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Seasonal Regulation of Membrane and Intracellular Corticosteroid Receptors in the House Sparrow Brain

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Abstract

A number of studies have demonstrated seasonal regulation of the adrenocortical response to stress, or of corticosteroid binding globulins, but very few studies have examined seasonal regulation of corticosteroid receptor levels. As a result, there have been few attempts to produce an integrated picture of seasonal plasticity of the stress response. We measured baseline and stress-induced corticosterone (CORT), corticosteroid binding globulin and neuronal cytosolic and membrane corticosteroid receptor levels in male and female, wild-caught house sparrows (*Passer domesticus*) during three different seasons over the annual cycle (nesting, molting and winter). We identified three neuronal corticosteroid receptors in the house sparrow brain: two intracellular receptors and one membrane-associated receptor. Little is known about corticosteroid receptors in neuronal membranes of avian and mammalian species, but we found that the levels of membrane corticosteroid receptors varied seasonally, being lowest during the nesting season. Cytosolic corticosteroid receptor numbers (both low and high affinity receptors) also varied seasonally. In contrast to the membrane bound receptors, however, the numbers of low and high affinity cytosolic receptors were lowest during winter. In addition, mean levels of total basal and stress-induced CORT in the plasma varied seasonally. Both basal and stress-induced levels of total CORT were significantly higher during nesting than during winter or molt. Finally, corticosteroid binding globulin levels in plasma were also seasonally regulated, in a pattern similar to total CORT, so that estimated free CORT levels did not vary between seasons. These data indicate that multiple components of the stress response are seasonally regulated in birds obtained from wild populations. Interactions between these regulated components provide a basis for seasonal differences in behavioural and physiological responses to stress.

Seasonal animals face very different challenges throughout the year. For example, in birds, metabolic needs and behavioural patterns vary depending on whether animals are raising young, defending a territory or molting. Glucocorticoids secreted in response to stress can affect both behavioural and metabolic processes (1). The ability to seasonally regulate effects of glucocorticoids could help an animal survive and reproduce in variable and/or unpredictable environments. The vertebrate stress response may be regulated at multiple levels. For example, stress-induced corticosteroid release varies seasonally in birds (2–4), mammals (5), reptiles (6) and amphibians (7), and corticosteroid binding globulin (CBG) capacity in plasma is regulated on a seasonal basis in a number of different vertebrates (2, 8–13). It is likely that these robust seasonal changes in glucocorticoid and CBG levels are accompanied by changes in target cell sensitivity to glucocorticoids. However, very little is known about seasonal regulation of corticosteroid receptors. Photoperiod alters [^{3}H]corticosterone (CORT) binding capacity in livers from captive *Xenopus laevis* (14) and protein and mRNA levels of one subtype of corticosteroid receptor in the hamster brain (15, 16). However, we know nothing directly concerning seasonal changes in receptor levels in animals living in naturalistic conditions.

In mammals, the best described corticosteroid receptors are intracellular, ligand-activated transcription factors. One

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subtype of intracellular receptor displays high affinity for corticosteroids, such as CORT and cortisol, and high affinity for the mineralocorticoid aldosterone. These receptors, known as type I or mineralocorticoid receptors (MR), are enriched in the mammalian hippocampus, kidney and colon. The second major subtype of intracellular receptor, type II or glucocorticoid receptor (GR), is ubiquitous and has an approximately 10-fold lower affinity for corticosteroids than does MR. It is not clear how closely the pharmacology of mammalian receptors matches that of avian receptors. Radioligand binding studies have identified two intracellular corticosteroid receptors in the duck and the chicken (17, 18), and a mammalian GR antibody recognizes GR-like proteins in the quail brain (19). In addition to these intracellular transcription factors, corticosteroids also bind to specific receptors in the plasma membranes of neurones and other tissues (20–26). In most vertebrates, these membrane-associated receptors are poorly characterized.

In order to understand seasonal plasticity in stress responsiveness, the dynamic regulation of glucocorticoid secretion, plasma levels of steroid binding globulins and tissue levels of intracellular and membrane receptors for corticosteroids need to be examined. A number of studies in mammals have examined several of these variables in order to provide an integrated picture of the stress response (27–33). For example, Dhabar *et al*. (33) measured plasma CORT and CBG levels to compare free CORT levels between rat strains with varied stress responses, and showed that strains with higher free CORT also have higher GR occupancy under stressful conditions.

We were interested in gaining an integrated perspective of the stress response in a free-living, seasonal animal. Toward this end, we measured baseline and stress-induced CORT titres, CBG capacity, and cytosolic and membrane-associated neuronal corticosteroid receptor number in wild-caught house sparrows (*Passer domesticus*) at three different stages during the annual cycle.

Materials and methods

Animals

House sparrows (*Passer domesticus*) were collected with mist nets in the vicinity of Tempe, AZ, between 05.30 h and 08.00 h at three different stages in the annual cycle: molt (24–28 August 1999; 19 males and 16 females; all animals in full body molt), winter (14–16 December 1999; 19 males and 20 females), and nesting (4–7 April 2000; 19 males and 22 females; near the beginning of the breeding season, all females had brood patches). Upon capture, birds were either sacrificed immediately by decapitation, or bled (for CORT and CBG titres) and then moved to animal facilities on campus for mitotane treatment. All procedures were in compliance with university and federal regulations.

