

## Exposure to chronic stress downregulates corticosterone responses to acute stressors

Erin L. Rich and L. Michael Romero

Department of Biology, Tufts University, Medford, Massachusetts

Submitted 21 June 2004; accepted in final form 5 February 2005

**Rich, Erin L., and L. Michael Romero.** Exposure to chronic stress downregulates corticosterone responses to acute stressors. *Am J Physiol Regul Integr Comp Physiol* 288: R1628–R1636, 2005. doi:10.1152/ajpregu.00484.2004.—We used captive European starlings (*Sturnus vulgaris*) to test whether corticosterone responses differed in birds held under normal laboratory conditions or conditions of chronic stress. Surprisingly, both basal corticosterone concentrations and corticosterone responses to acute stress were significantly reduced when birds were chronically stressed. To determine the mechanism underlying this reduced response, animals under both conditions were injected with lactated Ringer's solution (control), adrenocorticotropic (ACTH), arginine vasotocin (AVT), or dexamethasone (DEX). ACTH increased corticosterone concentrations above stress-induced levels in both cases, although maximum responses were lower in chronically stressed birds. AVT did not augment the corticosterone response under nonchronically stressed conditions, but it did under chronically stressed conditions. DEX reduced maximal corticosterone concentrations in both cases. Neither ovine nor rat corticotropin-releasing factor (CRF) altered normal stress responses. These data indicate that changes in responsiveness of the hypothalamic-pituitary-adrenal axis to ACTH and AVT serve to downregulate corticosterone responses during chronic stress. Furthermore, these data lead to the following hypothesis: ACTH output from the pituitary limits maximum corticosterone concentrations under normal conditions, but reduced AVT release from the hypothalamus regulates lower corticosterone concentrations under chronic stress conditions.

environmental stress; negative feedback; hypothalamic-pituitary-adrenal axis; conservation

GLUCOCORTICOIDS ARE STEROID hormones released from adrenal tissue in response to stressful stimuli. Upon perception of stress, the avian hypothalamus is activated to secrete arginine vasotocin (AVT, a congener of the mammalian arginine vasopressin) and corticotropin-releasing factor (CRF), which stimulate the pituitary to release adrenocorticotropic (ACTH), in turn, causing the release of glucocorticoids from the adrenals (7, 8). This pathway constitutes the hypothalamic-pituitary-adrenal (HPA) axis, and its activation is one component of the physiological stress response. This response is believed to be aimed at maintaining or restoring homeostasis, thereby helping the animal to survive a stressful episode (45, 50). The primary avian glucocorticoid is corticosterone (CORT) (22).

The presumed benefits of an acute activation of the HPA axis contrast with chronic activation. Various deleterious effects of chronic CORT elevation have been documented in many species, including suppression of reproductive function and behavior, immune system suppression, muscle wasting, growth suppression, and neuronal cell death (45, 49). Chronic stress generally produces chronic elevations in baseline CORT

concentrations. However, studies of chronically stressed rats indicate that facilitation of the HPA axis takes place to maintain responsiveness to acute stressors (11, 13, 25), perhaps by increasing the role of vasopressin over CRF in the release of ACTH (1, 16, 17). Therefore, animals must avoid chronic stress situations if they are to remain healthy, especially in the cases of endangered species, captive, domesticated, or hospitalized animals.

Studies in conservation biology have begun to focus on the effects of chronic stress in free-living populations. Examples include studies on the hormonal effects of oiling due to a tanker spill on a breeding colony of Magellanic penguins (*Spheniscus magellanicus*) and the efficacy of washing oiled birds in reducing stress (19); correlations between fecal steroids and predation threat, food accessibility, and individual social status in female ring-tailed lemurs *Lemur catta* (9); and measurements of CORT in Galapagos marine iguanas (*Amblyrhynchus cristatus*) under famine conditions of a severe El Niño (39). To fully understand the field data being collected, it is imperative that the physiology of chronic stress in nonmammalian species be characterized under controlled, laboratory conditions. It must be determined whether high CORT concentrations in wild animals are results of the presumed stressor per se, or reflections of natural variation (e.g., 12, 14, 34).

Our research subjects were European starlings (*Sturnus vulgaris*), a common species in North America that have been used in previous studies of avian stress (15, 36). In this study, our first goal was to compare CORT responses after acute stress under normal laboratory conditions to CORT responses under chronic stress conditions. This required developing a protocol for imposing chronic stress in the laboratory by using repeated unpredictable stressors to elicit multiple acute stress responses each day (13). All stressors used were tested initially to ensure that they elicited a rise in CORT.

Our second goal was to determine whether the different CORT responses under acute and chronic stress were associated with different HPA function. We tested three aspects of HPA function: pituitary responsiveness to exogenous AVT and CRF; adrenal tissue responsiveness to exogenous ACTH; and the efficacy of negative feedback using the synthetic glucocorticoid dexamethasone (DEX). The mechanism that alters CORT release could be a function of the adrenal tissue itself, through downregulation of ACTH receptors or a decreased ability to manufacture the steroids. On the other hand, the adrenal tissue could be unchanged, and the mechanism may lie at the level of the pituitary and a decreased secretion of ACTH. If pituitary release of ACTH is also unchanged; however, the

Address for reprint requests and other correspondence: L. M. Romero, Dept. of Biology, Tufts Univ., Medford, MA 02155 (E-mail: michael.romero@tufts.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

mechanism of regulation likely occurs above the pituitary, at the level of the hypothalamus and the secretion of AVT and/or CRF (33, 38). This latter case may reflect changes in CORT negative feedback.

## METHODS

Ten European starlings were trapped from the wild in early December 2000, using mist nets, and housed communally in an indoor flight aviary until commencement of the experiment. During all phases of the experiment, birds were housed individually within a common room and given food and water ad libitum. The light cycle in the isolation room was fixed at 13:11-h light-dark cycle. All tests involving sample collection began at midday (between 5 and 7.5 h after lights-on) to control for daily hormonal fluctuation (36). Birds were given approximately two weeks to acclimate to conditions in the isolation room before experimentation. Birds were a mixture of males and females, because earlier work indicated no sex differences in CORT stress responses in captive starlings (36). All experiments were conducted according to Association for Assessment of Laboratory Animal Care guidelines and approved by the Tufts Institutional Animal Care and Use Committee.

**Dose-response curves.** Dose-dependent CORT responses to injection of exogenous ACTH, CRF, or dexamethasone (DEX, a synthetic glucocorticoid) were measured in each of the 10 birds, and responses from appropriate doses were used to compare with responses during chronic stress. CORT responses to AVT were also measured using a 4- $\mu$ g subcutaneous injection. This dose was determined to be optimal in a previous experiment (B. Nephew, unpublished data). Intravenous injections were given in the jugular vein, and subcutaneous injections were given near the jugular vein. The jugular lies near the skin in this species and is easily visualized for injection. Since injection sites were similar, identical handling occurred in both cases, and the same intravenous Ringer solution injections were used as a control.

