

Development of stress reactivity in white-crowned sparrow nestlings: Total corticosterone response increases with age, while free corticosterone response remains low

Haruka Wada ^{a,*}, Thomas P. Hahn ^b, Creagh W. Breuner ^{a,c}

^a Integrative Biology, University of Texas at Austin, TX 78712-0253, USA

^b Section of Neurobiology, Physiology and Behavior, University of California Davis, CA 95616-8761, USA

^c Division of Biological Sciences, University of Montana, MT 59812, USA

Received 26 October 2005; revised 11 October 2006; accepted 21 October 2006

Available online 5 December 2006

Abstract

Activation of the adrenocortical response to stress during development can have fundamental consequences over the lifetime of the organism; as such, many organisms are less responsive to stress during critical developmental periods. In this study, we evaluated stress reactivity in nestling white-crowned sparrows, examining corticosterone and binding globulin levels in response to both restraint stress and ACTH challenge. Restraint stress induced a significant corticosterone response in both 4 to 6- and 7 to 9-day-old nestlings, but not in the youngest group (1–3 days); this non-significant increase in corticosterone in the youngest birds resembles the mammalian hyporesponsive period, wherein young animals are resistant to most stressors. Binding globulin levels appear to extend this period of low reactivity: when free corticosterone levels were calculated, only the oldest age group (7–9 day) showed a significant response to restraint. ACTH challenge data revealed that all ages of white-crowned sparrow nestlings responded to exogenous ACTH treatment with significant elevation of corticosterone, although early-stage nestlings did not reach adult levels of response.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Stress hyporesponsive period; Corticosteroid-binding globulin; Corticosterone; Stress; Ontogeny; Altricial; Nestling

1. Introduction

Free-living animals face a variety of challenges that can disrupt homeostasis. Activation of the hypothalamic–pituitary–adrenal (HPA) axis in response to these disruptions is a well-conserved phenomenon. In adults, acute increases in glucocorticoids are thought to increase fitness (reviewed in Sapolsky et al., 2000), but extended secretion can have deleterious consequences (Silverin, 1986; Wingfield and Silverin, 1986; Tokarz, 1987; Petitte and Etches, 1991). In developing animals, this trade-off appears more severe. While glucocorticoid elevation has been shown to benefit young in obtaining food and transitioning to independence (Heath, 1997;

Kitaysky et al., 2001a,b, 2003), there are potent, long-term negative effects, such as decreasing growth (Morici et al., 1997), immune function (reviewed in McEwen et al., 1997), and neuronal cell number (Howard and Benjamins, 1975). Hence, the far-reaching consequences of elevated glucocorticoid secretion during development require tight HPA-axis regulation in young as compared to adults.

Previous studies indicate the presence of a stress hyporesponsive period in rat pups (Haltmeyer et al., 1966; Butte et al., 1973; Martin et al., 1977; Schoenfield et al., 1980; Meaney et al., 1985; reviewed in Sapolsky and Meaney, 1986) and in rainbow trout (Barry et al., 1995). During this period, there is a reduction in the glucocorticoid response to stress. This reduction early in development is thought to minimize the long-term repercussions of elevated glucocorticoids. Although there is an extensive literature on the hyporesponsive period in rats, few studies address the phenomenon in birds.

* Corresponding author. Fax: +1 512 471 3878.

E-mail address: haruka@mail.utexas.edu (H. Wada).

In addition to suppression of the HPA axis, organisms have multiple mechanisms to regulate the amount of glucocorticoids reaching tissues. Corticosteroid-binding globulin (CBG) may provide one such mechanism. The majority of plasma steroid is bound by binding globulins, with only 5–10% circulating unbound, or ‘free’ (Ekins, 1990). According to the free hormone hypothesis, only free hormones can enter tissues or be broken down; the free fraction is, therefore, thought to be the biologically active form of the steroid (Ekins, 1990; Breuner and Orchinik, 2002). If the free hormone hypothesis is correct, increasing CBG early in development may limit bioavailability of glucocorticoids during this critical period (Breuner et al., 2003; Lynn et al., 2003). However, the role of CBG in regulating bioavailability of CORT is currently under debate. In this light, we examine both total and free CORT levels, allowing for interpretation of data based on either the total or free hormone hypotheses.

Toward this end, we explored the basic ontogeny of the adrenocortical response in nestling white-crowned sparrows, using standardized restraint stress (Wingfield, 1994). Furthermore, we performed adrenocorticotrophic hormone (ACTH) challenges to assess developmental progression of the adrenals. These data will allow us to evaluate how total and free hormone levels are regulated in relation to crucial developmental stages in white-crowned sparrow nestlings. Periods of low stress reactivity would suggest protection from the deleterious effects of elevated corticosterone during critical developmental stages.

2. Materials and methods

2.1. Study animals

Nuttall’s white-crowned sparrow nestlings (*Zonotrichia leucophrys nuttalli*) were taken from nests at Bodega Marine Laboratory, University of California at Davis. Stress series samples (see below) were collected April through May of 2004, and ACTH challenges were performed April through June of 2003 and April through May of 2004.

White-crowned sparrow nestlings fledge around 10 days of age (Morton, 2002). During this 10-day nestling period, young go through dramatic developmental changes which include nearly an order of magnitude increase in body mass, feather growth, and acquisition of thermoregulatory ability, alertness, and coordinated movements (Morton, 2002). In this study, the nestling period is divided into three stages: days 1–3 (early nestling stage), days 4–6 (middle nestling stage), and days 7–9 (late nestling stage) (see Section 4 for description of the development during each stage).

Age of nestlings was estimated by (1) noting date of hatch (when nests are found in the egg stage) or (2) comparing developmental measures collected on the nestlings to the developmental schedule described in Morton (2002) and to personal observations of known age nestlings. Nests were randomly assigned to one of the three age categories for sampling. Each nest was sampled only once, and only one nestling per nest was sampled to avoid unequal representation of siblings in the three age groups. Sex was determined for late-stage nestling samples.

