plasma testosterone (a), total plasma corticosterone (b), corticosteroid binding globulin, CBG (c), or free testosterone (d) levels. Bars indicate mean ± SE. Sample sizes are indicated above the bars.

constant) of 1.2 ± 0.1 nM for corticosterone and 9.1 ± 1.6 nM for testosterone (Fig. 1).

HORMONE LEVELS

Experimental males did not increase plasma testosterone in response to territorial challenge. Total levels of plasma testosterone did not differ between experimental males following territorial challenge and control males captured in Potter traps (unpaired t-test, $t_{17} = 0.1$, $P = 0.91$; Fig. 2a). Similarly, total plasma corticosterone levels did not differ between experimental and control males (unpaired t-test, $t_{15} = -0.1$, $P = 0.89$; Fig. 2b). Experimental males showed a trend toward greater CBG capacity relative to controls (unpaired t-test, $t_{15} = -2.0$, $P = 0.06$; Fig. 2c). Calculated values of free corticosterone and free testosterone did not differ between groups (unpaired t-tests, free corticosterone: $t_{15} = 0.9$, $P = 0.41$; free testosterone: $t_{15} = 0.8$, $P = 0.42$; Fig. 2d).

Among experimental males ($n = 8$), the duration of territorial challenges ranged from 6.3 to 26.5 min. Challenge duration was not related to levels of total plasma testosterone ($r^2 = 0.001$, $P = 0.95$), total plasma corticosterone ($r^2 = 0.2$, $P = 0.17$), free testosterone ($r^2 = 0.0001$, $P = 0.98$), or free corticosterone ($r^2 = 0.2$, $P = 0.20$). Additionally, CBG capacity did not relate to the length of the intrusion ($r^2 = 0.02$, $P = 0.78$).

DISCUSSION

Male Mountain White-crowned Sparrows responded rapidly and aggressively to simulated territorial intrusions. Although we did not measure behavior in the absence of a territorial challenge in our control males, the experimental males exhibited behaviors commonly associated with aggression, and rates of expression of these behaviors were similar to those documented in other studies (Petrinovich and Patterson 1981, Chilton et al. 1995).

We investigated two specific mechanisms by which male Mountain White-crowned Sparrows might modulate testosterone bioavailability following a territorial challenge: (1) direct modulation via increased testosterone secretion, and (2) indirect modulation via increased corticosterone secretion leading to an increase in free plasma testosterone. Although males in our study responded aggressively to territorial challenges, we did not find evidence for either mechanism. Interestingly, although the difference was not significant, there was a trend toward greater CBG capacity in experimental males relative to controls. However, plasma CBG levels did not relate to duration of simulated territorial intrusions, suggesting that this trend may be explained by individual differences in plasma CBG prior to territorial challenge and not by rapid changes in CBG in response to territorial challenge. Collectively, our results indicate that the response of males to territorial challenges and plasma testosterone levels were not positively associated during the early breeding season in this population.

Prior studies of other White-crowned Sparrow subspecies have indicated that testosterone and aggression may be uncoupled in severe environments when time to breed is limited. The low elevation, temperate-breeding subspecies (Z. l. pugetensis) modulates testosterone in response to territorial challenges (Wingfield and Hahn 1994), whereas the arctic-breeding subspecies (Z. l. gambelii) does not (Meddle et al. 2002). Our total testosterone data

support this pattern, as *Z. l. oriantha* breed in high-altitude meadows where weather can be severe and second broods are uncommon (Morton 2002).

Whereas ecological factors associated with a high-altitude breeding environment may constrain testosterone response in male Mountain White-crowned Sparrows, the possibility remains that plasma interactions of testosterone, corticosterone, and CBG may function to modulate free testosterone levels in other species. For example, testosterone and corticosterone are known to bind to the same binding globulin in at least one other bird species (Dark-eyed Junco; Deviche et al. 2001), an amphibian (Orchinik et al. 2000), a reptile (Jennings et al. 2000), and humans (Brien 1981, Cooke et al. 1996). Though few studies have simultaneously measured testosterone and corticosterone following a territorial challenge, recent work by Van Duyse et al. (2004) indicated that male Great Tits (*Parus major*) subjected to simulated territorial intrusions had higher corticosterone and lower testosterone following a territorial challenge than did controls at three stages of the breeding season. Within this framework, an increase in corticosterone would displace testosterone from CBG in the plasma, resulting in a greater tissue level of testosterone that could facilitate expression of aggressive behavior in appropriate contexts, followed by rapid clearance of testosterone from the bloodstream. Van Duyse et al. (2004) did not measure CBG levels, and thus could not calculate free testosterone; however, the decline in total testosterone they report could be explained by rapid clearance of free testosterone from the blood following a rise in corticosterone. In addition, increased corticosterone secretion has been documented during reproductive aggression in at least one other species (Northern Pintails [*Anas acuta*]; Sorenson et al. 1997, but see Silverin 1993, Wingfield and Lewis 1993). Thus, the hypothesis that plasma interactions of testosterone, corticosterone, and CBG may regulate testosterone activity at target tissues remains an intriguing possibility. Furthermore, additional factors such as fluctuations in other steroids (e.g., progesterone) may also be important in mediating aggressive responses during the breeding season. Without simultaneously measuring multiple factors following territorial challenges, such mechanisms may be easily overlooked.

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