Porcine ACTH doses evaluated were 50 IU/kg, 100 IU/kg, and 200 IU/kg, dissolved in lactated Ringer solution and injected intravenously (40). Ovine CRF (oCRF; 30, 47) in Ringer solution was given in doses of 3  $\mu$ g/kg, 6  $\mu$ g/kg, and 9  $\mu$ g/kg intravenously (38, 41), and 2  $\mu$ g, 4  $\mu$ g, and 8  $\mu$ g subcutaneously (a range of doses that corresponds to the 4- $\mu$ g subcutaneous dose of AVT previously shown to work; B. Nephew, unpublished data). DEX that was dissolved in EtOH and diluted with Ringer solution was injected intravenously in doses of 0.5  $\mu$ g/kg, 2  $\mu$ g/kg, 5  $\mu$ g/kg, and 10  $\mu$ g/kg (48). All weight-dependent doses were standardized for a 75-g bird, and given in 10  $\mu$ l. A 10- $\mu$ l intravenous injection of Ringer solution only served to control for the stress of injection. All hormones were from Sigma Chemical.

By using a protocol of injection followed by acute stress, we attempted to monitor the magnitude and duration of the CORT stress response. Passerine species take  $\sim$ 3 min to exhibit an appreciable rise in plasma CORT concentrations in response to acute stress (52). Therefore, blood samples collected within 3 min of disturbance (experimenter entering isolation room) are assumed to reflect CORT levels before disturbance. In all sampling sequences, initial samples were taken within 3 min of entrance into the experimental room to monitor basal CORT concentrations. Birds were then injected with either 10  $\mu$ l of lactated Ringer solution or hormone, always within 10 min of the initial disturbance and subsequently restrained in cloth bags to elicit an acute stress response. Blood samples were collected again at 30 min, after which birds were returned to their cages and the experimenter left the room, thus removing any further stressful stimuli. At 1 h and 4 h, a third and fourth blood sample was taken, each within 3 min of reentry into isolation room.

Two more stress protocols were used to further examine the effects of the exogenous hormones on CORT levels. In the "no-restraint" protocol, basal samples were collected, and birds were injected as previously described. Birds were then returned to their cages immediately after injection, and the experimenter left the room. At 30 min

and 60 min, samples were collected, each within 3 min of reentry. This protocol was carried out with intravenous injections of a Ringer solution (control), 9  $\mu$ g/kg oCRF, 9  $\mu$ g/kg rat CRF (rCRF), and subcutaneous injection of 4  $\mu$ g AVT.

Effects of DEX were further elucidated through the use of a 60-min restraint protocol. The rapid drop in CORT levels after acute stress in controls was somewhat unexpected and made it difficult to discern any further decreases in CORT due to DEX. We therefore used the 60-min restraint protocol, extending the time of stressful stimulation to allow negative feedback to take effect. Basal samples were collected, and injections were given as previously described. Birds were restrained in bags for 60 min, with samples collected at 30 and 60 min, and then returned to their cages. Samples were collected at 4 h to measure recovery. This protocol was carried out with intravenous injections of a Ringer solution control, 5  $\mu$ g/kg and 10  $\mu$ g/kg DEX, and 5  $\mu$ g/kg DEX followed by a second injection of 100 IU/kg ACTH at 30 min.

Sampling consisted of puncturing the brachial wing vein and collecting 60–100  $\mu$ l of blood in heparinized capillary tubes. Samples were centrifuged at 400 g for 10 min. Plasma was extracted and frozen until analyzed. Plasma CORT concentrations were measured by radioimmunoassay as described by Wingfield et al. (53). Data were analyzed using repeated-measures ANOVA.

This period of testing was spaced over 6 mo. Birds were sampled in groups of five, and the hormones and doses to be tested on a given day were selected at random. No bird was tested on two consecutive days or more than twice per week. Birds were weighed periodically for the first 4 mo.

**Stress tests.** "Stress tests" were performed to test the efficacy of various stimuli at eliciting a CORT stress response. Five starlings, not used in the rest of the experiment, were housed individually in the isolation room with the experimental birds. After acclimating to the room, they were tested with the following six stressors. 1) In the "restraint" treatment, birds were held in cloth bags for the 30-min stress period. This is the typical restraint stress used in other parts of the experiment. 2) In the "cage disturbances" treatment, birds were returned to their cages after basal samples were collected. The experimenter then remained in the room and disturbed each individual's cage by rattling, tapping the front, top, or sides, or opening and shutting the cage door. Each disturbance was brief but occurred approximately every 2 min for the 30-min period. 3) In the "crowding" treatment, all five birds were placed in a single novel cage after basal samples were collected. The cage measured  $\sim$ 34  $\times$  38  $\times$  45 cm, the same size as cages that the birds were housed in individually. 4) In the "music" treatment, birds were returned to their cages after basal samples were collected. A radio tuned to a local music station was placed in the isolation room with the volume set to approximate normal to loud talking for 30 min. 5) In the "tube" treatment, all birds were placed in a transparent tube  $\sim$ 13 cm in height and 10 cm in width. This allowed the birds to stand upright but not to fly, and to walk only the axis of the tube. The length was  $\sim$ 1 m, and five birds were put in the tube at once. 6) In the "roller cart" treatment, birds were returned to their cages after basal samples were collected. All cages were then placed on a two-level wheeled cart. The cart was rolled back and forth continuously for 30 min, so that birds had difficulty standing on the perches and frequently chose to sit on the floor of the cage. All six stressors were intended to elicit mild to moderate psychological stress without any physical discomfort.

In all cases, a basal blood sample was collected within 3 min of disturbance. Birds were then subjected to the stressor for 30 min, at which time a second sample was taken. If the treatment did not require an experimenter to be present, 30-min samples were taken within 3 min of re-entry to ensure that CORT responses were resulting from the intended stressor. The treatment was then terminated, and birds were left in their cages until a final sample was collected at 60 min, also within 3 min of disturbance. This period of testing was spaced over 3 weeks. No bird was tested on two consecutive days or more

than twice per week. Data were analyzed by repeated-measures ANOVA.

To ensure that any rise in CORT concentrations was due to the stressful stimuli rather than the stress of the sampling protocol itself, the previously described sampling sequence was done with no stress (control) during the first 30-min period. That is, basal samples were collected, birds were returned to their cages, and the experimenter left the room. Subsequently, 30-min and 60-min samples were taken, with birds returned to cages in between.