Chemicals

Radiolabelled [1, 2, 6, 7–3H] CORT (^{3}H-CORT; specific activity = 70 Ci/mM) was purchased from NEN, Boston, MA, USA; CORT was purchased from Steraloids Inc., Wilton, NH, USA; Mitotane (ortho, para′-DDD) and RU486 (mifepristone) were purchased from Sigma (St Louis, MO, USA).

Corticosterone assays

Blood sampled were obtained within 3.5 min of capture (baseline CORT sample). Birds were then held in a cloth bag until the 30 min 'stressed' sample was taken (34). To obtain the blood sample, the alar vein was punctured with a 26-gauge needle, and 40–100 µl of blood was collected into heparinized microcapillary tubes. Blood samples were kept on ice for 1–2 h until plasma was separated from the blood sample by centrifugation, and stored at −20°C.

Plasma CORT levels were determined following a combination of the methods of Wingfield *et al*. (2) and Moore *et al*. (35), as outlined in Breuner *et al*. (36). Briefly, samples were allowed to equilibrate overnight with 2000 c.p.m. of CORT for determination of individual recoveries. Each sample was extracted with 4.0 ml of dichloromethane, dried under nitrogen and resuspended in phosphate-buffered saline with 1% gelatin. Samples were assayed in duplicate, and assay values were corrected for plasma volume and individual recoveries following extraction. Standard curve range (2000–1.25 pg), detectability (5.0 pg/tube), accuracy (83%). Intraassay coefficients of variation were 3.6%, 14.6% and 2.3% (for three assays, one for each season); interassay coefficient of variation was 15%.

Corticosteroid receptor assays

Temperature, rinse volume, and tissue concentration were optimized for each receptor assay to maximize specific binding, and the time to reach equilibrium was empirically determined for each receptor. All assays contained 50 µl [^{3}H]CORT, 50 µl buffer or unlabelled CORT, and 50 µl tissue or plasma preparation. Non-specific binding was determined using 1 µM unlabelled CORT. All samples were run in triplicate. Bound and free radioligand were separated using rapid vacuum filtration over glass fibre filters (Brandel Harvester). After filtration, radioactivity bound to filters was measured by standard liquid scintillation spectroscopy. Protocols for these experiments were based on methods used in Orchinik *et al*. (37). To avoid interassay variation, receptor number (for a given receptor) was determined for all individuals in the same assay.

Cytosolic receptors

Animals

To prevent endogenous CORT from occupying high affinity receptors, we injected sparrows with mitotane, a chemical which reduces CORT production to below detectable levels in house sparrows (36). Mitotane [4.5 mg of mitotane in 100 µl peanut oil (180 mg/kg)] was injected into the pectoralis muscle every 12 h beginning the evening of capture for a total of five injections. Birds were housed 3–4/cage in an environmental chamber (2.2 × 2.4 × 2.0 m; under natural daylength for each season; 25°C), and supplied with commercial bird seed, apples and 0.75% saline *ad libitum* (in case mitotane decreased circulating aldosterone levels). On the morning of day 4 (approximately 12 h after the final mitotane injection) baseline and stressed blood samples were obtained to ensure the mitotane treatment was successful. Sparrows were then anaesthetized with nembutal (0.09 mg/kg) and perfused transcardially with heparinized saline. Brains were removed, rapidly frozen on dry ice, and stored at −75°C.

Cytosol preparation

Brains were cut longitudinally along the midline and homogenized in TEGMD buffer (10 mM Tris, 1 mM EDTA, 10% glycerol, 20 mM molybdic acid, 5 mM dithiothreitol) with a glass-Teflon homogenizer and centrifuged at 104 000 *g* for 1 h at 4°C. The resulting supernatant (cytosol) was used in the assay at a protein concentration of 4–8 mg/ml (determined using Bradford reagent and a standard curve of BSA). Competition and saturation experiments were performed using tissue pooled from multiple individuals. For single point assays, each 1/2 brain was processed separately.

Estimation of binding parameters

We followed the methods of Szuran *et al*. (38), and Orchinik *et al*. (37) with minor modifications. Briefly, for equilibrium saturation binding analysis, cytosol was incubated with [^{3}H]CORT ranging from 0.05–12 nM. Incubations included radioligand and either buffer, or 10 nM RU486, a GR antagonist that occupies lower-affinity CORT receptors, or 1 µM unlabelled CORT to define nonspecific binding. Therefore, at each [^{3}H]CORT concentration, we had measures of total specific binding to all cytosolic corticosteroid receptors and specific binding to high-affinity receptors (in the presence of 10 nM unlabelled RU486). We calculated binding to lower-affinity receptors as the difference between the total specific binding and specific binding to high-affinity sites. For the competition analysis, cytosol was incubated with 2.5 nM [^{3}H]CORT, and unlabelled CORT or RU486 at concentrations ranging from 1 µM to 0.1 nM. Incubations were carried out at room temperature (22°C) for 4.5 h and terminated by rapid filtration over Whatman GF/B filters. Filters

were soaked in TEM buffer with 0.3% polyethylenimine (PEI; Sigma) for 1 h prior to filtering. Tissue on filters was rapidly rinsed with 9 ml ice cold TEM (three rinses of 3 ml each).