2.2. Stress protocol

To measure reactivity of the HPA axis, we used a standardized capture and handling protocol described in Wingfield (1994). Plasma corticosterone levels are known to rise significantly within 3 min of capture (Wing-

field et al., 1982). However, in this study corticosterone had still not begun to rise by 4 min after nest disturbance (Fig. 1). Therefore, blood samples collected within 4 min of nest disturbance are used as baseline samples. After the initial sampling, nestlings were placed in a cloth bag and additional samples were collected at 15, 30, and 60 min. To avoid potential complications caused by changing nestling body temperature under different field conditions, all were kept inside a jacket to keep them near human body temperature. To collect blood samples, the alar vein was punctured with a 26G needle and 20–30 μ L of blood were collected into heparinized microhematocrit capillary tubes. The blood samples were kept cold on ice until the end of the day when they were centrifuged to separate plasma from the cellular fraction. The plasma samples were then kept at -20°C or below until the assays.

In addition to blood samples, developmental measurements (tarsus and wing length, body mass, feather development, and alertness) were taken. To minimize the time nestlings were away from the nest, those developmental measurements were collected between the 15 and 45 min samples.

2.3. ACTH challenges

Similar to the stress response protocol, baseline samples were collected within 4 min of capture. After the initial sampling, nestlings and adults were weighed and injected with ACTH (Sigma: 100 IU/kg body weight) in saline or saline alone into the jugular vein. The post-treatment samples were collected 30 min after the injection.

2.4. Measurement of corticosterone: EIA assay

Serum corticosterone levels were detected using Enzyme Immunoassay (EIA) kits (Cat No. 901–097, Assay Designs). In this assay, raw plasma is added directly to the wells without extraction; steroid displacement buffer (SDB) is added prior to the assay to degrade binding globulins. For every new species we optimize the assay using a protocol that is a bit more complicated than the usual RIA optimization method. To ensure that plasma is not interfering with hormone measurements, we strip plasma to remove any endogenous hormone and spike to a known amount of CORT. This way, as we dilute plasma (into a buffer containing the same amount of CORT) we will be able to measure directly how much interference is caused by the plasma, outside of variance introduced by unknown levels of hormone in the plasma. An additional complication is that Assay Designs provides a ‘steroid displacement buffer’ to degrade binding globulins. This SDB is a protease, and we have found that it can produce significant interference if used at too high a concentration. Therefore, our optimization incorporates plasma dilutions at multiple concentrations of SDB.

Optimization Protocol: Plasma dilution and SDB concentration were optimized using stripped, spiked plasma (stripped with 1% norit A Charcoal and 0.1% dextran in water, then spiked to ≈ 500 pg/ml with CORT standard from the assay) diluted into a buffer of known corticosterone concentration (~ 500 pg/ml). Samples were run against a standard curve at plasma dilutions of 1:10, 1:20, 1:40 and 1:60, each with 0, 1, and 2% SDB (% of raw plasma volume). Results are shown in Fig. 2. Each point represents the value of CORT given by the assay at each plasma dilution for each concentration of SDB. The grey box represents the mean \pm SEM measured from the dilution buffer alone (the CORT-spiked buffer used to dilute the plasma, so representing the expected CORT level in the samples), giving a range of acceptable values for CORT from the assay.

For the corticosterone EIA of white-crowned sparrow plasma, a plasma dilution of 1:40 or greater with 1% SDB (per raw plasma volume) eliminated measurable effects of plasma on the assay (Fig. 2). Optimizing SDB concentration is critical, as higher concentrations appear to degrade antibody activity in the assay, artificially increasing estimated corticosterone amounts in wells.

To determine corticosterone levels, 10 μ L 1:100 dilution of SDB buffer were added to 10 μ L plasma. After 5 min, 380 μ L assay buffer were added to each sample, vortexed, and aliquoted to individual wells in the assay plate

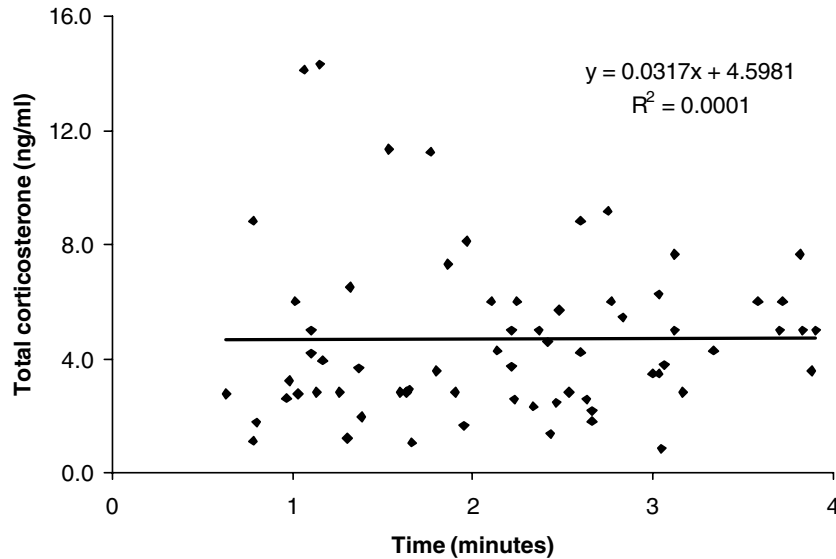


Fig. 1. Baseline corticosterone levels in relation to time after capture.