**Chronic stress.** For the duration of the chronic stress experiment, the 10 experimental birds were maintained under conditions identical to the dose-response experiments. The birds were subjected to one of the six stressors previously described for 30 min four to five times per day. No stressor was used twice in one day on the same bird. The order of the stressors was chosen at random each day, and time schedules were not rigidly kept, all in effort to maintain the unpredictability of the events (11, 13, 43). Stressors were usually 2 to 3 h apart, beginning between 1.5 and 3.5 h after lights-on. At least one stress per day occurred after lights-off. Stressors performed during lights-off were done under blue light, which cannot penetrate the avian skull to stimulate photoreceptors on the pineal body (29).

During *days 1* through *10* of the chronic stress period, blood samples were taken to monitor changes in the CORT response over time. Sampling was always done at midday (between 5.5 and 7.5 h after lights-on) and at least 2 h after the previous stressor. Baseline samples were collected within 3 min of disturbance. Birds were then held in cloth bags for 30 min, at which time a 30-min sample was taken to measure the acute stress response. Birds were then returned to their cages, and the experimenter left the room. A final sample was collected at 60 min, within 3 min of reentry, to measure duration of the acute response. Birds were sampled in groups of five every other day, omitting every fifth day for extra recovery. On the day that a group was not being sampled, they were weighed to monitor each individual for significant weight loss.

**Comparing acute response with and without chronic stress.** During *days 11* through *20* of the chronic stress period, the protocol remained the same, but the blood sampling occurred in conjunction with hormone injections. Basal samples were collected, and injections of intravenous Ringer solution (control), 100 IU/kg iv ACTH, or 4  $\mu$ g sc AVT were given within 10 min. These doses were chosen from the results of the dose-response experiments (see RESULTS section). Birds were then held in cloth bags for 30 min, sampled again, and released to their cages. The experimenter left the room and returned to take a blood sample at 60 min. The previously described 60-min restraint protocol was used with injections of Ringer solution control and 5  $\mu$ g/kg DEX.

The two groups of five birds were sampled and weighed on alternating days. The order of hormones tested was random. However, each hormone was administered to one group of five birds during *days 11* through *15* and to the remaining group during *days 16* through *20*. This was done so that each hormone would be tested in the middle and at the end of the chronic stress period, reducing any effects of progressive changes that might occur during the course of *days 11* through *20*.

After *day 20*, the chronic stress protocol ended. All birds were then bled and weighed three further times to monitor their recovery.

**Data analysis.** All data were analyzed by repeated-measures ANOVA. For *days 1* through *10*, CORT data from 2 days were grouped, because half of the birds were sampled each day. For injection data, the 10 birds injected with the same hormone were grouped, although injections may have occurred days apart. CORT data for basal samples were compared over the entire 20-day chronic stress period, as they were not affected by injection. Thirty- and sixty-minute samples were also analyzed over the first 10 days for trends associated with the onset and progression of chronic stress. CORT responses to hormone injection were compared with controls

and to the same injections under nonchronically stressed conditions. (Note that each bird was tested under both conditions).

Weight data were grouped similarly to CORT data, so that mean weight was found for 10 birds measured over 2 days, compared with the same 10 birds weighed over the following 2 days, and so on. The statistical analysis was performed with raw weight data, but weights were converted to percentages of starting body weights (weight on *days 1* and 2) for graphical presentation.

## RESULTS

**Dose-response curves.** There were no significant differences in basal CORT levels (taken within 3 min of entering isolation room) across all dose-response trials ( $F_{13,117} = 0.76$ ,  $P = 0.70$ ).

**ACTH.** Injections of exogenous ACTH (Fig. 1) significantly elevated CORT levels above controls in all three doses (overall effect of dose  $F_{3,36} = 6.92$ ,  $P < 0.001$ ; effect of time after injection  $F_{3,108} = 115$ ,  $P < 0.0001$ ; interaction between dose and time  $F_{9,108} = 6.76$ ,  $P < 0.0001$ ). Elevated CORT concentrations were measured at 30 min and 60 min with ACTH injection, but CORT levels were unaffected at 4 h. Doses of 50 IU/kg and 100 IU/kg produced equal-intensity CORT responses at 30 and 60 min, whereas 200 IU/kg produced a somewhat blunted response. We chose to use 100 IU/kg ACTH as the dose in the chronic stress study because it was effective and has been used in previous studies (see DISCUSSION).

**CRF and AVT.** Injections of exogenous ovine CRF did not affect the CORT response compared with controls in six concentrations given either intravenously (3  $\mu$ g/kg, 6  $\mu$ g/kg, 9  $\mu$ g/kg iv, overall effect of dose  $F_{3,36} = 0.02$ ,  $P = 0.99$ ; effect of time after injection  $F_{3,108} = 46$ ,  $P < 0.0001$ ; interaction between dose and time  $F_{9,108} = 0.37$ ,  $P = 0.95$ , Fig. 2A) or subcutaneously (0.5  $\mu$ g, 4  $\mu$ g, 8  $\mu$ g sc, overall effect of dose  $F_{3,36} = 1.72$ ,  $P = 0.09$ ; effect of time after injection  $F_{3,108} = 64.1$ ,  $P < 0.0001$ ; interaction between dose and time  $F_{9,108} = 1.86$ ,  $P = 0.07$ , Fig. 2B). Both oCRF and rCRF were also ineffective at a dose of 9  $\mu$ g/kg administered intravenously when handling was not accompanied by prolonged restraint (no-restraint protocol), compared with a Ringer solution injection.

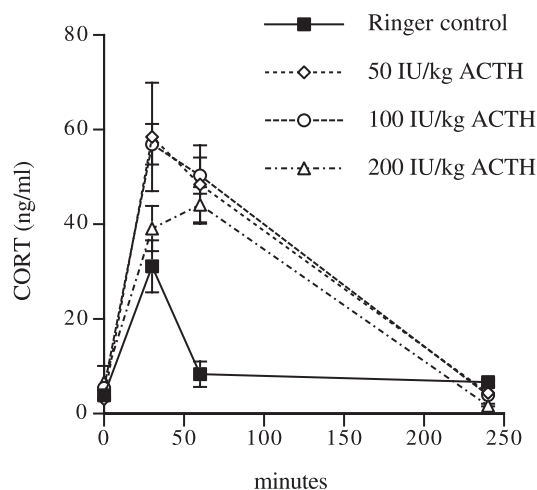


Fig. 1. Corticosterone (CORT) responses to 30 min of restraint (indicated by solid bar) with injection of Ringer control or adrenocorticotropicin (ACTH), over a 4-h period. All injections were intravenous, and birds were released to cages at 30 min. Each point represents mean CORT concentrations  $\pm$  SE.  $n = 10$  for all doses. \*Different from controls (see text).