Individual point sample analysis

To estimate receptor number for high-and low-affinity receptors in individual birds, we incubated cytosol from individual brains with 10 nM [^{3}H]CORT in the presence of either buffer, 10 mM RU486, or 1 μM CORT to define nonspecific binding. Based on affinity estimates derived from our equilibrium saturation analysis, mass action predicts that 10 nM [^{3}H]CORT should occupy >95% of high affinity receptors and approximately 63% of lower-affinity receptors. Since receptor affinity does not change seasonally, we used this assay to estimate differences in receptor number between season and sex, while also obtaining an estimate of individual variation.

Membrane receptors

Animals

House sparrows were decapitated after capture in the field (these sparrows did not receive mitotane injections as endogenous CORT did not influence determination of receptor number; C. Breuner and M. Orchinik, unpublished data). Most brains were collected within 5 min of capture, although it took up to 1 h in some cases. Neither time after capture (up to 1 h of restraint in a cloth bag) nor saline perfusion affected the apparent affinity or capacity of the membrane corticosteroid receptor (C. Breuner and M. Orchinik, unpublished data). After decapitation, brains were rapidly frozen on dry ice, and subsequently stored at −75°C.

Tissue preparation

Brains were cut longitudinally along the midline for use in experiments. In the single point assays, each 1/2 brain was processed separately. In the saturation experiments, tissue was pooled from multiple individuals of the same sex and season. Well-washed neuronal membranes were prepared as described in Orchinik *et al*. (37), except that tissue was incubated in hypoosmotic buffer (5 mM Hepes, 5 mM EDTA) for 30 min instead of 2 h. After membrane preparation, the final pellet was stored at −75°C until use. The day of the assay, the pellet was resuspended in 25 mM Hepes with 6 mM $MgCl_2$ and 6 mM $CaCl_2$ to a protein concentration of 4–8 mg/ml.

Equilibrium saturation analysis

Membrane resuspensions were incubated with 0.95 nM to 30 nM [^{3}H]CORT with or without unlabelled CORT to define NSB at 8°C for 4 h and the reactions were terminated by rapid filtration over Whatman GF/C filters. Filters were soaked in 25 mM Tris for 1 h prior to filtering. Tissue on filters was rapidly rinsed with 6 ml 25 mM Tris (two rinses of 3 ml each).

Individual point sample analysis

Receptor number was estimated by incubating membranes with 20 nM [^{3}H]CORT. Based on affinity estimates derived from equilibrium saturation analysis, this concentration of [^{3}H]CORT will bind to approximately 40% of receptors. Since the affinity of the receptor does not change significantly between seasons, we used specific binding at 20 nM [^{3}H]CORT as an approximate measure of differences in total receptor capacity between individuals.

Corticosteroid binding globulin

Plasma collection and preparation

Plasma collected to determine baseline CORT levels was also used to measure CBG affinity and capacity. To remove endogenous CORT, individual plasma samples were incubated with two volumes (vol/vol) of dextran-coated charcoal solution (0.1% dextran, 1% norit A charcoal in 50 mM Tris) for 20 min at room temperature. Suspensions was centrifuged for 10 min at 4500 r.p.m. and 4°C. Plasma samples were assayed at a final dilution of 1 : 900. Point sample analysis was run on individual plasma samples, whereas saturation analyses were run on pooled samples.

Equilibrium saturation analysis

Plasma was incubated with 0.25 nM to 12 nM [^{3}H]CORT in the presence or absence of unlabelled CORT at 4°C for 2 h, and the reactions were terminated by filtration over Whatman GF/B filters. Filters were soaked in 25 mM Tris with 0.3% PEI for 1 h prior to filtering. Filters were rapidly rinsed with 9 ml 25 mM ice cold 25 mM Tris (three rinses of 3 ml each).

Individual point sample analysis

An estimate of CORT binding capacity in individual plasma samples was determined using 8 nM 3H-CORT. Based on affinity estimates derived from equilibrium saturation analysis, this ligand concentration should occupy approximately 83% of total binding sites.

Statistical analysis

Binding parameter estimates from the competition and saturation analyses were obtained by fitting untransformed data to appropriate equations using iterative, least-squares curve-fitting techniques (GraphPad Prism, San Diego, CA, USA). Equilibrium dissociation constant values, K_ds, presented in Table 1 were obtained by averaging the K_d estimates from multiple saturation experiments from each season. Differences in K_ds were tested with a factorial analysis of variance (ANOVA: StatView 5, SAS Institute Inc., Cary, NC, USA). All individual point sample data sets were determined to have a normal distribution using the K-S-test for normalcy. There was no effect of sex, so male and female data were combined for statistical analysis. Hormone and CBG data were log transformed [log(X + 1)] to correct for heteroscedasticity. Basal and stress-induced CORT samples were analysed separately, each with a two-way factorial ANOVA. All individual point sample analyses were also analysed with a two-way factorial ANOVA followed by Fisher's PLSD post-hoc analysis. A familywise $\alpha = 0.05$ significance level was used for all tests, and the Bonferroni method was used to control for simultaneous testing.

