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Testosterone, Corticosterone, and Photoperiod Interact to Regulate Plasma Levels of Binding Globulin and Free Steroid Hormone in Dark-Eyed Juncos, *Junco hyemalis*

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The pharmacology and regulation of corticosteroid binding globulins (CBG) in Dark-eyed Juncos, *Junco hyemalis*, was investigated. The equilibrium dissociation constant for [^{3}H]corticosterone (CORT) binding to plasma was < 5 nM. This binding site had a similar high affinity for progesterone, approximately fivefold lower affinity for androgens, and negligible affinity for estradiol. The following data suggested that plasma CBG levels are regulated by both testosterone and day length: (1) CBG binding capacity in free-living adult males was greater in early than in late breeding season and greater in males than in females and (2) CBG levels were higher in testosterone-treated, castrated males than in castrated males receiving no testosterone and still higher in testosterone-treated males exposed to long days than in similar males exposed to short days. Birds apparently lack a sex steroid-specific binding globulin, but it was estimated that more than 90% of testosterone in junco plasma should bind to CBG. An increase in plasma CORT, such as occurs during a stress response, was judged to acutely increase free testosterone levels as much as fivefold. Corticosterone and testosterone may thus interact in a complex manner in species that lack sex hormone binding proteins.

Key Words: androgen; castration; CBG; Dark-Eyed Junco; seasonality; sex hormones; transcortin.

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INTRODUCTION

In plasma, steroid hormones can bind reversibly to binding globulins (BG) with high affinity and to albumin with low affinity. Steroid binding proteins in blood regulate the bioavailability of free steroids to tissues and the metabolic clearance rate of these steroids (Siiteri *et al.*, 1982). There are also additional potential roles for BGs (see Fortunati, 1999; Hammond, 1995). For example, cell membrane receptors for BGs have been identified and partially characterized, and some effects of steroids on target cells may result from their interaction with membrane-bound BGs (Maitra *et al.*, 1993; Nakhla *et al.*, 1999; Rosner *et al.*, 1999). BGs may also control the delivery of steroids into some target cells (Hammond, 1995). Thus, BGs probably play an active, rather than only a passive, role in mediating effects of circulating steroid hormones. Studying the mechanisms that regulate the production and binding characteristics of these proteins is warranted to fully understand the cellular actions of steroids in general.

The plasma of several avian species contains a corticosteroid binding protein (CBG; Assenmacher *et al.*, 1975; Gould and Siegel, 1978; Klukowski *et al.*, 1997; Kovacs and Peczely, 1983; Silverin, 1986; Wingfield *et al.*, 1984), but the mechanisms that

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regulate CBG production in birds are not well known. Corticosterone (CORT) binding protein capacity did not differ between breeding and molting (postbreeding) Snow Buntings, *Plectrophenax nivalis* (Romero *et al.*, 1998a). In contrast, Pekin ducks, *Anas platyrhynchos* (Assenmacher *et al.*, 1975; Daniel *et al.*, 1981), Pied Flycatchers, *Ficedula hypoleuca* (Silverin, 1986), White-crowned Sparrows, *Zonotrichia leucophrys* (Romero and Wingfield, 1998), and Lapland Longspurs, *Calcarius lapponicus* (Romero *et al.*, 1998b) had a higher CBG binding capacity at the beginning than at the end or after the breeding season. Male ducks and Pied Flycatchers also have a higher binding capacity than conspecific females (Daniel *et al.*, 1981; Silverin, 1986). Circulating testosterone (T) of gonadal origin may regulate these season- and sex-related differences since plasma T levels are generally higher in males than in females, and CBG binding capacity decreases after castration and increases following T administration (Assenmacher *et al.*, 1975; Daniel *et al.*, 1984; Klukowski *et al.*, 1997; Silverin, 1986).

In the birds studied, CBG binds glucocorticoids and progesterone with high affinity and T and estradiol with lower affinity (Wingfield *et al.*, 1984). Since bird plasma apparently does not contain high-affinity sex hormone binding proteins (SHBG; Wingfield *et al.*, 1984) similar to those found in other vertebrate classes, it is assumed that, in adults birds, T circulates in the plasma either unbound or bound to albumin with very low affinity. However, embryonic chicken blood contains proteins that bind T and CORT with equally high affinity (Savu *et al.*, 1986). Similarly, T and CORT can bind to the same plasma BG in an amphibian (Orchinik *et al.*, 2000) and in a reptilian (Jennings *et al.*, 2000) species. Therefore, CBG-like molecules may bind physiological concentrations of gonadal androgen also in birds.

The current study examined the potential regulation of CBG levels in Dark-eyed Juncos, *Junco hyemalis*, by T and photoperiod. In addition, an attempt was made to resolve issues related to the presence of gonadal steroid binding proteins in avian blood. Specifically, two possibilities were investigated: (1) junco plasma contains a gonadal steroid hormone-specific globulin and (2) a CBG-like molecule can bind a significant percentage of circulating androgen.

MATERIALS AND METHODS

Subjects and Plasma Sample Collection

Dark-eyed Juncos from a local population in Fairbanks (Alaska; 64°50′N, 147°50′W) were captured by use of Japanese mist nets and seed-baited Potter traps. Birds were sampled in the field and released at the capture site or brought into the laboratory (see below). Blood (approximately 200 μl) was collected from a wing vein into heparinized microhematocrit tubes within minutes of capture or removal from a trap. Tubes were sealed at one end, kept on ice, and within hours were centrifuged at 4° for 10 min. Plasma was stored at −20 or −80° until processed.