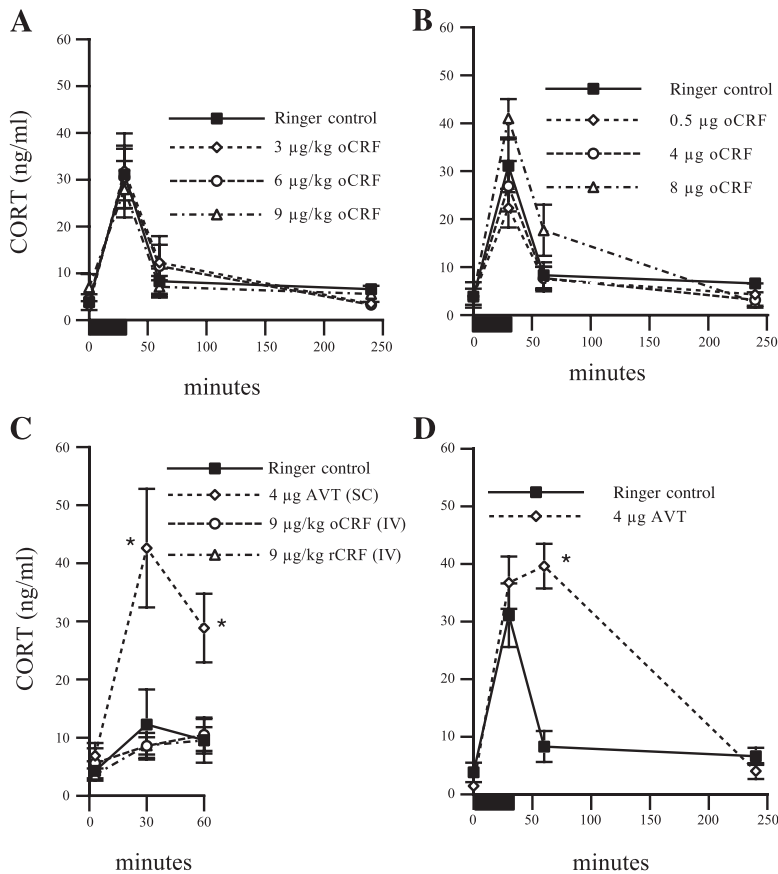


Fig. 2. CORT responses to injection of corticotropin-releasing factor (CRF) and arginine vasotocin (AVT). *A*: intravenous injection of oCRF. *B*: subcutaneous injection of ovine CRF (oCRF). *C*: injection of oCRF, rat CRF (rCRF), or AVT subcutaneously with no restraint. *D*: injection of AVT subcutaneously. All Ringer injections were intravenous. For *A*, *B*, and *D*, birds were subjected to 30 min of restraint (indicated by solid bar) and released to cages. For *C*, birds were not restrained, except to take samples and give injections. Each point represents mean CORT concentrations  $\pm$  SE.  $n = 10$  for all doses. \*Significantly different from controls (see text).

tion by the same no-restraint protocol (overall effect of dose  $F_{2,26} = 0.08$ ,  $P = 0.92$ ; effect of time after injection  $F_{2,52} = 6.78$ ,  $P < 0.005$ ; interaction between dose and time  $F_{4,52} = 0.49$ ,  $P = 0.75$ , Fig. 2C). Because CRF was ineffective at changing CORT levels, it was omitted during the chronic stress experiments (see below).

A known-effective dose of AVT (4 µg) administered subcutaneously caused a significant increase in CORT release over time, compared with controls (overall effect of AVT  $F_{1,18} = 9.0$ ,  $P < 0.01$ ; effect of time after injection  $F_{3,54} = 52$ ,  $P < 0.0001$ ; interaction between AVT and time  $F_{3,54} = 14.8$ ,  $P < 0.0001$ , Fig. 2D). CORT levels after AVT injection were not significantly higher than stress-induced levels at 30 min, but remained at the same elevated levels at 60 min, unlike controls. Four micrograms of AVT that were administered in the no-restraint protocol also succeeded in significantly elevating CORT levels above controls (overall effect of AVT  $F_{1,17} = 7.48$ ,  $P < 0.02$ ; effect of time after injection  $F_{2,34} = 12.3$ ,  $P < 0.0001$ ; interaction between AVT and time  $F_{2,34} = 4.21$ ,  $P < 0.03$ , Fig. 2C).

**DEX.** Injections of three doses of exogenous DEX (0.5 µg/kg, 2 µg/kg, 5 µg/kg iv administered) were ineffective at significantly lowering stress-induced or poststress CORT levels compared with controls (overall effect of dose  $F_{3,36} = 1.71$ ,  $P = 0.18$ ; effect of time after injection  $F_{3,108} = 65$ ,  $P < 0.0001$ ; interaction between dose and time  $F_{9,108} = 0.78$ ,  $P = 0.63$ , Fig. 3A). CORT appeared to decrease at 60 min after injection of 0.5 µg/kg and 5 µg/kg DEX; however, the rapid return to near-basal levels in controls made detection of negative feedback effects impossible. Doses of 5 µg/kg and 10

µg/kg DEX significantly lowered stress-induced CORT at 60 min, compared with controls, when the stress response was maintained by an extended period of restraint (overall effect of hormone  $F_{2,27} = 3.70$ ,  $P < 0.04$ ; effect of time after injection  $F_{3,81} = 128$ ,  $P < 0.0001$ ; interaction between hormone injection and time  $F_{6,81} = 4.24$ ,  $P < 0.001$ , Fig. 3B). There was no dose-dependent difference in response under the extended-restraint protocol, and administration of 100 IU/kg ACTH at 30 min after 5 µg/kg DEX injection reversed the CORT depression (Fig. 3B).