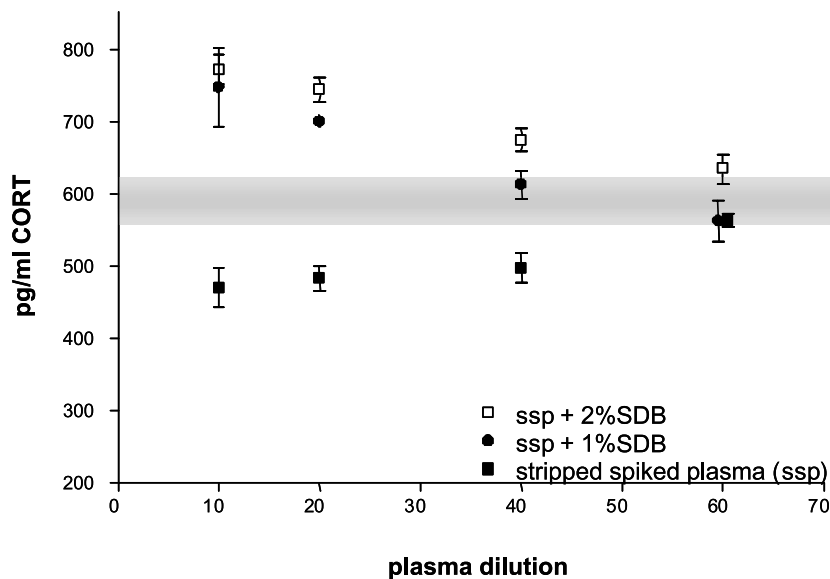


Fig. 2. Optimization of EIA corticosterone assay for white-crowned sparrows testing plasma dilution and % steroid displacement buffer (SDB). Plasma was stripped and spiked to a known amount prior to the assay, and plasma was diluted into a buffer with the same value of CORT. As the dilution factor increases, the values become closer to the actual corticosterone levels added. The grey box represents means \pm SEM measured from the dilution buffer alone (the CORT-spiked buffer used to dilute the stripped, spiked plasma, so representing the expected CORT level in the samples), giving a range of acceptable values for CORT from the assay. A plasma dilution of 1:40 or higher, with 1% SDB, eliminated any measurable effects of plasma in the assay.

(100 μ l sample per well, each in triplicate). The standard curve was measured in triplicate as well, with six standards ranging from 2000 to 15.63 pg/ml (100 μ l/well). A separate, external standard of 200 pg/ml corticosterone was run in triplicate on every plate. For the first incubation with conjugated corticosterone and antibody, the plate was shaken for 2 h in a 26 $^{\circ}$ C incubator. For the second incubation with substrate solution, the plate was incubated at the same temperature for 1 h without shaking. After adding stop solution, the plate was read immediately on a Multiskan Ascent microplate reader at 405 nm corrected at 595 nm. Samples were completely randomized within and across plates. Inter- and intra-plate coefficient of variations were 21.5% and 7.98%, respectively, and detectability was 6.5 pg/well (2.6 ng/ml). The detection limit was determined by taking two standard deviations away from the mean of

the blank wells. In EIA assays, total-binding wells (B0) receive only buffer, conjugate, and antibody. Thus we used these total-binding wells as blanks to determine the detection limit. This limit was assigned to undetectable samples in the assay.

2.5. Measurement of corticosteroid-binding globulin

Serum CBG levels were measured using a ligand-binding assay with tritiated corticosterone (described in Breuner et al., 2003). Previously, the CBG assay was characterized for white-crowned sparrow plasma (Lynn et al., 2003); optimal plasma dilution was 1:900 with an incubation period of 2 h at 4 $^{\circ}$ C. In these assays, total binding was determined using 50 μ L buffer, 50 μ L 3 H corticosterone, and 50 μ L stripped plasma.

Non-specific binding was determined using 1 μM unlabeled corticosterone instead of buffer. The affinity (K_d) of corticosterone for CBG at different ages was determined by equilibrium saturation binding assay. This was done using plasma pools for the three age classes and ^3H corticosterone between 0.30 and 13.25 nM. CBG capacity in individual samples was identified with a point sample assay using 18.9 nM ^3H corticosterone. Based on characterization assays, this ligand concentration should occupy 84.5% of the total binding sites. Therefore, we adjusted the CBG capacities to 100% for the free hormone analysis. Since CBG levels do not change within 1 h of corticosterone elevation in white-crowned sparrows (Breuner et al., 2006), plasma from any time point within 60 min was used for the CBG assay. Intraassay variation for the point sample assay was 14.6%.

Free hormone levels were estimated using an equation by Barsano and Baumann (1989):

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

where K_a is $1/K_d$ (nM), K_d is affinity of corticosterone for CBG, B_{max} is total CBG capacity, and H_{total} is total plasma hormone concentration. K_d was determined in equilibrium binding analysis using pooled plasma.

2.6. Statistics

The statistical analysis was done using SPSS 11.5 and GraphPad Prism 3.02, except for the 2-segment breakpoint analysis on free CORT (Fig. 8), which was completed in S+7. The effects of handling, age, and ACTH treatment on total and free corticosterone levels were determined by repeated measures ANOVA, followed by Bonferonni correction (stress response and ACTH studies were analyzed separately). CBG capacities were log transformed to correct for heteroscedasticity and the effect of age was determined using one-way ANOVA, followed by Tukey HSD. One-way ANOVA was used to compare the K_d s from three age groups. Data were considered to be significant when $P \leq 0.05$. Data are presented as means \pm SE.

When total corticosterone exceeds CBG capacity, it results in greatly elevated free corticosterone estimation. When the resultant free levels fell outside of two standard deviations of the mean, those values were excluded from the analysis (three out of 94 total samples).

3. Results

3.1. Total corticosterone levels

Overall, handling ($F_{3,18} = 17.23$, $P < 0.001$), age ($F_{2,19} = 20.38$, $P < 0.001$), and the interaction between handling and age ($F_{6,15} = 4.95$, $P < 0.001$) all contributed significantly to total corticosterone levels (Fig. 3). Pairwise comparison results show that the corticosterone levels of earlier two stages (D1–3 and 4–6) are significantly different from the later stage, D7–9 (D1–3 and D4–6, $P < 0.05$; see Table 1 for corrected and non-corrected P values). By 30 min, D7–9 corticosterone levels were significantly higher than those of the earlier two stages. Furthermore, while early-stage nestlings (D1–3) show no significant increase in corticosterone over the 60 min protocol (15, 30, and 60 min samples against 0 min sample: pairwise comparison $P > 0.05$; see Table 1 for pairwise comparison and Bonferonni correction P values), nestlings from latter two stages responded to handling stress with significant increase in corticosterone (D4–6 and D7–9; $P < 0.05$; again, see Table 1). Baseline levels did not differ between age groups ($P > 0.05$). The low n in this study (combined with the Bonferonni corrections) increases the potential for a Type II error. In addition, it is difficult to determine whether an absence of a statistically significant CORT response repre-