Castration and T Replacement in Short or Long Day-Exposed Males

Twenty-seven hatching-year male Dark-eyed Juncos were caught in September 1997 and housed in groups of 8–12 in indoor flight cages, together with juncos that were part of a separate study. Birds were exposed to short days (SD; 8L:16D; lights on at 0800 h AST) until March 11, 1998, when they were then adults. They were moved to individual cages in which they were visually, but not acoustically, isolated from each other. All individuals were then castrated under general anesthesia (Methoxyflurane inhalation; Metofane; Pitman-Moore Inc., Mundelerin, IL) between March 18 and 20. Birds then either remained exposed to SD ($n = 15$) or were transferred to a photostimulating light regime (LD; $n = 12$). Juncos belonging to the LD group were exposed to gradually increasing day length by the addition of 1 h of light per day until the photoperiod reached 20 h (lights on at 0400 h AST). On March 26, 8 SD and 5 LD males (SD-T; LD-T) were treated with two subcutaneous T-filled Silastic capsules. Each capsule consisted of a 10-mm-long piece of Silastic tubing (Konigsberg Instruments, Inc., Pasadena, CA; internal diameter, 1.5 mm; external diameter, 2 mm) filled with crystalline hormone (Sigma Chemical Co., St. Louis, MO) and sealed with silicone adhesive (Dow Corning, Midland, MI). Capsules were incubated in 0.9% NaCl solution at 37° for 24 h prior to implantation to initiate release of the steroid. The remaining males (SD-C, $n = 7$; LD-C, $n = 7$) received control (empty) capsules. A blood

sample was collected from each male 32 or 33 days after administration of the capsules. A portion of each sample was used to determine plasma T concentrations and the remainder to measure ^{3}H-CORT binding activity. All birds were killed on May 6 or 7 and at that time, body cavities were examined to ensure that castrations were complete. Throughout the study juncos received Mazuri parrot and small bird pelleted food (PMI Nutrition Int., St. Louis, MO) and Avi-Con vitamin-treated water (Vet-A-Mix Inc., Shenandoah, IA) *ad libitum*. The Institutional Animal Care and Use Committee of the University of Alaska Fairbanks approved all experimental procedures.

Binding Assays

Binding experiments used either individual plasma samples or pools of plasma from several birds and, unless specified otherwise, were performed using steroid-free plasma. Endogenous steroids were eliminated from plasma (Wingfield *et al.*, 1984). Specifically, individual plasma samples (15 μl) were diluted 20 times with 50 mM Tris–HCl buffer (pH 7.4) and then another three times with buffer containing 1% charcoal and 0.1% dextran. Samples were incubated in a water bath at 40° for 30 min and centrifuged at 4° for 10 min at 4500 rpm (2000*g*). The supernatant was collected, diluted with buffer to reach a plasma dilution of 1/250, and either used immediately or stored at −20° until used. Plasma pools were diluted three times with the dextran–charcoal suspension in buffer and then incubated and centrifuged as described. The supernatant was collected and diluted in buffer to reach a plasma dilution of 1/250 unless specified otherwise.

Radioligand binding assays for CBG (Orchinik *et al.*, 2000) were performed with minor modifications. Briefly, [1,2,6,7-^{3}H(N)]CORT (^{3}H-CORT; specific activity: 88 Ci/mmol; New England Nuclear, Boston, MA) was diluted in buffer and dispensed into polypropylene tubes, followed with 50 μl of assay buffer with or without unlabeled competitor (see Results) and 50 μl of diluted steroid-free plasma (final dilution 1:750 unless specified otherwise). Samples were incubated on ice for 1 h, unless indicated otherwise. Free and bound ^{3}H-CORT were separated by rapid filtration of the samples over Whatman GF/B glass fiber filters previously soaked for 1 h in ice-cold buffer containing 0.3% polyethylenimine (Sigma Chemical Co.). After filtration, filters were immediately rinsed three times with 3 ml of ice-cold 25 mM Tris–HCl buffer and placed into liquid scintillation glass vials. Filter-bound radioactivity was quantified by standard liquid scintillation spectroscopy using a β-counter (Beckman, LS 6500). Nonspecific binding (NSB) is defined as the binding of ^{3}H-CORT that occurred in the presence of 1 μM unlabeled CORT. All samples were assayed in duplicate or triplicate.

Testosterone Assays

Total T concentrations were assayed in aliquots of plasma (25 μl/assay tube) using a direct solid-phase commercial radioimmunoassay system (Diagnostic Products Corp., Los Angeles, CA). This assay has been previously used and validated to measure T in Dark-eyed Juncos (Gulledge and Deviche, 1998). It is sensitive (lower detection limit: 10 pg/tube) and specific (cross-reactivity: 3% with 5α-dihydrotestosterone, 0.02% with 17β-estradiol). All samples were assayed in duplicate and in a single series. The intraassay coefficient of variation was 5.8%.

Statistical Analyses

Binding parameter estimates were calculated by the fitting of appropriate mathematical models to the data by use of nonlinear regression analysis and by assumption of the law of mass action (Prism 2 or 3; GraphPad Software, San Diego, CA). Equilibrium dissociation constants (K_d), concentration of binding sites (B_{max} of ^{3}H-CORT in plasma), and concentrations of nonradioactive steroids that displace 50% of the specific binding (EC_{50}) for ^{3}H-CORT or ^{3}H-estradiol were determined. The EC_{50} estimates were converted to inhibition constants (K_i) by use of the equation of Cheng and Prusoff (1973): $K_i = EC_{50}/(1 + [^3\text{H-CORT}]/K_d)$. Two-tailed Student's *t* tests, also with the Prism program, were employed to compare best-fit values obtained from nonlinear regressions.

One-way analysis of variance (1ANOVA) followed with Student–Newman–Keuls pair-wise comparison tests allowed the identification of sex- and season-related differences in ^{3}H-CORT binding activity in free-living birds. A two-way analysis of variance (2ANOVA) assessed the effects of T administration

and photoperiod on plasma T concentrations and ^{3}H-CORT binding activity in captive castrates. All results are expressed as means ± standard errors and the significance level of all statistical tests was set at $P = 0.05$.

RESULTS

Effect of Plasma Dilution on ^{3}H-CORT Binding

Plasma obtained from males sampled in the field early in the breeding season (May 1998) was incubated at a final dilution of 1/50, 1/100, 1/200, or 1/400 for 2 h in the presence of 0.64 nM ^{3}H-CORT. Specific and nonspecific ^{3}H-CORT binding decreased linearly as plasma dilution increased (SB: linear regression coefficient, $R = 0.692$, $P = 0.013$; NSB: $R = 0.945$, $P < 0.001$). Nonspecific binding consistently represented less than 5% of TB.

Time Course of CORT Association to Plasma Binding Sites

Plasma (final dilution: 1/500) was incubated for 1 to 60 min in the presence of 1.2 nM ^{3}H-CORT. The specific binding of ^{3}H-CORT increased as a function of the time of incubation and the binding reaction reached equilibrium after approximately 20 min (Fig. 1). The observed rate constant was 0.52 ± 0.04 min^{-1}. Nonspecific binding did not change over time and, at equilibrium, represented approximately 3% of TB.