**Stress tests.** There were no significant differences in basal CORT concentrations for all treatments ( $F_{6,28} = 1.70$ ,  $P = 0.16$ ), and these levels reflected basal levels of the 10 experimental birds (Fig. 4). Controls showed slightly elevated CORT levels at 30 min, but had returned to basal levels by 60 min. All stressors elevated CORT above controls at 30 min. High stress-induced CORT levels were achieved with restraint (overall effect of treatment  $F_{1,8} = 21$ ,  $P < 0.002$ ; effect of time after treatment  $F_{2,16} = 19$ ,  $P < 0.0001$ ; interaction between treatment and time  $F_{2,16} = 10.2$ ,  $P < 0.002$ ), tube (overall effect of treatment  $F_{1,8} = 103$ ,  $P < 0.0001$ ; effect of time after treatment  $F_{2,16} = 203$ ,  $P < 0.0001$ ; interaction between treatment and time  $F_{2,16} = 128$ ,  $P < 0.0001$ ), and roller cart (overall effect of treatment  $F_{1,8} = 9.30$ ,  $P < 0.02$ ; effect of time after treatment  $F_{2,16} = 21$ ,  $P < 0.0001$ ; interaction between treatment and time  $F_{2,16} = 9.98$ ,  $P < 0.002$ ), while lower levels were found with cage disturbance (overall effect of treatment  $F_{1,8} = 2.52$ ,  $P = 0.15$ ; effect of time after treatment  $F_{2,16} = 57$ ,  $P < 0.0001$ ; interaction between treatment and time  $F_{2,16} = 13.35$ ,  $P < 0.0005$ ), music (overall

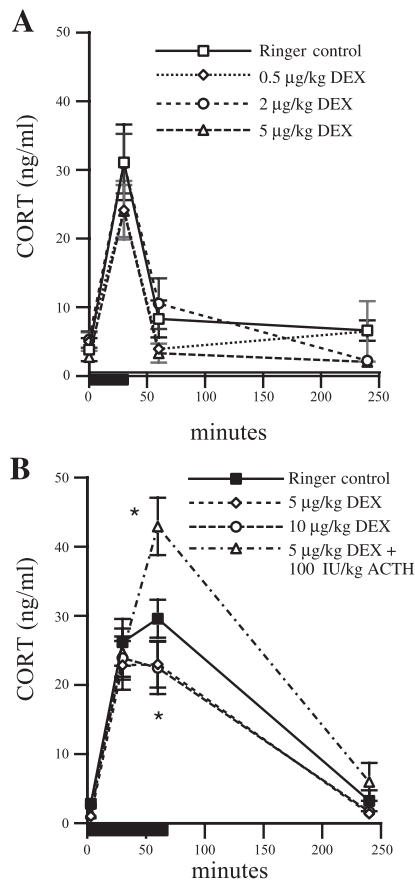


Fig. 3. CORT responses to 30 min of restraint (A) or 60 min of restraint (B) after injection of Ringer control, dexamethasone (DEX), or DEX + ACTH. Restraint period indicated by solid bar. All injections were intravenous, and birds were released to cages at the end of restraint. Each point represents mean CORT concentrations  $\pm$  SE.  $n = 10$  for all doses. \*Significantly different from controls (see text).

effect of treatment  $F_{1,8} = 0.61$ ,  $P = 0.46$ ; effect of time after treatment  $F_{2,16} = 45$ ,  $P < 0.0001$ ; interaction between treatment and time  $F_{2,16} = 3.88$ ,  $P < 0.05$ ), and crowding (overall effect of treatment  $F_{1,8} = 9.65$ ,  $P < .02$ ; effect of time after treatment  $F_{2,16} = 21$ ,  $P < 0.0001$ ; interaction between treat-

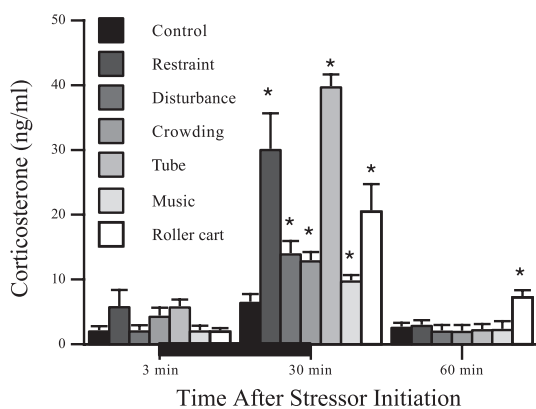


Fig. 4. CORT responses to handling followed by a 30-min stressor (indicated by solid bar). Birds were released to cages, and stressor was terminated at 30 min. Control birds were bled at 3 min and released to cages with no stressor. Each point represents mean CORT concentrations  $\pm$  SE and  $n = 5$  for all points. \*Significantly different from controls (see text).

ment and time  $F_{2,16} = 4.02$ ,  $P < 0.04$ ). CORT levels remained slightly elevated at 60 min (30 min poststress) with the roller cart, but they returned to basal levels with all of the other stressors.

*Chronic stress.* Basal CORT decreased over the full 20-day period of chronic stress and recovered slowly ( $F_{8,72} = 2.12$ ,  $P < 0.05$ , Fig. 5A). During the first 10 days of chronic stress, the CORT response to 30 min of restraint also decreased over

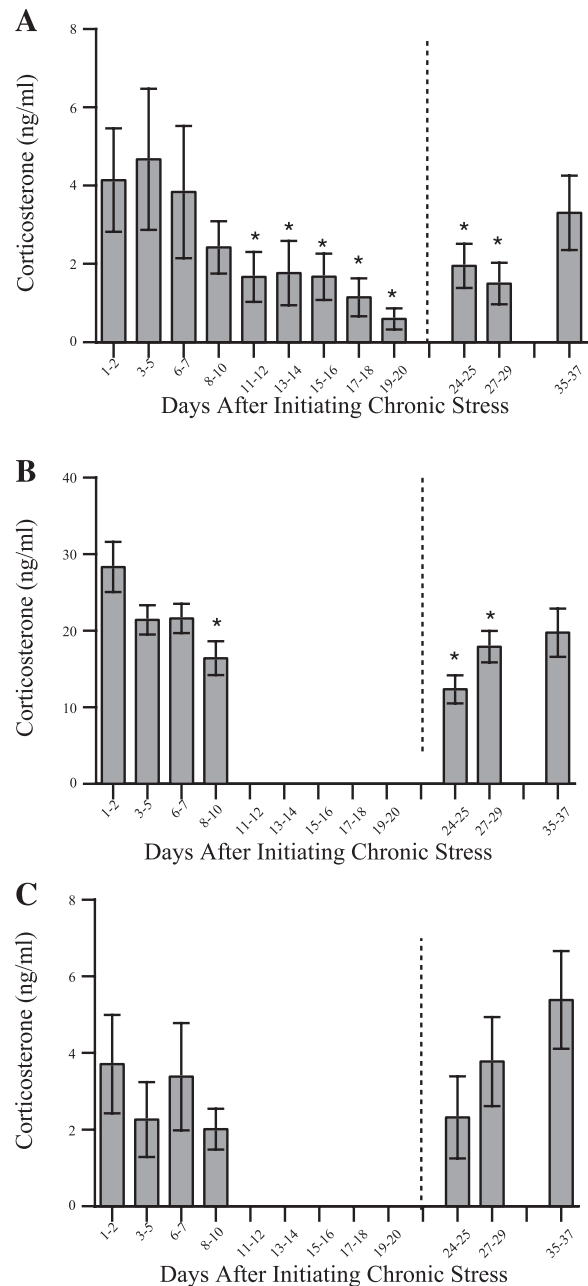


Fig. 5. CORT concentrations measured within 3 min of disturbance (A), after 30 min of restraint (B), and after 30 min of recovery from restraint (C) during chronic stress. Chronic stress began on day 1 and ended on day 20 (indicated by vertical line). Values at 30 and 60 min from days 11–20 were not collected since hormones were injected during this time. Each bar represents the mean of 10 birds measured once during the 2 or 3 days indicated. Error bars represent means  $\pm$  SE. Note differences in scale. \*Significantly different from controls (see text).

time ( $F_{3,27} = 7.35, P < 0.001, \text{Fig. } 5B$ ), but there was no change at 60 min (30 min poststress,  $F_{3,27} = 0.74, P = 0.54, \text{Fig. } 5C$ ). Note that hormone injections then prevented examining these responses after *day 10*.