Free CORT titres were estimated using the equation of Barsano and Baumann (39):

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

where $K_a = 1/K_d$ (nM).

Baseline CORT, stress-induced CORT and CBG capacity were measured in each individual (from blood samples taken at capture), so free CORT estimations could be made for each individual, and then a mean and standard error figured for each sex in each season. Individual CBG capacity estimations represent approximately 83% of B_{max}, so capacity values were increased to 100% for free CORT calculations. Free CORT levels were log transformed [log(X + 1)] to correct for heteroscedasticity, and then tested for group differences with a factorial ANOVA.

Results

Cytosolic receptors

Mitotane treatment brought circulating CORT levels to below detectable levels in all sparrows (data not shown). We found evidence for two corticosteroid receptor subtypes in house sparrow brain cytosol. In the saturation analysis, [^{3}H]CORT specific binding data from April females were best fit by a two-site model yielding K_d of 0.37 ± 0.13 nM and 11.49 ± 12.56 nM, and B_{max} of 41.97 ± 9.7 and 59.89 ± 19.17 fmol/mg prot, respectively (Fig. 1, [^{3}H]CORT curve). The same experiment repeated in December males produced similar affinity estimates with K_d values of 0.23 ± 0.03 nM, and 8.98 ± 1.11 nM. Data from the competition analysis (Fig. 2, CORT curve) were also best fit by a two-site model, yielding an IC_{50} of 1.8 ± 0.73 nM and 30.8 ± 18.65 nM, respectively. IC_{50} values were not converted to K_d values because the data did not describe a simple bimolecular reaction. RU486 displaced approximately 30% of [^{3}H]CORT specific binding with high affinity (Fig. 2, RU486 curve). In the presence of 8 nM RU486, equilibrium saturation data are best fit by a one-site model

TABLE 1. Dissociation Constants (K_d) for Corticosteroid Binding Sites in House Sparrow Brain and Plasma (Data, Mean ± SE, Were Averaged From Multiple (n) Experiments).

	Brain		Plasma	
	High affinity cytosolic	Low affinity cytosolic	Membrane associated	CBG
Nesting	0.25 ± 0.07 (7)	4.5 ± 1.0 (4)	27.1 ± 2.0 (9)	3.2 ± 0.5 (5)
Molting	0.26 ± 0.10 (5)	3.7 ± 1.4 (2)	25.5 ± 3.9 (8)	1.7 ± 0.2 (4)
Wintering	0.10 ± 0.05 (4)	6.7 ± 1.0 (4)	27.8 ± 3.1 (7)	2.8 ± 0.14 (2)
ANOVA	F = 1.1, P = 0.37	F = 1.9, P = 0.21	F = 0.14, P = 0.87	F = 4.1, P = 0.06

CBG, corticosteroid binding globulin.

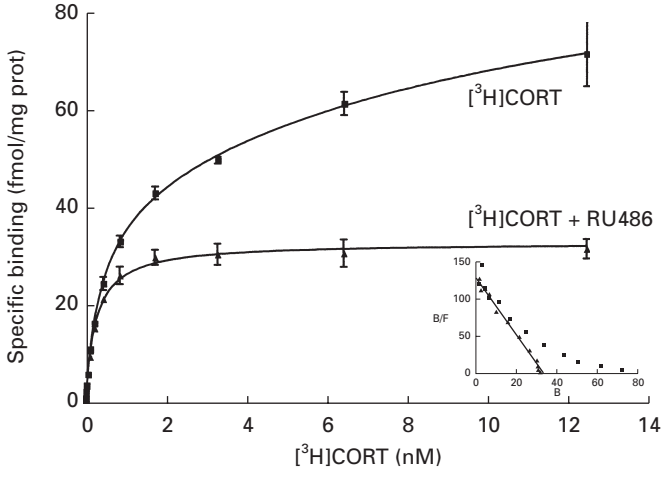

FIG. 1. Intracellular corticosteroid receptors: equilibrium saturation binding of [^{3}H]CORT to house sparrow neuronal cytosol. Data shown are specific binding (means ± SEM at each concentration) of [^{3}H]CORT in the presence (triangles) or absence (squares) of 10 nM RU486. Without RU486, the data is best fit by a two-site model with a K_d of 0.37 ± 0.13 nM and 11.49 ± 12.56 nM, respectively In the presence of RU486, binding was described by a one-site model with K_d = 0.25 ± 0.02 nM. Inset: Scatchard-Rosenthal replot of the data.