Equilibrium Saturation Binding of ^{3}H-CORT

The equilibrium binding parameters for the specific binding of ^{3}H-CORT were determined. For this, plasma samples were pooled from adult birds belonging to the following three groups: males at the beginning of the breeding season (April 25 to May 4, 1998; $n = 15$), when plasma T concentrations are elevated (Deviche *et al.*, 2000); males at the end of the breeding season (June 26–29, 1998; $n = 10$), when circulating plasma T levels are low; females (May 21 to June 2, 1998; $n = 9$), which on average have undetectable plasma T (Deviche and Gulledge, 2000). The combination of samples from birds in each group resulted in three plasma pools that were incubated in the presence of ^{3}H-CORT at concentrations varying between 0.04 and 18.7 nM (Fig. 2A). A one-site model best described the data for each group. Scatchard–Rosenthal plots of transformed data were linear (Fig. 2B), consistent with a homogenous population of sites in each plasma pool. For each group, ^{3}H-CORT bound to presumed CBG in plasma with a similar high affinity. K_d estimates were 2.84 ± 0.07 nM (early season males), 3.08 ± 0.13 nM (late season males), and 2.75 ± 0.15 nM (females).

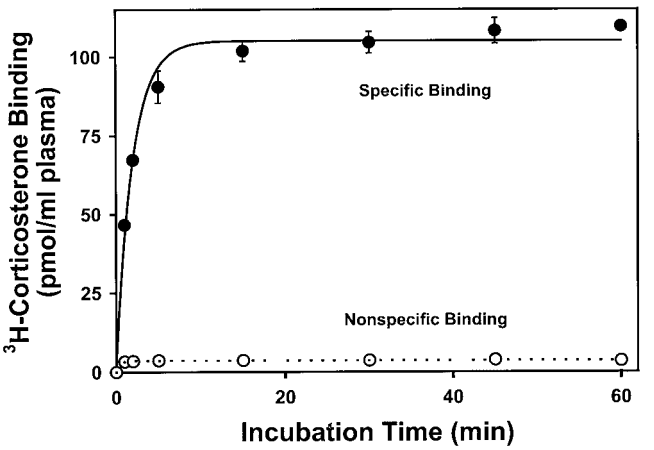

FIG. 1. Association curves depicting the specific and nonspecific binding (mean ± standard error) of [^{3}H]cortisosterone to Dark-eyed Junco plasma as a function of the duration of incubation at 4°.

In contrast to K_ds, B_{max} estimates differed among the three groups. The binding capacity was higher in males caught at the beginning (365 ± 3 pmol/ml plasma) than at the end (294 ± 4 pmol/ml plasma) of the breeding season (Student t test: $P < 0.001$). Male plasma contained a higher concentration of specific binding sites than female plasma (117 ± 2 pmol/ml plasma; comparison with either group of males: $P < 0.001$).

Sex- and Season-Related Differences in Individual Plasma Samples

Differences in plasma CBG levels between seasons and sexes were also examined using individual plasma samples from the birds used in the previous experiment. Samples were incubated with 15.5 nM

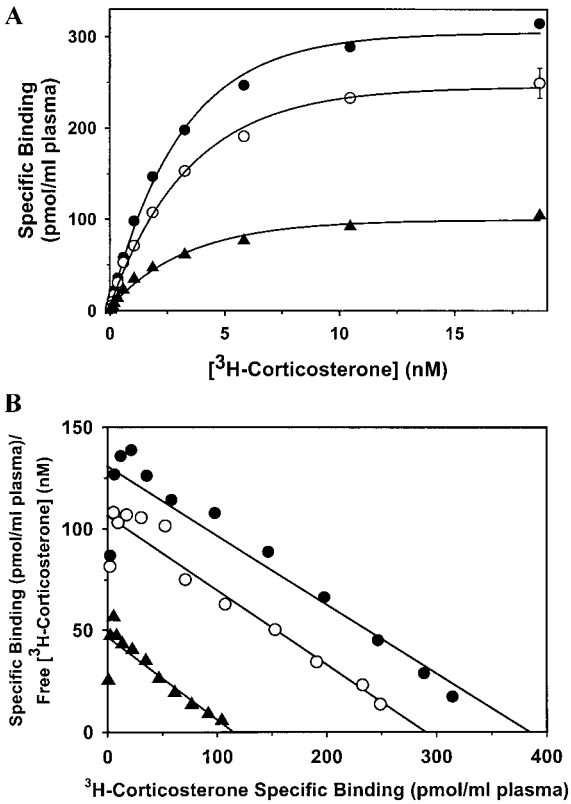

FIG. 2. (A) Equilibrium saturation binding isotherms at 4° illustrating the specific binding (mean ± standard error) of [^{3}H]corticosterone to Dark-eyed Junco plasma as a function of the concentration of radioligand in the incubation medium. *Filled circles,* adult males sampled at the beginning of the breeding season; *empty circles,* adult males sampled at the end of the breeding season; *triangles,* adult females sampled during the breeding season. (B) Scatchard–Rosenthal plots generated from the data in (A). A linear regression line was fitted to each set of experimental points.

^{3}H-CORT. This concentration of ^{3}H-CORT should occupy more than 80% of plasma binding sites, based on mass-action calculations. In agreement with the differences described above, the amounts of specifically bound ^{3}H-CORT differed among the groups (1ANOVA: $F_{2,33} = 36.3$, $P < 0.001$): early season males, 338 ± 21 pmol/ml plasma; late season males, 280 ± 22 pmol/ml plasma; females, 91 ± 11 pmol/ml plasma. Multiple pair-wise comparison tests revealed that SB was significantly higher in either group of males than in females ($P < 0.05$). The difference between the means of individual samples from early and late season males approached statistical significance ($P = 0.06$).

Effects of T Treatment and Photoperiod on CORT Plasma Binding

Testosterone was undetectable in plasma obtained from castrates, whereas T-treated juncos had elevated circulating levels of the steroid that were within the range of those measured in free-living intact males at the beginning of the breeding season (Deviche *et al.*, 2000). Hormone levels in T-treated juncos did not differ between birds exposed to SD or to LD (Fig. 3A).