Changes in body weight over the 20-day chronic stress period paralleled changes in CORT ( $F_{11,99} = 16.2, P < 0.0001, \text{Fig. } 6$ ). No single bird lost more than 10% of its starting body weight, and the average loss did not drop below 5% for the entire stress period. Starting body weight recovered within days of the termination of chronic stress.

**HPA function during chronic stress.** Injection of 100 IU/kg ACTH in chronically stressed birds significantly increased CORT response to acute stress at 30 min and 60 min (overall effect of treatment  $F_{1,18} = 150, P < 0.0001$ ; effect of time after treatment  $F_{2,36} = 39.6, P < 0.0001$ ; interaction between treatment and time  $F_{2,36} = 16.1, P < 0.0001, \text{Fig. } 7A$ ). The pattern of elevation mimicked the response to ACTH in the birds under nonchronically stressed conditions, except the magnitude of CORT response was smaller. Four micrograms of AVT significantly affected CORT responses in chronically stressed birds (overall effect of treatment  $F_{1,18} = 9.86, P < 0.01$ ; effect of time after treatment  $F_{2,36} = 30.8, P < 0.0001$ ; interaction between treatment and time  $F_{2,36} = 2.79, P = 0.075, \text{Fig. } 7B$ ). This pattern of effect was opposite the pattern found without chronic stress, in that CORT levels increased above controls in response to AVT at 30 min, but returned to near basal levels by 60 min.

DEX administered to chronically stressed birds significantly affected CORT responses to 1 h of restraint (overall effect of treatment  $F_{1,18} = 0.27, P = 0.61$ ; effect of time after treatment  $F_{3,54} = 39.2, P < 0.0001$ ; interaction between treatment and time  $F_{3,54} = 5.10, P < 0.005, \text{Fig. } 7C$ ). The pattern of CORT depression was similar in chronically stressed birds and non-chronically stressed birds, with 60 min CORT levels lower than controls. However, all birds injected with Ringer or with DEX had lower CORT responses under chronic stress than they did with no chronic stress. Chronically stressed birds injected with DEX had higher 30-min CORT levels than controls.

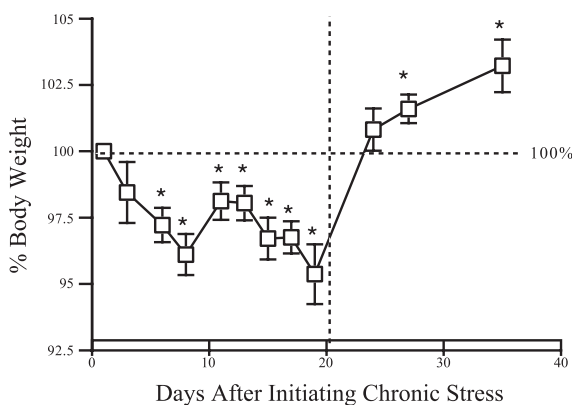


Fig. 6. Percent body weight was calculated during and after the chronic stress period. Chronic stress began on *day 1* and ended on *day 20* (indicated by vertical line). Each point represents the mean of 10 birds, measured once over 2 to 3 days. Error bars represent means  $\pm$  SE. \*Significantly different from controls (see text).

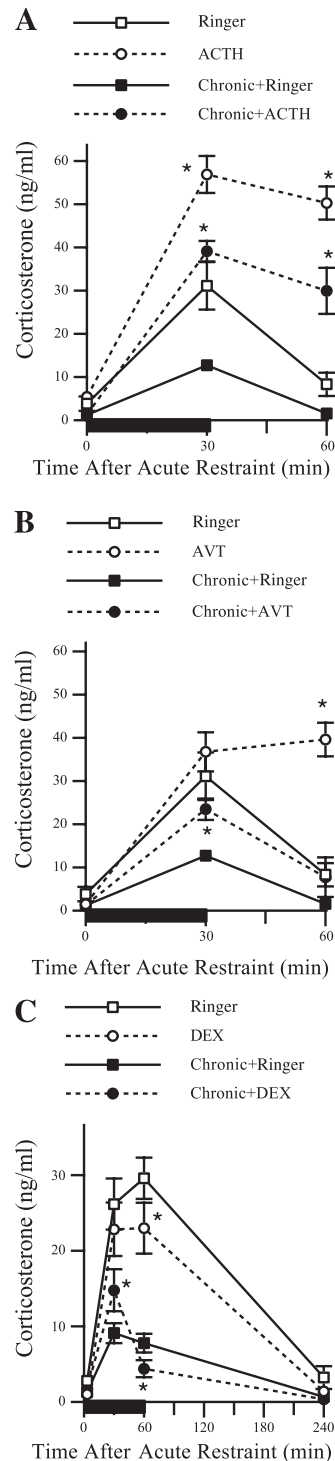


Fig. 7. CORT concentrations measured in response to either 30 min (A and B) or 60 min (C) restraint and injection of Ringer solution or 100 IU/kg ACTH (A), 4  $\mu$ g AVT (B), or 5  $\mu$ g/kg DEX (C). All hormones were injected under both no-chronic-stress and chronic stress conditions. Data without chronic stress are from the same birds and repeated from Fig 1 (A), Fig. 2D (B), and Fig. 3B (C). Each point represents the mean of 10 birds  $\pm$  SE. \*Significantly different from controls in same chronic stress state (see text).

DISCUSSION

**Dose-response curves.** The ACTH dose-response curve showed appreciable CORT responses to all doses tested. This

indicates that the adrenal tissue has reserve capacity to secrete CORT and is not maximally stimulated by endogenous ACTH released during stress (33). Although the two lower doses (50 and 100 IU/kg) were indistinguishable, the highest dose was slightly less effective. This could be due to increased negative feedback or a mild toxicity of the extremely high dose. We chose to proceed with the 100 IU/kg dose because it has been used in previous studies (38, 40, 41), and it did not suggest any negative effects.