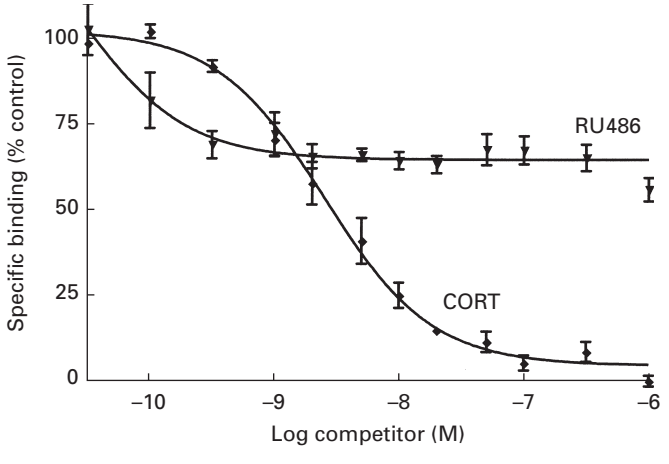

FIG. 2. Inhibition of 2 nM [^{3}H]CORT binding to house sparrow neuronal cytosol by unlabelled CORT and RU486. Shown are specific binding data, expressed as the percentage of [^{3}H]CORT specific binding in the absence of competitor.

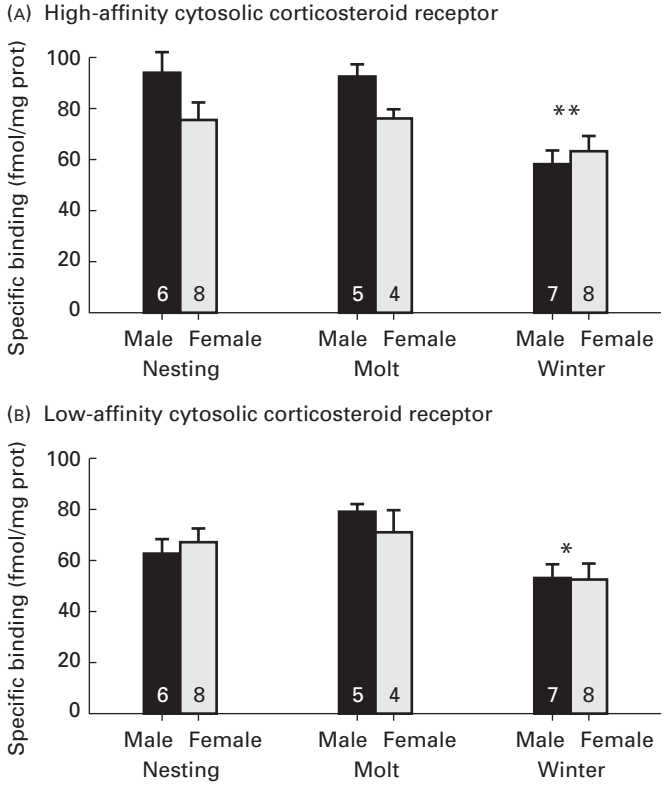

FIG. 3. Seasonal point sample analysis for the high-affinity (A) and low-affinity (B) cytosolic receptors. Data represent means ± SE of specific binding of 10 nM [^{3}H]CORT to house sparrow cytosol (corrected for protein content). High-affinity receptor capacity was determined in tubes containing 10 nM unlabelled RU486. Low-affinity receptor capacity was estimated by subtracting high-affinity receptor capacity from total specific binding. Each individual was assayed in triplicate. Number of individuals in each group is stated at the bottom of the bar. In both receptors, capacity is lower during winter than during nesting or molting (high affinity: **P = 0.0013, low affinity: *P < 0.002).

describing a high-affinity binding site (K_d = 0.25 ± 0.02 nM, B_{max} = 33.0 ± 0.72 fmol/mg prot; Fig. 1, [^{3}H]CORT + RU486 curve).

There were seasonal changes in receptor number in both high-and low-affinity cytosolic receptors (as determined by point sample analysis: ANOVA, high affinity: F = 8.038, P = 0.0013; low affinity: F = 7.52, P < 0.002), but no seasonal change in affinity (Table 1). In both receptor types, receptor number was significantly lower during winter than during

molting or nesting (Fig. 3A,B). There was a trend towards fewer high-affinity receptors in females than in males ($F = 3.60$, $P = 0.069$).

Membrane receptor

We found evidence for a membrane-associated corticosteroid receptor in the house sparrow brain. Equilibrium saturation data from April males were best fit by a one-site model (Fig. 4; $K_d = 19.0 \pm 2.2$ nM, $B_{max} = 174.6 \pm 11.7$ fmol/mg prot). While there was no seasonal change in affinity (Table 1), receptor number (as determined by individual point samples) was significantly lower during the nesting stage than during molting or wintering stages (Fig. 5; ANOVA, $F = 4.62$; $P < 0.015$; there was no effect of gender). B_{max} data combined from multiple saturation experiments mirror these differences (breeding: 222 ± 49 fmol/mg prot; molting: 354 ± 66 fmol/mg prot; wintering: 289 ± 22 fmol/mg prot).