Plasma from individual castrates held on LD or SD, with or without T replacement, was incubated with 15.5 nM ^{3}H-CORT. Specific binding of ^{3}H-CORT increased in response to T administration (2ANOVA: $F_{1,26} = 41.8$, $P < 0.001$) and was influenced by photoperiod ($F_{1,26} = 8.56$, $P = 0.008$), but there was a statistically significant hormone treatment × photoperiod interaction ($F_{1,26} = 5.57$, $P = 0.027$; Fig. 3B). Multiple pair-wise comparison tests indicated that LD exposure increased binding beyond that induced by T treatment, but LD exposure had no effect in C males. Specific binding of ^{3}H-CORT approached that measured in free-living males sampled at the beginning of

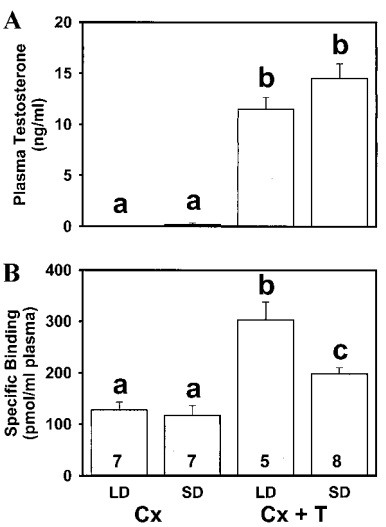

FIG. 3. (A) Total plasma testosterone concentrations (mean ± standard error) of adult castrated Dark-eyed Juncos treated (Cx + T) or not treated (Cx) with testosterone for 4 weeks and exposed to short or long days (SD, LD). Columns with a different superscript letter differ statistically ($P < 0.05$; Student–Newman–Keuls multiple pair-wise comparison tests). (B) Specific binding (mean ± standard error) of [^{3}H]corticosterone to plasma of the same males as in (A). Numbers inside columns indicate sample sizes.

TABLE 1
Results of Two Independent Competition Studies (S1, S2) Showing the Affinity, Expressed as Inhibition Constants (K_is; Mean ± SE), of Various Unlabeled Steroids for [^{3}H]Corticosterone Binding Sites in Dark-Eyed Junco Plasma

Unlabeled Competitor		K_i (nM)	% inhibition at 1 μM
Progesterone	S1	1.72 ± 0.15	100
	S2	2.76 ± 0.16	100
Mean		2.24	100
Corticosterone	S1	4.15 ± 0.34	100
	S2	6.40 ± 0.77	100
Mean		5.28	100
Dexamethasone	S1	11.20 ± 1.19	100
	S2	13.10 ± 1.11	99
Mean		12.15	100
Testosterone	S1	17.31 ± 1.92	99
	S2	28.53 ± 2.38	98
Mean		22.92	99
5α-Dihydrotestosterone	S1	29.53 ± 2.19	99
	S2	22.51 ± 3.85	97
Mean		26.02	98
17β-Estradiol	S1	$>10^6$	49
	S2	$>10^6$	45
Mean		$>10^6$	47

Note. The percentage inhibition of [^{3}H]corticosterone binding in the presence of 1 μM each competing steroid is also shown.

the breeding season only in LD-exposed, T-treated castrates.

Specificity of Plasma Binding Sites for CORT

The abilities of unlabeled steroids (T, 5α-dihydrotestosterone, CORT, dexamethasone, 17β-estradiol, and progesterone) to compete for specific ^{3}H-CORT binding sites were also investigated. For this, plasma obtained from adult males was incubated with 0.6 nM or 1.04 nM ^{3}H-CORT in the presence of unlabeled steroid concentrations varying between 1×10^{-6} and 1×10^{-10} M. ^{3}H-CORT bound to a site that displayed moderate to high affinity for all steroids tested except estradiol (Table 1). The potency of unlabeled steroids to displace ^{3}H-CORT binding was progesterone $\geq$ CORT $>$ dexamethasone $>$ T $\simeq$ 5α-dihydrotestosterone $\gg$ estradiol (Fig. 4A).

Plasma Binding Sites for Sex Steroids

The affinity of CORT for its specific binding sites in junco plasma was 5 to 10 times greater than was the affinity of T and 5α-dihydrotestosterone for these sites, whereas estradiol had negligible affinity. Whether junco plasma contains separate binding sites with high and specific affinity for both T and estradiol, similar to those found in mammals and other classes of vertebrates, was assessed. For this, plasma from free-living adults (four males, one female) sampled early in the breeding season (April 29–May 10) was incubated in the presence of 6.0 nM [2,4,6,7,16,17-^{3}H(N)]estradiol (^{3}H-estradiol; New England Nuclear, Boston, MA; specific activity: 118 Ci/mmol). Nonspecific binding was measured in the presence of 1 μM estradiol in the incubation medium. To maximize

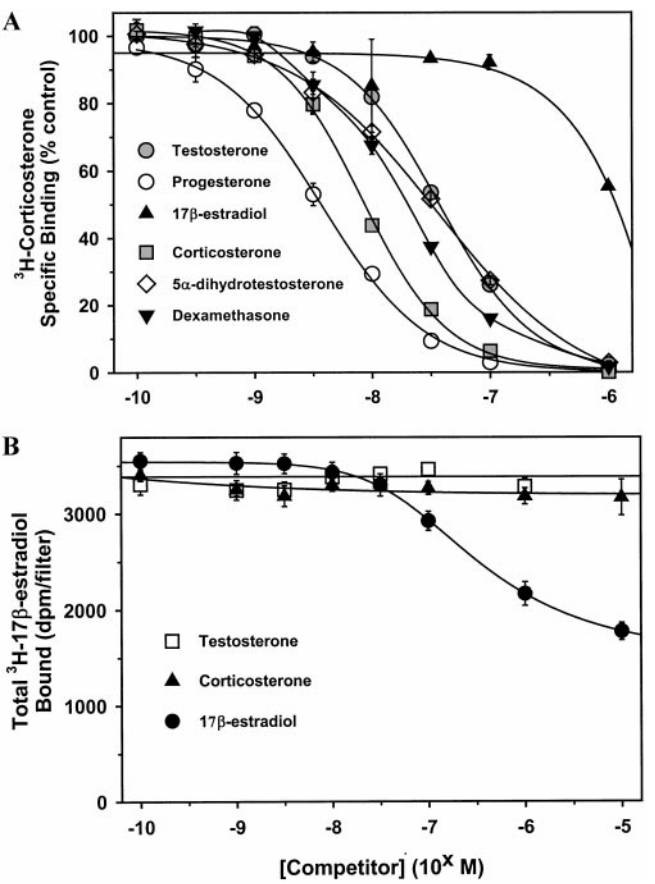

FIG. 4. (A) Competition curves illustrating the specific binding (% control; mean ± standard error) of [^{3}H]cortisosterone to Dark-eyed Junco plasma as a function of the concentration of various unlabeled competitors in the incubation medium. (B) Competition curves showing the total binding (mean ± standard error) of [^{3}H]17β-estradiol to Dark-eyed Junco plasma as a function of the concentration of unlabeled 17β-estradiol, testosterone, or corticosterone in the incubation medium.

chances of detecting specific ^{3}H-estradiol binding sites, the effect of stripping temperature and of stripping itself on specific binding was examined.