The oCRF dose-response curve showed no responses to either intravenous or subcutaneous injections. Although neither CRF nor AVT is thought to stimulate CORT release directly, they can stimulate CORT release indirectly by stimulating endogenous ACTH release (33). Therefore, the lack of a response to oCRF could be interpreted in two ways: 1) the pituitary cannot respond with increased ACTH output, and therefore, no augmented CORT response is observed; or 2) the oCRF itself is ineffective. We tried two experiments to try to tease apart these two possibilities. First, we used the no-restraint protocol to ensure that the pituitary could produce ACTH, at least to normal stress-induced levels, if the oCRF itself were effective. Second, we tried injecting rCRF. Because we again found no response to either treatment, CRF must be ineffective at elevating ACTH above basal levels. However, this again allows two possible interpretations: 1) only oCRF and rCRF were tested, which starling CRF receptors may not recognize; or 2) CRF is not an ACTH secretagogue in this species. There is evidence that CRF may not be the primary ACTH secretagogue in other avian species (8, 33). Because no effect was found, CRF was not used in the chronic stress part of the study. In its place, we used AVT as the ACTH secretagogue, which was found to effectively elevate CORT to normal stress-induced levels (Fig. 2, C–D).

DEX was expected to decrease CORT levels by negative feedback (6, 26, 44). The effect of DEX was significant, although not as strong as expected from earlier studies (e.g., 2, 48) and not dose dependent. Regardless, we can conclude that negative feedback is taking place, although the system does not appear to be very sensitive to DEX in this species.

Together, these dose-response curves provided a picture of “normal” HPA function in acutely stressed starlings and were used as the basis for comparison when birds were chronically stressed.

**Stress tests.** A wide variety of natural stressors are known to elicit a CORT response (34, 45, 50). We used stressors not expected to affect aspects of physiology beyond the stress response (as might food or water deprivation, forced exercise, etc.; see Ref. 37). All stressors used in this study effectively elicited a CORT response after 30 min with a clear demonstration of graded stress responses, dependent on the potency of the stressor, as seen in other species (21). Bag restraint, a traditional acute stressor for avian species, produced one of the highest responses.

**Chronic stress.** This study clearly demonstrated a decrease in basal and stress-induced CORT levels with the onset and progression of chronic stress. This was contrary to expectations (e.g., 1, 13, 25, 49). For example, Moore et al. (27) reported slight increases in basal CORT concentrations in tree lizards (*Urosaurus ornatus*) over 3 wk of stress due to captivity and repeated handling. In addition, Dunlap and Schall (18) found no increase in basal CORT levels in Western fence lizards

(*Sceloporus occidentalis*) that were infected with a malarial parasite, although infected animals had significantly higher CORT responses to acute stress. Also, Fowler et al. (19) found significantly elevated basal CORT in Magellanic penguins (*S. magellanicus*) that had been oiled and then washed after a tanker spill. These and other studies consistently suggest that chronic stress is associated with an elevation of CORT at basal and/or stress induced levels.

An attenuation of the stress response with chronic stress could indicate one of three situations: 1) habituation, 2) exhaustion, or 3) downregulation. It is highly unlikely that the birds were habituating to the chronic stress treatment. Every measure was taken to ensure that the treatments occurred unpredictably, both in timing and sequence. Unpredictability and lack of control are classic psychological elements of chronic stress (23). All birds showed an immediate drop in body weight with the onset of the chronic stress protocol, and decreasing body condition often reflects chronic stress (e.g., 10, 20, 39). Also, changes in HPA responsiveness to hormone challenge suggest that the CORT decline originates from changes in physiology, not in the perception or cognitive interpretation of the stressors.

It is also unlikely that the chronic stress protocol stressed the birds to the point of physiological exhaustion. By exhaustion, we mean the state in which the animal can no longer compensate for sustained stress and effects become life-threatening, as originally proposed by Selye (46). First, there were no radical changes in CORT response, such as complete obliteration or extreme suprphysiological responses to acute stress. The simple damped effect suggests a more controlled response than exhaustion might entail. Also, while weight loss was significant, it was carefully monitored to ensure that there were no dangerous declines, and weights quickly recovered after termination of chronic stress, even though basal CORT levels did not. This pattern of weight and basal CORT fluctuation also suggests a controlled physiological response.

A controlled physiological response is further suggested by studies on laboratory rats. Studies of chronic stress in rats typically show some attenuation of corticosterone responses over the period of chronic stress, although rarely to the extent shown in this study (reviewed in Ref. 13). Researchers often interpret this attenuation as indicating habituation because the rats continue to elevate corticosterone to new stimuli, although few of these studies stress rats to the point of weight loss. When the stimuli are more severe and weight loss does occur, evidence of habituation is generally absent (reviewed in Ref. 13). Therefore, the observed attenuation in this study is most likely a controlled downregulation of HPA activity in response to chronic stress. This response could be aimed at minimizing the deleterious effects of chronic CORT release to prevent CORT itself from disrupting normal functions (35, 45, 50).

It is interesting to speculate, however, what mechanism might be responsible for the observed weight loss, as this effect is often attributed to chronically elevated CORT, whereas these data show attenuated CORT. CORT implants in male song sparrows (*Melospiza melodia*) increased fat scores without changing body mass, suggesting reciprocal loss of muscle mass (51) and led to wasting of pectoral muscles in dark-eyed juncos (*Junco hyemalis*) (20). However, observations suggested that the birds in this study were losing fat reserves, while pectoral muscles seemed less affected. Another function of CORT in

birds appears to be suppression of metabolic rates at night, with expected energetic savings of 20% over a 16-h night (5). The lower CORT levels observed in this study may reduce energy savings, resulting in increased burning of fat reserves. This would be consistent with our observations of body composition and weight loss.

Also, corticosteroid binding globulin (CBG) levels should be measured for changes in response to chronic stress. CBG is a plasma protein that binds CORT, with only an unbound steroid believed to be available to CORT receptors. Consequently, changes in CBG levels may alter the amount of CORT actually reaching target tissues. Chronic stress is known to decrease CBG concentrations in the blood (3). However, it is still unresolved whether CBG acts primarily as a way to buffer the animal from high CORT concentrations or as a carrier to transport CORT to its target tissues, so whether CBG causes an increase or decrease in physiological activity is not yet confirmed (3, 42).