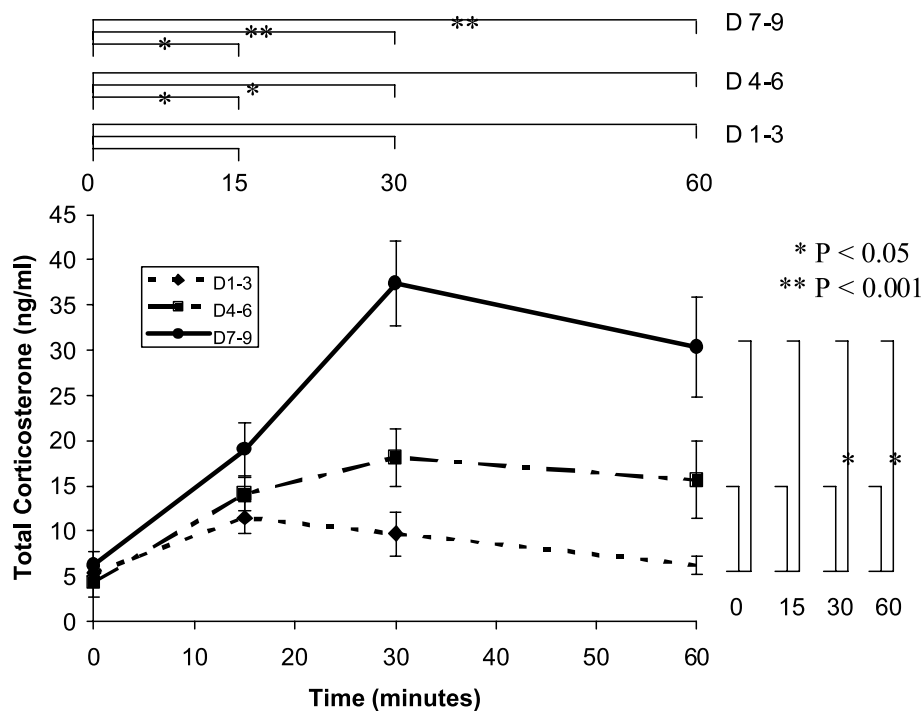


Fig. 3. Changes in total corticosterone levels in response to handling stress. Data are means \pm SE ($n = 9, 8, \text{ and } 7$). Both handling and age had significant effects on total corticosterone levels.

Table 1
Bonferonni corrected and non-corrected *P* values

		Bonferonni	Non-corrected
<i>Total corticosterone</i>			
Age	D1–3 < D7–9	<0.001	<0.001
	D4–6 < D7–9	0.003	0.001
Time (B0 to others)	D1–3	0.215, 1.00, and 1.00	0.036, 0.0190, and 0.816
	D4–6	0.035, 0.010, and 0.056	0.006, 0.002, and 0.009
	D7–9	0.006, <0.001, and <0.001	0.001, <0.001, and <0.001
Baseline	D1–3 = D4–6 = D7–9	1.000, 1.000, and 1.000	0.662 and 0.808
<i>Free corticosterone</i>			
Time	D1–3	0.100, 0.329, and 1.00	0.017, 0.055, and 0.966
	D4–6	0.557, 0.141, and 0.114	0.093, 0.023, and 0.019
	D7–9	1.00, 0.004, and 0.147	0.646, 0.001, and 0.025
<i>ACTH challenge</i>			
Age (overall)	D1–3 = D4–6	0.247	0.041
	D1–3, D4–6 < D7–9	<0.001 and 0.016	<0.001 and 0.003
	D7–9 < Adult	0.012	0.002
Age (ACTH response)	D1–3 < D7–9	0.002	<0.001
	D7–9 = Adult	1.000	0.204
ACTH vs. Saline	D1–3	0.063	0.063
	D4–6	0.005	0.005
	D7–9	0.001	0.001

sents an absence of effect at the biological level (what increase in CORT is biologically relevant for the animal?). In this light, we choose to discuss the data in terms of ‘limited stress reactivity’ in the younger age groups, as opposed to ‘no stress response’ as indicated by the statistics.

3.2. Corticosteroid-binding globulin

The affinity (K_d) of corticosterone for CBG did not change throughout the nestling period (Fig. 4, day 2–3,

4–6, and 7–9 nestlings = 3.13 ± 0.60 nM, 3.12 ± 0.33 nM, and 4.19 ± 0.67 nM, respectively; $P = 0.304$). Additionally, while mean CBG capacity increased with age, this difference was not significant ($F_{2,21} = 2.06$, $P = 0.15$, Fig. 5).

3.3. Free corticosterone levels

There were significant overall effects of time (0–60 min: $F_{2,17} = 5.97$, $P = 0.001$) and interaction between time and age ($F_{6,13} = 2.53$, $P = 0.03$; Fig. 6), but no main effect of

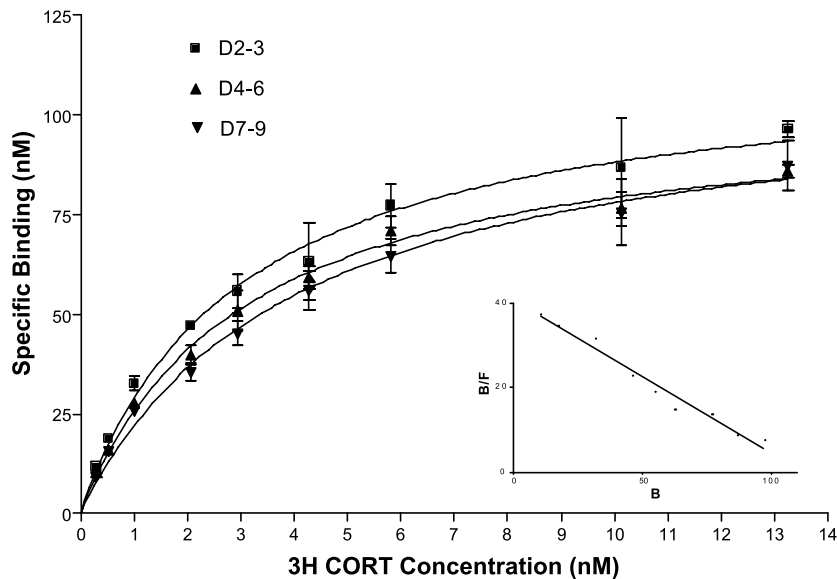


Fig. 4. Equilibrium saturation binding of [³H]CORT to nestling plasma at D2–3, D4–6, and D7–9. Data shown are specific binding (means ± SE at each concentration). Data are best fit by a one-site model, K_d s do not differ between age groups. Inset: Scatchard–Rosenthal replot of the data (for clarity, only D2–3 data is shown).