Specific binding of ^{3}H-estradiol to stripped plasma equaled 9.0 ± 2.3 pmol/ml plasma at 0° and 13.7 ± 5.4 pmol/ml plasma at 40°. Specific binding to non-stripped plasma was only 0.9 ± 0.7 pmol/ml plasma at 0° and 2.5 ± 0.8 pmol/ml plasma at 40°. As shown by 2ANOVA for repeated measures, the specific binding of ^{3}H-estradiol, although minimal, was increased by stripping ($F_{1,19} = 43.11, P = 0.003$) and was higher in samples stripped at 40° than at 0° ($F_{1,19} = 9.56, P = 0.037$).

Therefore, plasma was stripped of endogenous steroids at 40°, and then incubated with 1.56 nM ^{3}H-estradiol in the presence or absence of unlabeled estradiol, T, or CORT at concentrations varying between 0.1 and 10,000 nM. Unlabeled 17β-estradiol displaced a fraction of ^{3}H-estradiol binding sites with low affinity ($EC_{50} > 200$ nM; Fig. 4B). Neither T nor CORT displaced ^{3}H-estradiol specific binding sites at concentrations up to 10 μM. Taken together, these studies provide no evidence for the presence of a high-affinity plasma binding site specific for sex steroids similar to mammalian SHBG.

Regulation of Free T Levels by "CBG"

Testosterone bound to plasma with a lower affinity than did CORT ($K_d = 22.92$ nM vs 2.8 or 5.28 nM, respectively), but the B_{max} for "CBG" (365 nM) was well in excess of the circulating levels of either steroid (Deviche *et al.*, 2000). Since there was no evidence for the presence of a SHBG in plasma, the possibility that a significant proportion of circulating T may normally bind to "CBG" was investigated. For this, the amount of free T under different physiological conditions was calculated by use of the mass action-based equation of Barsano and Baumann (1989)

$$H_{free} = 0.5\left[H_{total} - B_{max} - \frac{1}{K_a} \pm \sqrt{\left(B_{max} - H_{total} + \frac{1}{K_a}\right)^2 + 4\left(\frac{H_{total}}{K_a}\right)}\right],$$

$$\text{in which } K_a = \frac{1}{K_d}.$$

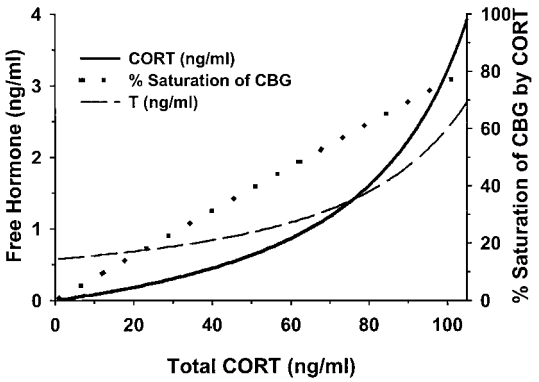

FIG. 5. Model of CORT and T interaction with "CBG." Curves were generated by use of the equation of Barsano and Baumann (1989) for estimating free hormone levels (ng/ml) from total hormone levels. We estimated the free CORT levels at concentrations of total CORT ranging from 1 to 125 ng/ml. Free T levels were calculated for the same range of total CORT, assuming a constant level of total T (9 ng/ml; levels in free-living juncos at the beginning of the breeding season). At 9 ng/ml, T will saturate less than 10% of CBG (7.8%), so we ignored this factor (% saturation of CBG by T) in the estimations.

This model estimates the amount of free T in junco plasma in the presence of baseline (Deviche *et al.*, 2000) or stress-induced CORT levels.

At the beginning of the breeding season, free-living male juncos have approximately 9 ng/ml total circulating T (Deviche *et al.*, 2000). Given the binding parameters above, and initially ignoring CORT, it was estimated that 93.6% of the total T would be bound to "CBG," leaving only 0.58 ng/ml hormone free in plasma or bound to albumin with very low affinity. To take into account the effect of average baseline circulating levels of CORT on free T, the fraction of CBG that would be occupied by 15 ng/ml of CORT (11.8% of CBG) was subtracted. This correction had very little effect on the estimate of free T (0.65 ng/ml). However, as shown in Fig. 5, if circulating CORT concentrations increase from 20 to 100 ng/ml plasma in response to acute stress (Klukowski *et al.*, 1997; Schoech *et al.*, 1999; Silverin *et al.*, 1997), and the fraction of CBG bound by 100 ng/ml CORT (76.5%) is subtracted from the total CBG present, the estimate of free T levels in plasma increased approximately fivefold.

DISCUSSION

These studies revealed three new observations about plasma binding globulins. First, plasma CBG

levels in free-living birds are regulated in a complex manner by both T and day length. Second, avian CBG, which is typically thought of as a primarily CORT-binding protein, also binds T with sufficiently high affinity that more than 90% of this androgen may normally be bound to the protein. Finally, since CORT and T bind to the same plasma binding globulin, changes in plasma levels of CORT, such as those experienced during a stress response, are likely to acutely alter free plasma T levels.