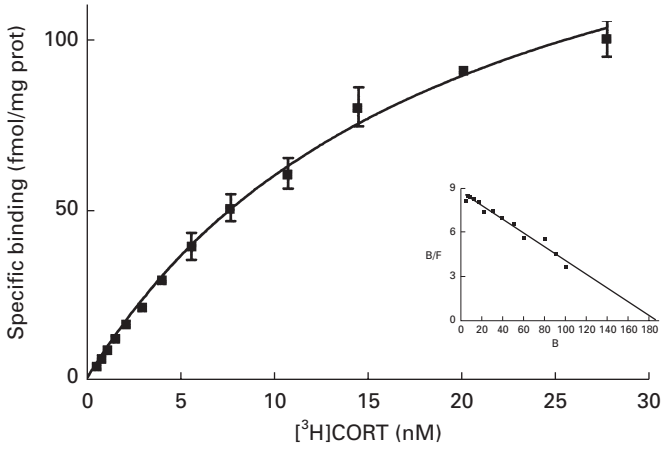

FIG. 4. Membrane-associated corticosteroid receptor: equilibrium saturation binding of radiolabelled cortocosterone [^{3}H]CORT to male house sparrow neuronal membranes in the nesting season. Data shown are specific binding (means and SEM at each concentration), corrected for protein concentration. Inset: Scatchard-Rosenthal replot of the data.

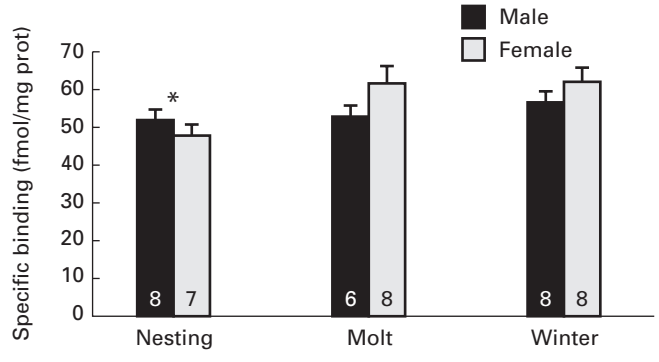

FIG. 5. Seasonal point sample analysis for the membrane-associated corticosteroid receptor. Data represent means ± SE of specific binding at 20 nM [^{3}H]CORT to house sparrow neuronal membranes. Each individual was assayed in triplicate. The number of individuals in each group is stated at the bottom of the bar. Capacity is significantly lower during nesting than during molt or winter (*$P < 0.015$).

CBG

Saturation data from molting males were best fit by a one-site model yielding a K_d of 1.8 ± 0.19 nM and B_{max} of 65.2 ± 2.3 nM (Fig. 6). The individual single point assays indicated that capacity was significantly different in each season tested. Capacity was highest during nesting, lower during winter, and lowest during molting (Fig. 7, ANOVA, $F = 28.0$, $P < 0.0001$). The trend towards a seasonal change in affinity was not significant (Table 1).

Corticosterone titres

Both baseline and stress-induced titres of total CORT differed seasonally. Baseline and stress-induced titres of total CORT were significantly higher during nesting than during molt and winter. (Fig. 8; ANOVA, baseline: $F = 6.39$, $P < 0.005$; stress-induced: $F = 34.37$, $P < 0.0001$).

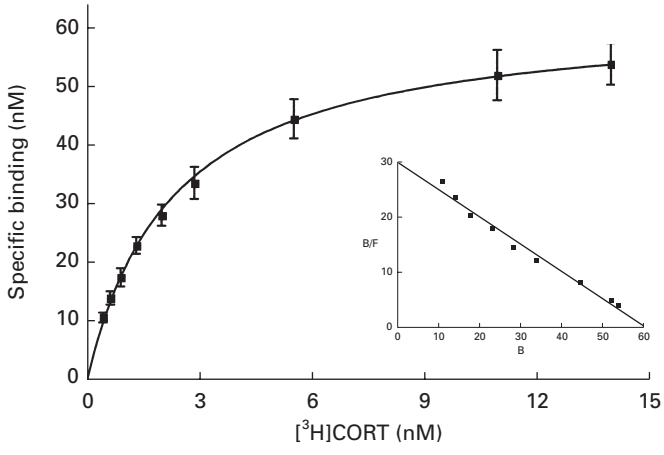

FIG. 6. Corticosteroid binding globulin: equilibrium saturation binding of radiolabelled cortocosterone [^{3}H]CORT to house sparrow plasma. Data shown are specific binding (means ± SE at each concentration). Data are best fit by a one-site model. Inset: Scatchard-Rosenthal replot of the data.

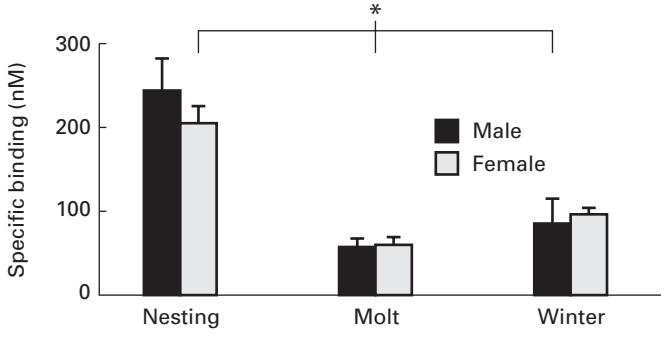

FIG. 7. Seasonal point sample analysis for corticosteroid binding globulin. Data represent means ± SE of specific binding at 8 nM [^{3}H]CORT. Each individual was assayed in triplicate, number of individuals in each group is listed at the bottom of the bar. corticosteroid binding globulin capacity is significantly different at each season tested. *$P < 0.0001$.