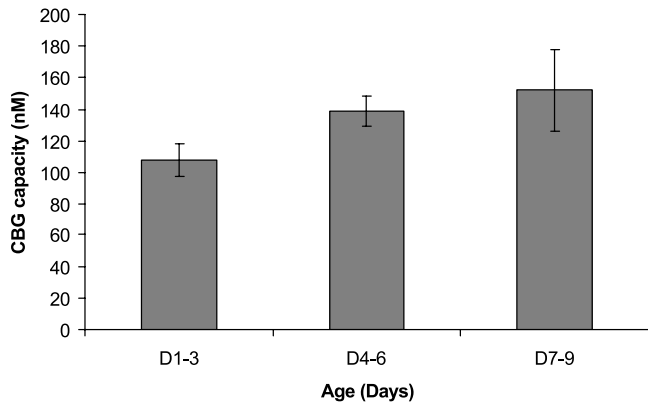


Fig. 5. Corticosteroid binding globulin capacity during 10-day nestling period. Data are means \pm SE ($n = 9, 8,$ and 7). There was a trend towards increasing CBG with age.

age ($F_{2,17} = 0.68, P = 0.52$). Pairwise comparisons determined that early- and middle-stage nestlings showed no significant response in free CORT levels (D1–3 $P > 0.05$; D4–6 $P > 0.05$; see Table 1), while late-stage nestlings showed a robust response ($P < 0.05$).

3.4. ACTH challenges

Overall, time, age, and treatment had significant effects on total corticosterone levels in nestlings challenged with ACTH ($F_{1,43} = 140.88, P < 0.001$; $F_{3,41} = 13.04, P < 0.001$; $F_{1,43} = 21.68, P < 0.001$, respectively, Fig. 7). In addition, there were significant interactions between time and age ($F_{3,41} = 7.77, P < 0.001$), and time and treatment ($F_{1,43} = 19.90, P < 0.001$).

All ages responded to ACTH injection with a significant increase in corticosterone ($F_{1,43} = 21.68, P < 0.001$). Nonetheless, the response in the early-stage nestlings was significantly lower than that of the late-stage nestlings

($P < 0.05$). Furthermore, the response in the late-stage nestlings was not significantly different from that of adults ($P > 0.05$). When ACTH and saline treatment groups were compared, ACTH elicited a significantly stronger response than saline in the later two stages ($P < 0.05$). The early-stage nestlings show a strong trend as well.

4. Discussion

White-crowned sparrow nestlings show an extremely diminished corticosterone response to restraint during the first 3 days post-hatch. In addition, CBG capacity increases with age. This CBG increase with age further diminishes the glucocorticoid response to stress, especially in the middle age group. As a result, white-crowned sparrow nestlings show low stress-reactivity (in free CORT) lasting through the first six days of the 10-day nestling period.

It is difficult to visually evaluate this extension of low reactivity from Figs. 3 and 6 (total and free CORT at 0, 15, 30, and 60 min). To more clearly exemplify the shift in patterns of CORT secretion between total and free, we have calculated an integrated measure of CORT.

Integrated CORT is a measure of the total amount of CORT secreted over the hour of restraint stress (expressed as ng/ml/hr); with only one value for each individual, it simplifies the comparisons between individuals, ages, and total or free (as used in Breuner et al., 1998). Regression analysis of total integrated CORT by age shows a linear increase over the 9 days of the nesting cycle (Fig. 8A: $R^2 = 0.66$, data were best fit by a linear model). However, regression analysis of free integrated CORT shows that CORT response to stress remains low over the first 6 days of development (Fig. 8B: $R^2 = 0.64$, 2-segment linear regression (following Duggleby and Ward, 1991) identifies the best-fit breakpoint at 6.49 days). Hence, whether one favors the total or free hormone hypothesis will lead to vastly different conclusions

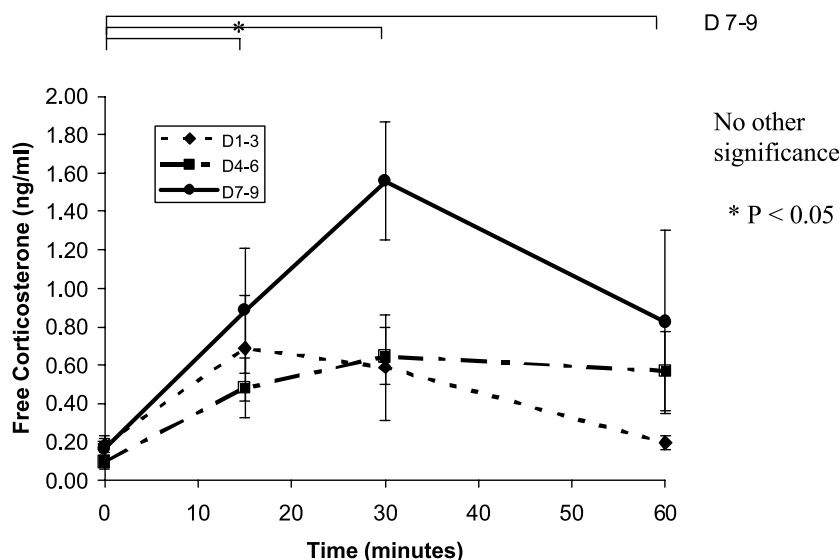


Fig. 6. Changes in free corticosterone levels in response to handling stress during the 10-day nestling period. $N = 9, 7,$ and 6 .

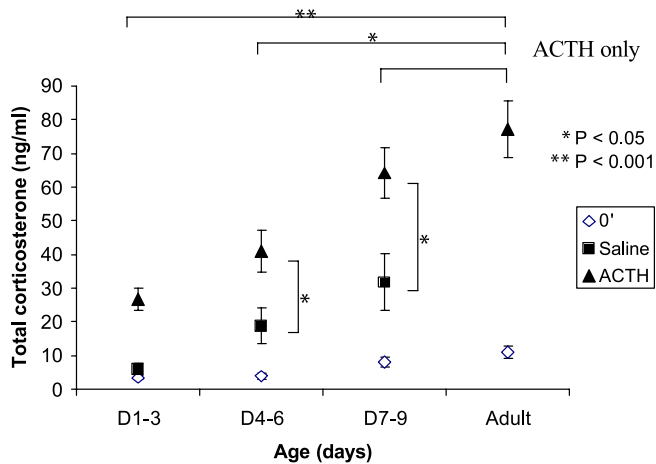


Fig. 7. Corticosterone secretion in response to jugular injection of saline or ACTH. Data shown are means \pm SE (sample size for 0', saline, and ACTH groups are: early nestling stage, 11, 6, and 6; middle nestling stage, 19, 9, and 11; late nestling stage, 15, 9, and 7; adult, 5 and 5, respectively). This graph combines 0' from both treatments, however, they were evaluated separately for statistics.

on the development of stress reactivity in nestling white-crowned sparrows.