Regulation of CBG Binding Capacity by T and Photoperiod

Elevated circulating T concentrations are associated with high CBG levels in some, although apparently not in all avian species (Peczely, 1979; Peczely and Daniel, 1979). For example, CBG capacity is higher during the breeding season than at the end or after the breeding season in male Lapland Longspurs (Romero *et al.*, 1998b), White-crowned Sparrows (Romero and Wingfield, 1998), and Pied Flycatchers (Silverin, 1986) and is higher in male than in female Pekin Ducks (Daniel *et al.*, 1981). In some avian species, this correlation reflects a causal relationship, as castration decreases CBG binding capacity (ducks: Daniel *et al.*, 1984), and androgen administration to gonadally intact (Klukowski *et al.*, 1997) or castrated (Daniel *et al.*, 1984) males has the opposite effect. In the current study, equilibrium saturation binding analysis of pooled plasma indicated that the concentration of CBG was greater in males sampled early than in males sampled late in the breeding season (results of an individual point sample experiment were consistent with this at the $P = 0.06$ level) and greater in males than in females. In addition, CBG binding capacity increased following T treatment to castrated birds. These results confirm those of Klukowski *et al.* (1997) and support the hypothesis that T upregulates CBG concentration in Dark-eyed Juncos. Testosterone administration increased binding capacity without altering the affinity of CORT for CBG, as was also the case in the Klukowski *et al.* (1997) study. A direct transcriptional effect of androgen on CBG production may account for increased CBG binding associated with elevated plasma T levels. Alternately, androgen-mediated changes in CBG may be due to alterations of CBG metabolism or to changes in CORT levels. In support of the latter hypothesis, baseline CORT levels in juncos (Deviche *et al.*, 2000) and other passerines (Silverin and Wingfield, 1982; Wingfield and Farner, 1978) are elevated early in the breeding season, when circulating T concentrations are also high. It is interesting that administration of T to juncos increased baseline (Ketterson *et al.*, 1991) and stress-induced (Schoech *et al.*, 1999) CORT levels. The hypothesis that T affects CBG by elevating CORT levels has not been investigated in birds. In adult mammals, elevated CORT usually decreases these levels (Cole *et al.*, 1999; Feldman *et al.*, 1979; Marissal-Arvy *et al.*, 2000; Zhao *et al.*, 1997), although CORT may stimulate CBG production during fetal life (Berdusco *et al.*, 1993).

In addition to being stimulated by T (directly or indirectly), CBG binding capacity in castrated males increased in response to LD exposure. However, this photoperiod-induced increase occurred only in the presence of T. Several observations suggest that the influence of LD exposure on CBG levels in captive juncos is physiologically relevant in captive and wild male birds. First, of the experimental groups, the LD-exposed, T-treated castrates had CBG levels (303 ± 35 pmol/ml plasma) closest to those measured in free-living males sampled at the end of April–early May (338 ± 21 pmol/ml plasma), when males are naturally exposed to LD and to high circulating T levels (Deviche *et al.*, 2000). Second, CBG binding capacity in free-living males decreased between May and the end of June, coincident with a large decrease in plasma T concentrations. During this time, however, photoperiod increased from 16.7 h (early May) to 21.8 h (end of June), consistent with our finding that exposure to LD increased CBG binding site concentrations only when plasma T levels were concurrently elevated. CBG binding capacity during the breeding season was higher in males than in females. It is possible that this sex difference resulted from females having low or undetectable plasma T levels (Deviche and Gulledge, 2000). In addition, CBG binding activity during the breeding season may be unaffected by LD exposure in females while it is increased in males. Studies investigating the influence of T treatment to females exposed to various light conditions are warranted to investigate this possibility.

The mechanism mediating the stimulating influence of LD exposure on CBG in the presence of elevated plasma T is unknown, but we note that photoperiod

modulates other physiological functions in birds independent of changes in gonadal steroid levels (Bentley *et al.,* 1999). For example, transfer of photosensitive birds from SD to LD initially stimulates, but subsequently inhibits, the hypothalamo–pituitary–gonadal axis as these birds become photorefractory (Nicholls *et al.,* 1988). Refractoriness to LD develops in gonadally intact as well as in castrated (Wingfield *et al.,* 1980), pinealectomized, or bilaterally enucleated (Wilson, 1991) birds, demonstrating that it does not depend on the presence of gonadal steroids, the pineal gland, or the eyes. In oscines, transfer from SD to LD increases the size of brain regions responsible for vocal behavior perception and production, and this increase is likewise partly gonadal androgen independent (Bernard *et al.,* 1997; Dloniak and Deviche, 2001; Smith *et al.,* 1997).

Specificity of CBG Binding

Consistent with studies on other avian species (Silverin, 1986; Wingfield *et al.,* 1984), we found no evidence that junco plasma contains a typical SHBG with high affinity for estradiol and T similar to that seen in mammals (Hammond *et al.,* 1996; Petra, 1991). However, the affinity of T for plasma "CBG" and the abundance of CBG in junco plasma are sufficiently high for a large proportion of the circulating androgen to normally bind to the protein. Similar observations about a lack of specificity between androgen and CORT binding to plasma binding protein have been made in other species (Jennings *et al.,* 2000; Orchinik *et al.,* 2000; Savu *et al.,* 1986) including birds (Wingfield *et al.,* 1984). Therefore, the claim that androgens do not bind to specific binding proteins other than albumin in avian plasma appears to be unwarranted.

The present data and other results indicate that large interspecific differences exist with respect to the binding specificity of CBG for circulating steroids. For example, avian (Gould *et al.,* 1978; Murakami, 1991; Savu *et al.,* 1986; present results), but not mammalian (Maitra *et al.,* 1993), CBG has high affinity for the synthetic glucocorticoid, dexamethasone. Also, the binding characteristics of CBG differ across species in primates (Robinson *et al.,* 1985) and other mammals (Gayrard *et al.,* 1996). The evolutionary significance of these differences and their physiological relevance to the control of free vs bound circulating steroid concentrations (see below), hence to the biological actions of steroids in general, warrant further investigations.

Regulation of Free Steroid Hormone Concentrations

Estimating from baseline levels of CORT, T, and CBG in early and late breeding season and from the respective affinity of these steroids for CBG, a mass action-based equation was used to predict free CORT and T levels. As expected, most baseline CORT (99%) was predicted to be bound to CBG in all seasons. However, when the binding of CORT to CBG was ignored, over 90% of plasma T was also predicted to be bound to the globulin in the early and late breeding season (93.6 and 92.7% bound, with free T levels of 0.58 and 0.07 ng/ml, respectively). This result was unexpected based on the literature. Taking into account the binding of CORT to CBG by subtraction of the bound fraction from CBG total binding capacity, there was little effect of baseline CORT on the levels of free T in either season. In contrast, when free T levels were estimated at increasing plasma CORT concentrations with increasing occupancy of CBG by CORT, the concentration of free androgen increases. As illustrated in Fig. 5, an increase in plasma CORT concentrations from 20 to 100 ng/ml, as is seen in juncos exposed to acute stressors (Schoech *et al.,* 1999), would produce approximately a fivefold increase in free T concentration. A somewhat similar phenomena has been described for the relationship between plasma cortisol and free T in humans (Cooke *et al.,* 1996). Thus, avian "CBG" apparently functions as a T and as a CORT binding protein, and plasma CORT levels regulate circulating concentrations of free (and bound) T. This conclusion may have important implications for the understanding of the functional relationships between the pituitary–adrenal and the pituitary–gonadal axes.