Using our affinity and capacity estimates for CBG, free CORT titres were estimated for each individual sparrow (Fig. 9). In contrast to total CORT, there were no significant seasonal differences in free CORT titres in either basal or stressed conditions (ANOVA, free baseline CORT: $F=1.6$, $P=0.22$; free stress-induced CORT: $F=0.048$, $P=0.95$).

Discussion

We have identified seasonal differences in several components on the stress response in house sparrows. The numbers of high and low affinity cytosolic corticosteroid receptors in house sparrow brain, presumably corresponding to MR- and GR-like molecules, respectively, were significantly lower during the winter season, whereas membrane-associated corticosteroid receptor number was significantly lower during the breeding season. This is the first study to demonstrate seasonal changes in the number of neuronal membrane-associated corticosteroid receptors, and these data provide a possible explanation for seasonal differences in rapid behavioural and physiological components of the stress response (40). We also found seasonal differences in basal and stress-induced levels of plasma CORT, and seasonal changes in the plasma concentrations of CBG.

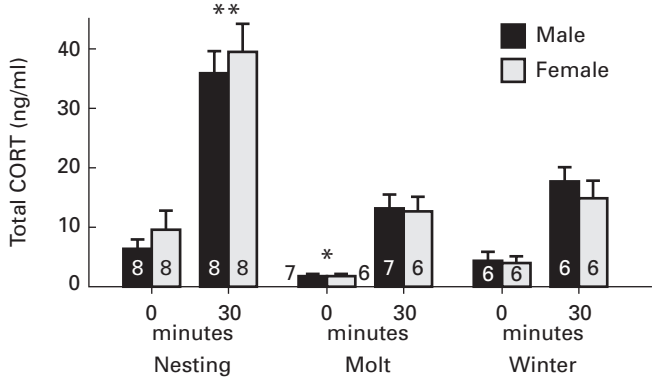

FIG. 8. Total baseline (0–3.5 min) and stressed (30 min) corticosterone (CORT) titres in male and female house sparrows over three seasons. Baseline and stress-induced samples were analysed separately. All data were log transformed prior to analysis. Baseline CORT is significantly lower during molt than during nesting or winter. Baseline and stress-induced CORT is significantly higher during nesting than during molting or winter. *$P<0.005$, **$P<0.0001$.

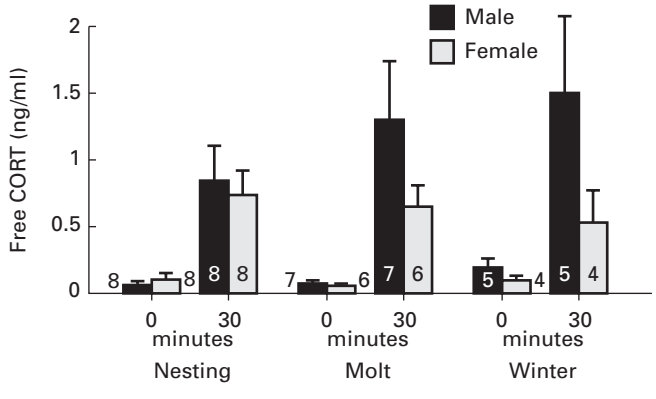

FIG. 9. Free baseline (0–3.5 min) and stressed (30 min) coerticosterone (CORT) titres in male and female house sparrows over three seasons. Using total CORT titres and affinity and capacity data for corticosteroid binding globulin, free CORT titres were estimated for each individual sparrow. Data are reported as means ± SE. Number of individuals in each group is listed at the bottom of the bar. There are no significant seasonal differences in free CORT.

Corticosteroid receptors

Cytosolic and membrane receptor numbers were regulated in opposite directions. This contrast suggests that the membrane receptors may serve different functions from the intracellular receptors. It is possible that membrane corticosteroid receptors in the brain mediate rapid behavioural responses to stress, whereas the intracellular receptors mediate many more enduring organizational, structural, synaptic and behavioural changes. It is also likely that the two receptors systems work together to regulate numerous brain functions.

This contrast in seasonal regulation also suggests that different mechanisms regulate the expression of membrane and cytosolic receptors. Corticosteroids regulate intracellular receptor number in rats over the short-term (hours to days), although the data are complex (41–44). However, CORT does not appear to be the primary regulator of the seasonal changes in receptor capacity in the house sparrow because there is no clear positive or negative correlation between baseline CORT and intracellular receptors. While reproductive hormones have strong seasonal patterns, they cannot be entirely responsible for seasonal regulation of corticosteroid receptors, as corticosteroid receptor levels change between molting and winter, whereas reproductive hormones do not. Two other hormones, melatonin and thyroxine, have highly seasonal secretion patterns. It is possible that either of these hormones may play a role in seasonal regulation of corticosteroid receptor capacity.