The hyporesponsive period has been described mainly in mammals and some in fish. In rainbow trout, larvae are resistant to handling and cold shock stress until two weeks post-hatch (Barry et al., 1995). In rats, baseline cortisol levels decline after birth and remain low until the middle of the second week (Martin et al., 1977; Meaney et al., 1985; reviewed in Sapolsky and Meaney, 1986). At the same time, rat pups fail to respond to various stressors such as histamine (Butte et al., 1973), shock and heat (Haltmeyer et al., 1966), and ether (Schoenfield et al., 1980) during this time.

The patterns of CBG in postnatal development have been investigated in some mammals, but the literature shows mixed results. In neonatal pigs, cortisol remains constant from day one through ten; however, due to an increase in CBG, free cortisol levels decline with age (Heo et al., 2003; note that it is not clear whether these samples represent baseline or stress-induced levels). Rat

pups, on the other hand, have extremely low levels of CBG pre-weaning. Six-day-old pup CBG is less than 3% of adult levels, but rises to 25% of adult levels by day 15 (Viau et al., 1996). Some studies suggest that cortisol and CBG follow very similar patterns resulting in similar changes in total and free cortisol (Henning, 1978). However, others indicate that an increase in CBG precedes the postnatal elevation of cortisol, which would keep the free levels low (Tinnikov, 1993).

In this study, we also observed a period of low stress reactivity (with no significant increase in CORT) in the first 3–6 days of development. This appears functionally equivalent to mammalian stress hyporesponsive period. This hyporesponsive period in young is thought to be adaptive due to the deleterious effects of glucocorticoids on growth and development (Sapolsky and Meaney, 1986). As noted above, chronically (weeks to months) elevated levels of glucocorticoids during development are known to suppress growth (Morici et al., 1997) and protein synthesis, damage neuronal cells (Howard and Benjamins, 1975), and alter development of cognition (Kitaysky et al., 2003) and the HPA axis (see Seckl, 2001). Thus, avoiding high levels of the hormone during a critical period in development may be adaptive (Sapolsky and Meaney, 1986). This may be especially important for species which undergo substantial developmental changes after birth/hatching.

In birds, a hyporesponsive period could be important for a variety of reasons. Many passerines gain 90% of adult body mass during the first 10–20 days of life (Ricklefs, 1968). Similarly, altricial white-crowned sparrow nestlings go through major developmental changes within the first 3 days of their lives (Morton, 2002). This includes eye development and maximum increase in body size (Morton and Carey, 1971). During this period, nestlings show a nearly logarithmic increase in body mass. Therefore, avoiding exposure to corticosterone may be needed and adaptive since young are increasing tissue mass and developing essential organs.

The middle stage (D4–6) is critical for development as well. During this period, nestlings open their eyes, complete

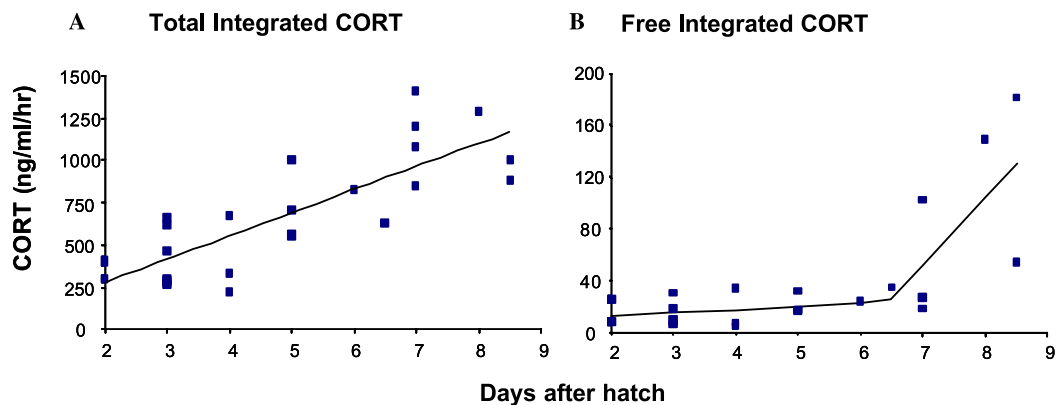


Fig. 8. Integrated corticosterone: each point represents a calculation of the cumulative amount of corticosterone [either total (A) or free (B) corticosterone] secreted in each individual nestling over the hour of restraint stress. Data are plotted by age of nestling. These data help illuminate the extension of low stress-reactivity (into days 4–6) when free CORT is considered.

body mass growth, and increase alertness and coordination of movements (Morton and Carey, 1971; Morton, 2002). By the end of this stage, coordination of the movements is nearly established and the transition from ectothermy to endothermy has been made. Based on total corticosterone, the 'hypo-responsive' period is most extreme during only the first three days post-hatch. However, when free corticosterone is estimated, corticosterone response is indistinguishable between early and middle-stage nestlings. Thus, CBG may be one component of a mechanism to further protect the vulnerable tissues from corticosterone exposure during development.

One of the beneficial effects of corticosterone in adults is an increase in activity levels (Breuner et al., 1998). In the early-stage nestlings, induction of escape behavior is not adaptive (Sims and Holberton, 2000). Interestingly, the only age group that shows a robust increase in free corticosterone is the late nestling stage (D7–9). By this age, nestlings can fledge if they are threatened and can move away from the nest (Morton, 2002).

Corticosterone secretion in both sexes of late-stage nestlings appears to be identical until 30 min of handling (data not shown). The sample size is too small to run statistics here; however, it is an intriguing question to explore when sex difference in adrenocortical response appear in altricial species.