What is the potential functional significance of CORT and androgen binding to same plasma binding globulin? This phenomenon may be especially important physiologically in birds and other species for which high-affinity gonadal steroid binding proteins have not been identified in plasma. In these species, the release of T from "CBG" may protect against acute suppression of T levels or reproductive function in response to brief exposure to elevated CORT levels (Orr *et al.,* 1992). On the other hand, a sudden fivefold

increase in free T levels may enhance the rate of hepatic breakdown of T and/or the negative feedback effect of T on the hypothalamic–pituitary–gonadal axis. In some species, and depending upon the relative affinities of T and CORT for CBG, this interaction may be a mechanism by which T regulates free plasma CORT levels (Jennings *et al.*, 2000).

In conclusion, these data and other recent studies suggest that the dynamic regulation of CBG by gonadal steroids, adrenal steroids, and photoperiod warrants further investigation. Given the recent discoveries indicating that CBG plays a more active role in hormone action than just plasma transport, it is becoming increasingly important to determine the contributions of free and CBG-bound hormone. Finally, the competition between CORT and T for plasma binding sites in birds may have significant implications for the physiological, developmental, and behavioral actions of both steroids.

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REFERENCES

Assenmacher, I., Astier, H., Daniel, J. Y., and Jallageas, M. (1975). Experimental studies on the annual cycles of thyroid and adrenocortical functions in relation to the reproductive cycle of drakes. *J. Physiol. (Paris)* **70,** 507–520.

Barsano, C. P., and Baumann, G. (1989). Editorial: Simple algebraic and graphic methods for the apportionment of hormone (and receptor) into bound and free fractions in binding equilibria; or how to calculate bound and free hormone. *Endocrinology* **124,** 1101–1106.

Bentley, G. E., Van't Hof, T. J., and Ball, G. F. (1999). Seasonal neuroplasticity in the songbird telencephalon: A role for melatonin. *Proc. Natl. Acad. Sci. USA* **96,** 4674–4679.

Berdusco, E. T., Hammond, G. L., Jacobs, R. A., Grolla, A., Akagi, K., Langlois, D., and Challis, J. R. (1993). Glucocorticoid-induced increase in plasma corticosteroid-binding globulin levels in fetal sheep is associated with increased biosynthesis and alterations in glycosylation. *Endocrinology* **132,** 2001–2008.

Bernard, D. J., Wilson, F. E., and Ball, G. F. (1997). Testis-dependent and -independent effects of photoperiod on volumes of song control nuclei in American tree sparrows (*Spizella arborea*). *Brain Res.* **760,** 163–169.

Cheng, Y., and Prusoff, W. H. (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* **22,** 3099–3108.

Cole, T. J., Harris, H. J., Hoong, I., Solomon, N., Smith, R., Krozowski, Z., and Fullerton, M. J. (1999). The glucocorticoid receptor is essential for maintaining basal and dexamethasone-induced repression of the murine corticosteroid-binding globulin gene. *Mol. Cell. Endocrinol.* **154,** 29–36.

Cooke, R. R., McIntosh, J. E. A., and Murray-McIntosh, R. P. (1996). Effect of cortisol on percentage of non-sex-hormone-bound steroid: Implications for distribution of steroids on binding proteins in serum. *Clin. Chem.* **42,** 249–254.

Daniel, J. Y., Malaval, F., and Assenmacher, I. (1981). Evidence of a sex related difference of transcortin level in adult ducks. *Steroids* **38,** 29–34.

Daniel, J. Y., Malaval, F., and Assenmacher, I. (1984). Androgen control of transcortin binding capacity in adult male ducks. *Steroids* **43,** 363–369.

Deviche, P., and Gulledge, C. C. (2000). Vocal control region sizes of an adult female songbird change seasonally in the absence of detectable circulating testosterone concentrations. *J. Neurobiol.* **42,** 202–211.

Deviche, P., Wingfield, J., and Sharp, P. J. (2000). Year-class differences in the reproductive system, plasma prolactin and corticosterone concentrations, and onset of prebasic molt in male Dark-eyed Juncos (*Junco hyemalis*) during the breeding period. *Gen. Comp. Endocrinol.* **118,** 425–435.

Dloniak, S. M., and Deviche, P. (2001). Effects of testosterone and photoperiodic condition on song production and vocal control region volumes in adult Dark-eyed Juncos (*Junco hyemalis*). *Horm. Behav.*, in press.

Feldman, D., Mondon, C. E., Horner, J. A., and Weiser, J. N. (1979). Glucocorticoid and estrogen regulation of corticosteroid-binding globulin production by rat liver. *Am. J. Physiol.* **237,** E493–E499.

Fortunati, N. (1999). Sex hormone-binding globulin: Not only a transport protein. What news is around the corner? *J. Endocrinol. Invest.* **22,** 223–234.

Gayrard, V., Alvinerie, M., and Toutain, P. L. (1996). Interspecies variations of corticosteroid-binding globulin parameters. *Domes. Anim. Endocrinol.* **13,** 35–45.

Gould, N. R., and Siegel, H. S. (1978). Partial purification and characterization of chicken corticosteroid-binding globulin. *Poult. Sci.* **57,** 1733–1739.

Gulledge, C. C., and Deviche, P. (1998). Photoperiod and testosterone independently affect vocal control region volumes in adolescent male songbirds. *J. Neurobiol.* **36,** 550–558.

Hammond, G. L. (1995). Potential functions of plasma steroid-binding proteins. *Trends Endocrinol. Metab.* **6,** 298–304.

Hammond, G. L., and Bocchinfuso, W. P. (1996). Sex hormone-binding globulin: Gene organization and structure/function analyses. *Horm. Res.* **45,** 197–201.

Jennings, D. H., Moore, M. C., Knapp, R., Matthews, L., and Orchinik, M. (2000). Plasma steroid-binding globulin mediation of differences in stress reactivity in alternative male phenotypes in Tree Lizards, *Urosaurus ornatus.* Unpublished.

Ketterson, E. D., Nolan, V., Jr., Wolf, L., Ziegenfus, C., Dufty, A. M., Jr., Ball, G. F., and Johnsen, T. S. (1991). Testosterone and avian life histories: The effect of experimentally elevated testosterone on corticosterone and body mass in Dark-eyed Juncos. *Horm. Behav.* **25,** 489–503.

Klukowski, L. A., Cawthorn, J. M., Ketterson, E. D., and Nolan, V., Jr. (1997). Effects of experimentally elevated testosterone on plasma corticosterone and corticosteroid-binding globulin in dark-eyed juncos (*Junco hyemalis*). *Gen. Comp. Endocrinol.* **108,** 141–151.