Very few studies have investigated the seasonal regulation of corticosteroid receptors. In a seasonal mammal, the golden hamster, a change from long-day to short-day photoperiod increases protein and mRNA levels of MR (15, 16), but not GR (15), in the hippocampus and hypothalamus. The increase in MR on short-days is accompanied by an apparent increase in the negative feedback in the hypothalamic-pituitary-adrenal axis (HPA) axis, seen in a more rapid termination of the stress response in short day hamsters. During winter, house sparrows show a blunted stress response to handling, but with only two timepoints of CORT secretion (0 and 30 min), we cannot determine if the low stress-induced CORT level is due to a less active HPA-axis, or an increase in negative feedback. In addition, high-affinity (MR-like) receptors in the house sparrow brain are at their lowest number during winter, suggesting that negative feedback mechanisms would be weakest at this point. Humans [nonseasonal animals, with no seasonal variation in glucocorticoid secretion (45–47)] show no seasonal change in glucocorticoid receptor capacity in lymphocytes (48). In captive female *Xenopus* held under natural photoperiods, there is a peak in [^{3}H]CORT binding capacity in the liver during August, concurrent with the lowest

level of baseline CORT (14). House sparrows also show higher levels of cytosolic receptors during long days (breeding and molt), but cytosolic receptor numbers show no correlation with basal or stress-induced total CORT levels.

We estimated seasonal differences in membrane receptor number through point sample experiments on individual sparrow brains and through equilibrium saturation binding analysis using pooled tissue. The former method compares specific binding at one concentration of [^{3}H]CORT, allowing for a measurement of individual variation, while minimizing the amount of radioligand and tissue used relative to equilibrium saturation binding experiments. Point sample experiments are only appropriate when there are no seasonal changes in receptor affinity. In contrast, equilibrium saturation binding analysis provides a more accurate measure of B_{max} (receptor capacity), but it is not feasible to perform such studies on multiple, individual brains. We used a nonsaturating concentration of radioligand (20 nM) in point sample assays for the membrane receptor because CORT and the plasma membrane lipid bilayer are both amphipathic, so at concentrations of [^{3}H]CORT above the K_d for house sparrow (26 nM), nonspecific binding of CORT to the membrane becomes a problem. At an estimated occupancy of 40% of membrane receptors, specific binding of 20 nM [^{3}H]CORT reflects a conservative estimate of changes in receptor number.

Corticosterone and CBG

As has been reported in many other species, total CORT titres (basal and stress-induced) varied seasonally in the house sparrow. Total basal and stress-induced CORT titres were significantly higher during nesting than during molting and winter. CBG was also seasonally regulated. In fact, CBG levels changed in parallel with total CORT levels, similar to the direction of regulation seen in a number of bird species (2, 8, 9, 12). Consequently, there were no significant seasonal differences in estimations of basal or stressed free CORT in house sparrow plasma. If protein-bound steroids are not biologically active [the 'free hormone hypothesis' (49)] then the large seasonal change in total CORT may not be physiologically relevant, and seasonal changes in receptor number may be a primary regulator in the seasonal variation of the behavioural response to stress.

However, studies in mammalian systems suggest a more complex role for CBG. Several studies indicate that the CBG–CORT complex itself is biologically active. In this case, increases in total CORT during the nesting season would result in higher levels of CBG–CORT-induced activity. There are specific binding sites for CBG in rat and human tissues (50–53), and the CBG-CORT complex can increase adenylate cyclase activity (50). The CBG–CORT complex may be internalized upon binding to the membrane receptor on MCF-7 cells (50, 54), potentially increasing the concentration of free CORT inside the cell. Alternatively, the CBG molecule may be proteolytically cleaved at specific sites, such as sites of inflammation, increasing the local concentration of free CORT (55–57). Taken together, CBG potentially regulates steroid action at several levels and warrants further study.

CBG levels can also change dramatically in response to a stressor. Food restriction, prolonged exercise, or social conflict can rapidly (within hours or days, depending on the stressor) decrease CBG levels by half (58–60) (S. Lynn, C. Breuner, and J. Wingfield, unpublished data on food restriction in sparrows). If the CBG–CORT complex is biologically active, less CBG will result in less biological activity (in a stressed house sparrow during nesting, a 50% reduction in CBG decreases the number of CORT-bound CBG molecules by approximately 20%). However, it also causes higher levels of free CORT (a 50% reduction in CBG causes a six-fold increase in free CORT in a stressed, nesting house sparrow). With lower total CORT during molt, a 50% cut in CBG capacity would only increase free CORT by three-fold. Hence, seasonal changes in total CORT may be physiologically relevant because (i) it would result in seasonal changes in CBG–CORT activity, and (ii) rapid decreases in CBG differentially affect free CORT levels in different seasons.

There were no significant gender-related differences in hormone levels, CBG capacity, or intracellular and membrane receptor levels. In species where parental care is highly gender specific, there can be differences between sexes in the sensitivity of the stress response during the nesting period (61). However, in the house sparrow, both sexes participate equally in nest building, incubation, brooding and feeding of fledglings (62).

In conclusion, we have demonstrated seasonal regulation of multiple components of the stress response in the house sparrow. While regulation of total CORT and CBG levels affect hormone availability to all tissues, regulation of receptor levels at the target organ could allow for a local change in sensitivity to stress. For example, an decrease in low-affinity cytosolic receptors in the liver may decrease the metabolic sensitivity to CORT, while at the same time an increase in membrane receptor levels in the song-control system may increase the sensitivity of song production to environmental perturbations. Integrated studies such as these should further our understanding of the functional significance of the stress response in free-living, seasonal animals.

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