The developmental status of newly hatched nestlings varies among species. Precocial nestlings, for instance, can walk, feed, and thermoregulate. On the other hand, altricial nestlings are nest-bound and completely dependent on parents for feeding and brooding. Considering the degree of development these nestlings go through after hatching, suppressed stress responses may be more important in altricial than precocial young. Several studies support this expectation. Semialtricial nestlings gradually increase adrenocortical response to handling with age, and show adult-like responses immediately before fledging (American kestrel *Falco sparverius*, Love et al., 2003; Magellanic penguin, *Spheniscus magellanicus*, Walker et al., 2005). In contrast, altricial Northern mockingbird nestlings (*Mimus polyglottos*, Sims and Holberton, 2000) show little or no stress response any time during the nestling period. In this study, we found that altricial nestlings *could* respond to restraint stress before fledging, however responses did not reach adult-like levels before fledging.

4.1. ACTH challenge

Early-stage white-crowned sparrow nestlings secreted little to no corticosterone in response to handling stress. ACTH challenge was used to understand the role of the adrenal during this hypo-responsive period. Early stage adrenals were unable to produce adult-like responses to ACTH, whereas late-stage adrenals could. These data indicate adrenals contribute to the low stress reactivity seen in these early-stage nestlings. However, early-stage adrenals did show a significant corticosterone response to ACTH,

where little to no response occurred with restraint stress alone (similar to mammals; Hiroshige and Sato, 1970; Cote and Yasumura, 1975). This indicates that the low reactivity is also due to dampened up-stream control of pituitary, hypothalamus, or neuronal input to hypothalamus.

We observed some discrepancy in baseline total corticosterone data in the different parts of our study. Stress series data suggest there was no increase in baseline corticosterone with age. However, ACTH data indicate that baseline levels increase significantly between the earlier two stages (D1–6) and the late stage (D7–9). Group means between the two studies are similar however variation is greater in the stress series study. When all the baseline data from two studies are pooled the baseline corticosterone levels increase significantly between D1–6 and D7–9 (one-way ANOVA, $F_{2,66} = 5.77$, $P = 0.005$).

5. Conclusions

This is one of the first studies to demonstrate that altricial nestlings can respond to a stressor before fledging. Nonetheless, white-crowned sparrow nestlings have a period of extremely low stress reactivity during the first three days of 10-day nestling period. This period is extended through day six when considering free corticosterone, due to increasing CBG. This may be adaptive to further protect developing tissue from excessive exposure to corticosterone.

Is this period of low stress reactivity due to specific regulation of corticosterone and binding globulins, or simply to the developmental progression of the HPA axis and liver? We are unable to assess whether the low levels of CORT after hatch are a suppression of a previously able adrenal, since we do not have CORT responses to stress pre-hatch. Chickens shows a decline in responsiveness just post-hatch (Freeman and Manning, 1984), but evidence from a precocial species is difficult to transfer to an altricial one. In white-crowned sparrows, we hypothesize that the increasing stress reactivity is primarily due to development of the organs; however, as a result of their differential rates of development (CBG levels increase at a faster rate than total CORT), free CORT shows a more extreme hypo-responsive period later in development.

Acknowledgments

We thank Bodega Marine Laboratory for providing laboratory space, an access to the reserve, and housing. We also thank Leslie Jarmon for editorial assistance, Glennis Julian for assistance in EIA assays, Art Woods and Dave Patterson for statistical assistance, L. Michael Romero for assistance in ACTH challenge techniques, and Terri Pope and Alaina Thomas for their help in the field. This research was funded by the National Science Foundation: IBN 0236536 to C.W. Breuner, and IBN-0235911 to T.P. Hahn.