Kovacs, K., and Peczely, P. (1983). Phase shifts in circadian rhythmicity of total, free corticosterone and transcortine plasma levels in hypothyroid male Japanese quails. *Gen. Comp. Endocrinol.* **50,** 483–489.

Maitra, U. S., Khan, M. S., and Rosner, W. (1993). Corticosteroid-binding globulin receptor of the rat hepatic membrane: Solubilization, partial characterization, and the effect of steroids on binding. *Endocrinology* **133,** 1817–1822.

Marissal-Arvy, N., Ribot, E., Sarrieau, A., and Mormede, P. (2000). Is the mineralocorticoid receptor in brown Norway rats constitutively active? *J. Neuroendocrinol.* **12,** 576–588.

Murakami, T. (1991). Positive cooperativity of [^{3}H]dexamethasone binding to chick corticosteroid-binding globulin. *Comp. Biochem. Physiol. A* **100,** 361–364.

Nakhla, A. M., Leonard, J., Hryb, D. J., and Rosner, W. (1999). Sex hormone-binding globulin receptor signal transduction proceeds via a G protein. *Steroids* **64,** 213–216.

Nicholls, T. J., Goldsmith, A. R., and Dawson, A. (1988). Photorefractoriness in birds and comparison with mammals. *Physiol. Rev.* **68,** 133–176.

Orchinik, M., Matthews, L., and Gasser, P. J. (2000). Distinct specificity for corticosteroid binding sites in amphibian cytosol, neuronal membranes, and plasma. *Gen. Comp. Endocrinol.* **118,** 284–301.

Orr, T. E., and Mann, D. R. (1992). Role of glucocorticoids in the stress-induced suppression of testicular steroidogenesis in adult male rats. *Horm. Behav.* **26,** 350–363.

Peczely, P. (1979). Effect of testosterone and thyroxine on corticosterone and transcortine plasma levels in different bird species. *Acta Physiol. Acad. Sci. Hung.* **53,** 9–15.

Peczely, P., and Daniel, J. Y. (1979). Reciprocal interactions in the testis–thyroid–adrenal axis of the Japanese male quail. *Gen. Comp. Endocrinol.* **39,** 164–173.

Petra, P. H. (1991). The plasma sex steroid binding protein (SBP or SHBG): A critical review of recent developments on the structure, molecular biology and function. *J. Steroid Biochem. Mol. Biol.* **40,** 735–753.

Robinson, P. A., Hawkey, C., and Hammond, G. L. (1985). A phylogenetic study of the structural and functional characteristics of corticosteroid binding globulin in primates. *J. Endocrinol.* **104,** 251–257.

Romero, L. M., and Wingfield, J. C. (1998). Seasonal changes in adrenal sensitivity alter corticosterone levels in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*). *Comp. Biochem. Physiol.* **119C,** 31–36.

Romero, L. M., Soma, K. K., and Wingfield, J. C. (1998a). Changes in pituitary and adrenal sensitivities allow the snow bunting (*Plectrophenax nivalis*), an Arctic-breeding songbird, to modulate corticosterone release seasonally. *J. Comp. Physiol. [B]* **168,** 353–358.

Romero, L. M., Soma, K. K., and Wingfield, J. C. (1998b) Hypothalamic–pituitary–adrenal axis changes allow seasonal modulation of corticosterone in a bird. *Am. J. Physiol.* **274,** 1338–1344.

Rosner, W., Hryb, D. J., Khan, M. S., Nakhla, A. M., and Romas, N. A. (1999). Sex hormone-binding globulin mediates steroid hormone signal transduction at the plasma membrane. *J. Steroid Biochem. Mol. Biol.* **69,** 481–485.

Savu, L., Zouaghi, H., Gerard, H., Gerard, A., and Nunez, E. A. (1986). High-affinity binding of testosterone in serum from normal developing chick embryos and during the graft-versus-host reaction. *Biochim. Biophys. Acta* **881,** 15–21.

Schoech, S. J., Ketterson, E. D., and Nolan, V. (1999). Exogenous testosterone and the adrenocortical response in Dark-eyed Juncos. *Auk* **116,** 64–72.

Siiteri, P. K., Murai, J. T., Hammond, G. L., Nisker, J. A., Raymoure, W. J., and Kuhn, R. W. (1982). The serum transport of steroid hormones. *In* "Recent Progress in Hormone Research," pp. 457–510. Academic Press, New York.

Silverin, B. (1986). Corticosterone-binding proteins and behavioral effects of high plasma levels of corticosterone during the breeding period in the pied flycatcher. *Gen. Comp. Endocrinol.* **64,** 67–74.

Silverin, B., and Wingfield, J. C. (1982). Patterns of breeding behaviour and plasma levels of hormones in a free-living population of Pied Flycatchers, *Ficedula hypoleuca. J. Zool. Lond.* **198,** 117–129.

Silverin, B., Arvidson, B., and Wingfield, J. (1997). The adrenocortical responses to stress in breeding Willow Warblers *Phylloscopus trochilus* in Sweden: Effects of latitude and gender. *Funct. Ecol.* **11,** 376–384.

Smith, G. T., Brenowitz, E. A., and Wingfield, J. C. (1997). Roles of photoperiod and testosterone in seasonal plasticity of the avian song control system. *J. Neurobiol.* **32,** 426–442.

Wilson, F. E. (1991). Neither retinal nor pineal photoreceptors mediate photoperiodic control of seasonal reproduction in American Tree Sparrows (*Spizella arborea*). *J. Exp. Zool.* **259,** 117–127.

Wingfield, J. C., and Farner, D. S. (1978). The annual cycle of plasma irLH and steroid hormones in feral populations of the White-crowned Sparrow, *Zonotrichia leucophrys gambelii. Biol. Reprod.* **19,** 1046–1056.

Wingfield, J. C., Follett, B. K., Matt, K. S., and Farner, D. S. (1980). Effect of day length on plasma FSH and LH in castrated and intact White-crowned Sparrows. *Gen. Comp. Endocrinol.* **42,** 464–470.

Wingfield, J. C., Matt, K. S., and Farner, D. S. (1984). Physiologic properties of steroid hormone-binding proteins in avian blood. *Gen. Comp. Endocrinol.* **53,** 281–292.

Zhao, X. F., Scrocchi, L. A., and Hammond, G. L. (1997). Glucocorticoids induce corticosteroid-binding globulin biosynthesis by immature mouse liver and kidney. *J. Ster. Biochem. Mol. Biol.* **60,** 163–169.