References

- Barry, T.P., Malison, J.A., Held, J.A., Parrish, J.J., 1995. Ontogeny of the cortisol stress response in larval rainbow trout. *Gen. Comp. Endocrinol.* 97, 57–65.
- Barsano, C.P., 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.
- Breuner, C.W., Greenberg, A.L., Wingfield, J.C., 1998. Non-invasive corticosterone treatment rapidly increases activity in Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*). *Gen. Comp. Endocrinol.* 111, 386–394.
- Breuner, C.W., Orchinik, M., 2002. Beyond carrier proteins: plasma binding proteins as mediators of corticosteroid action in vertebrates. *J. Endocrinol.* 175, 99–112.
- Breuner, C.W., Orchinik, M., Hahn, T.P., Meddle, S.L., Moore, I.T., Owen-Ashley, N.T., Sperry, T.S., Wingfield, J.C., 2003. Differential mechanisms for plasticity of the stress response across latitudinal gradients. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 285, 594–600.
- Breuner, C.W., Lynn, S.E., Julian, G.E., Cornelius, J.M., Heidinger, B.J., Love, O.P., Sprague, R.S., Wada, H., Whitman, B.A., 2006. Plasma binding globulins and the acute stress response. *Horm. Metab. Res.* 38, 260–268.
- Butte, C., Kakihana, R., Farnham, M.L., Noble, E.P., 1973. The relationship between brain and plasma corticosterone stress in developing rats. *Endocrinology* 92, 1775–1779.
- Cote, T.E., Yasumura, S., 1975. Effect of ACTH and histamine stress on serum corticosterone and adrenal cyclic AMP levels in immature rats. *Endocrinology* 96, 1044–1047.
- Duggleby, R.G., Ward, L.C., 1991. Analysis of physiological data characterized by 2 regimes separated by an abrupt transition. *Physiol. Zool.* 64, 885–889.
- Ekins, R., 1990. Measurement of free hormones in blood. *Endocr. Rev.* 11, 5–46.
- Freeman, B.M., Manning, A.C.C., 1984. Re-establishment of the stress response in *Gallus domesticus* after hatching. *Comp. Biochem. Physiol. A* 78, 267–270.
- Haltmeyer, G.C., Denenberg, V.H., Thatcher, J., Zarrow, M.X., 1966. Response of the adrenal cortex of the neonatal rat after subjection to stress. *Nature* 212, 1371.
- Heath, J., 1997. Corticosterone levels during nest departure of juvenile American kestrels. *Condor* 99, 806–811.
- Henning, S.J., 1978. Plasma concentrations of total and free corticosterone during development in the rat. *Am. J. Physiol.* 235 (Endocrinol. Metab. Gastrointest. Physiol. 4), E451–E456.
- Heo, J., Kattesh, H.G., Roberts, M.P., Schneider, J.F., 2003. Plasma levels of cortisol and corticosteroid-binding globulin (CBG) and hepatic CBG mRNA expression in pre- and postnatal pigs. *Domest. Anim. Endocrinol.* 25, 263–273.
- Hiroshige, T., Sato, T., 1970. Changes in hypothalamic content of corticotropin-releasing activity following stress during neonatal maturation in the rat. *Neuroendocrinology* 7, 257–270.
- Howard, E., Benjamins, J.A., 1975. DNA, ganglioside and sulfatide in brains of rats given corticosterone in infancy, with an estimate of cell loss during development. *Brain Res.* 92, 73–87.
- Kitaysky, A.S., Kitaiskaia, E.V., Wingfield, J.C., Piatt, J.F., 2001a. Dietary restriction causes chronic elevation of corticosterone and enhances stress response in red-legged kittiwake chicks. *J. Comp. Physiol. B* 171, 701–709.
- Kitaysky, A.S., Wingfield, J.C., Piatt, J.F., 2001b. Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. *Behav. Ecol.* 12, 619–625.
- Kitaysky, A.S., Kitaiskaia, E.V., Piatt, J.F., Wingfield, J.C., 2003. Benefits and costs of increased levels of corticosterone in seabird chicks. *Horm. Behav.* 43, 140–149.
- Love, O.P., Bird, D.M., Shutt, L.J., 2003. Corticosterone levels during post-natal development in captive American kestrels (*Falco sparverius*). *Gen. Comp. Endocrinol.* 130, 135–141.
- Lynn, S.E., Breuner, C.W., Wingfield, J.C., 2003. Short-term fasting affects locomotor activity, corticosterone, and corticosterone binding globulin in a migratory songbird. *Horm. Behav.* 43, 150–157.
- Martin, C.E., Cake, M.H., Hartmann, P.E., Cook, I.F., 1977. Relationship between foetal corticosteroids, maternal progesterone, and parturition in the rat. *Acta Endocrinol.* 84, 203–207.
- McEwen, B.S., Biron, C.A., Brunson, K.W., Bulloch, K., Chambers, W.H., Dhabhar, F.S., Goldfarb, R.H., Kitson, R.P., Miller, A.H., Spencer, R.L., Weiss, J.M., 1997. The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. *Brain Res. Rev.* 23, 79–133.
- Meaney, M.J., Sapolsky, R.M., McEwen, B.S., 1985. The development of the glucocorticoid receptor system in the rat limbic brain. I. Ontogeny and autoregulation. *Dev. Brain Res.* 18, 159–164.
- Morici, L.A., Elsey, R.M., Lance, V.A., 1997. Effects of long-term corticosterone implants on growth and immune functions in juvenile alligators, *Alligator mississippiensis*. *J. Exp. Zool.* 279, 156–162.
- Morton, M.L., Carey, C., 1971. Growth and the development of endothermy in the mountain white-crowned sparrow (*Zonotrichia leucophrys oriantha*). *Physiol. Zool.* 44, 177–189.
- Morton, M.L., 2002. Mountain White-Crowned Sparrow: migration and reproduction at high altitude. Cooper Ornithological Society.
- Petitje, J.N., Etches, R.J., 1991. Daily infusion of corticosterone and reproductive function in the domestic hen (*Gallus domesticus*). *Gen. Comp. Endocrinol.* 83, 97–405.
- Ricklefs, R.E., 1968. Patterns of growth in birds. *Ibis* 110, 419–451.
- Sapolsky, R.M., Meaney, M.J., 1986. Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. *Brain Res. Rev.* 11, 65–76.
- Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress response? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrinol. Rev.* 21, 55–89.
- Schoenfield, N.M., Leathem, J.H., Rabii, J., 1980. Maturation of adrenal stress response in the rat. *Neuroendocrinology* 31, 101–105.
- Seckl, J.R., 2001. Glucocorticoid programming of the fetus; adult phenotypes and molecular mechanisms. *Mol. Cell. Endocrinol.* 185, 61–71.
- Silverin, B., 1986. Corticosterone-binding proteins and behavioral effects of high plasma levels of hormones in a free-living population of pied flycatchers, *Ficedula hypoleuca*. *Gen. Comp. Endocrinol.* 64, 67–74.
- Sims, C.G., Holberton, R.L., 2000. Development of the corticosterone stress response in young Northern mockingbirds (*Mimus polyglottos*). *Gen. Comp. Endocrinol.* 119, 193–201.
- Tinnikov, A.A., 1993. On the role of corticosteroid-binding globulin (CBG) in modulating activity of glucocorticoids: developmental patterns for CBG, corticosterone, and a-Fetoprotein levels in the rat serum. *Jpn. J. Physiol.* 43, 247–251.
- Tokarz, R.R., 1987. Effects of corticosterone treatment on male aggressive behavior in a lizard (*Anolis sagrei*). *Horm. Behav.* 21, 358–370.
- Viau, V., Sharma, S., Meaney, M.J., 1996. Changes in plasma adrenocorticotropin, corticosterone, corticosteroid-binding globulin, and hippocampal glucocorticoid receptor occupancy/translocation in rat pups in response to stress. *J. Neuroendocrinol.* 8, 1–8.
- Walker, B.G., Wingfield, J.C., Boersma, P.D., 2005. Age and food deprivation affects expression of glucocorticoid stress response in Magellanic Penguin (*Spheniscus magallanicus*) chicks. *Physiol. Biochem. Zool.* 78, 78–89.
- Wingfield, J.C., 1994. Modulation of the adrenocortical response to stress in birds. In: Davey, K.G., Peter, R.E., Tobe, S.S. (Eds.), *Perspectives in Comparative Endocrinology*. National Research Council of Canada, Ottawa, pp. 520–528.
- Wingfield, J.C., Smith, J.P., Farner, D.S., 1982. Endocrine responses of white-crowned sparrows to environmental stress. *Condor* 84, 399–409.
- Wingfield, J.C., Silverin, B., 1986. Effects of corticosterone on territorial behavior of free-living male song sparrows *Melospiza melodia*. *Horm. Behav.* 20, 405–417.