**CORT modulation.** The dose-response tests established typical HPA function under nonchronically stressed conditions. Although exogenous AVT does not elevate CORT levels above physiological stress-induced levels, it does lengthen the response (Fig. 2D). This suggests that either ACTH or CORT secretion had already reached maximal levels. However, CORT secretion is not maximal because exogenous ACTH further elevates CORT secretion. Therefore, our data support pituitary release of ACTH as the rate-limiting step regulating CORT secretion under normal conditions, either because of limits in receptor capacity for AVT or limits in the ability to secrete ACTH.

Under chronic stress, however, HPA function appears to shift and hypothalamic release of AVT becomes the rate-limiting step. CORT secretion remains submaximal because the adrenal continues to respond to exogenous ACTH (Fig. 7A). However, endogenous ACTH release is no longer maximal, since exogenous AVT further augments the response (Fig. 7B). Consequently, the lower endogenous CORT response during chronic stress must result from the failure of the hypothalamus to send a larger AVT signal, thus making AVT release from the hypothalamus the rate-limiting step during chronic stress.

The CORT response to AVT injection, however, was not maintained at the same level through 60 min as it was in birds without chronic stress (Fig. 7B). This was not due to reduced adrenal tissue responsiveness. When pituitary function is bypassed with ACTH injection, adrenal tissue is capable of maintaining elevated CORT through 60 min (Fig. 7A). Instead, this may indicate that the pituitary has exhausted its reserves of ACTH and must increase synthesis before further CORT release can occur. Alternatively, a strong negative feedback signal at the level of the pituitary may shut down ACTH production. However, stronger negative feedback seems unlikely, since the DEX effectiveness remained similar during chronic stress (Fig. 7C).

In conclusion, modulation of CORT responses during chronic stress appears to be a regulated change in the HPA axis. Under normal conditions, the pituitary regulates the rate of ACTH release, which, in turn determines stress-induced CORT concentrations. Under chronic stress, the hypothalamus regulates AVT release, which presumably results in less ACTH leading to lower concentrations of stress-induced CORT. It is

important to note, however, that this study was done on captive birds held on a short-day photoperiod. Variations in stress responses have been found between wild and captive members of the same species (24, 40), and wild starlings have lower baseline but higher stress-induced CORT concentrations than the captive birds in this study (31). Furthermore, modulation can occur both seasonally (35) and daily (4, 32, 36). We predict, however, that chronic stress will elicit a similar drop in CORT concentrations in wild starlings.

As a whole, these data suggest that chronic stress is not always manifested in high CORT levels. Such observations are important to any study attempting to assess repeated or long-term stress and lend further support to the idea that accurate identification of chronically stressed populations cannot rely upon CORT measurement alone (28, 34).

#### ACKNOWLEDGMENTS

We thank B. Nephew, K. Tran, and N. Niruka, for help in administering chronic stressors.

#### GRANTS

Funding for this project was provided by National Science Foundation grants IBN-9975502 and IBN-0235044 to L. M. Romero.

#### REFERENCES

1. **Aguilera G, Rabadan-Diehl C, Lou X, and Kiss A.** Regulation of pituitary ACTH secretion during stress: role of corticotropin-releasing hormone and vasopressin. In: *Stress: Molecular Genetic and Neurobiological Advances*, edited by McCarty R, Aguilera G, Sabban E, and Kvetnansky R. New York: Gordon and Breach Science Publishers, 1996.
2. **Astheimer LB, Buttemer WA, and Wingfield JC.** Gender and seasonal differences in the adrenocortical response to ACTH challenge in an Arctic passerine, *Zonotrichia leucophrys gambelii*. *Gen Comp Endocrinol* 94: 33–43, 1994.
3. **Breuner CW and Orchinik M.** Beyond carrier proteins: plasma-binding proteins as mediators of corticosteroid action in vertebrates. *J Endocrinol* 175: 99–112, 2002.
4. **Breuner CW, Wingfield JC, and Romero LM.** Diel rhythms of basal and stress-induced corticosterone in a wild, seasonal vertebrate, Gambel's white-crowned sparrow. *J Exp Zool* 284: 334–342, 1999.
5. **Buttemer WA, Astheimer LB, and Wingfield JC.** The effect of corticosterone on standard metabolic rates of small passerine birds. *J Comp Physiol [B]* 161: 427–431, 1991.
6. **Carroll B, Feinberg M, and Greden J.** A specific laboratory test for the diagnosis of melancholia. *Arch Gen Psychiatry* 38: 15–23, 1981.
7. **Carsia RV, Weber H, and Perez FMJ.** Corticotropin-releasing factor stimulates the release of adrenocorticotropin from domestic fowl pituitary cells. *Endocrinology* 118: 143–148, 1986.
8. **Castro MG, Estivariz FE, and Iturriza FC.** The regulation of the corticosterone cell activity in Aves. II. Effect of various peptides on the release of ACTH from dispersed, perfused duck pituitary cells. *Comp Biochem Physiol* 83A: 71–75, 1986.
9. **Cavigelli SA.** Behavioural patterns associated with faecal cortisol levels in free-ranging female ring-tailed lemurs, *Lemur catta*. *Anim Behav* 57: 935–944, 1999.
10. **Cherel Y, Robin JP, Walch O, Karmann H, Netchitailo P, and Le Maho Y.** Fasting in king penguin. I. Hormonal and metabolic changes during breeding. *Am J Physiol Regul Integr Comp Physiol* 254: R170–R177, 1988.
11. **Dallman MF.** Stress update: Adaptation of the hypothalamic-pituitary-adrenal axis to chronic stress. *Trends Endocrinol Metab* 4: 62–69, 1993.
12. **Dallman MF, Akana SF, Cascio CS, Darlington DN, Jacobson L, and Levin N.** Regulation of ACTH secretion: variations on a theme of B. *Recent Prog Horm Res* 43: 113–173, 1987.
13. **Dallman MF and Bhatnagar S.** Chronic stress and energy balance: role of the hypothalamo-pituitary-adrenal axis. In: *Handbook of Physiology; Section 7: The Endocrine System; Volume IV: Coping with the Environment: Neural and Endocrine Mechanisms*, edited by McEwen BS and Goodman HM. New York: Oxford University Press, 2001, p. 179–210.



14. **Dallman MF, Strack AM, Akana SF, Bradbury MJ, Hanson ES, Scribner KA, and Smith M.** Feast and famine: critical role of glucocorticoids with insulin in daily energy flow. *Front Neuroendocrinol* 14: 303–347, 1993.
15. **Dawson A and Howe PD.** Plasma corticosterone in wild starlings (*Sturnus vulgaris*) immediately following capture and in relation to body weight during the annual cycle. *Gen Comp Endocrinol* 51: 303–308, 1983.
16. **De Goeij DCE, Binnekade R, and Tilders FJH.** Chronic stress enhances vasopressin but not corticotropin-releasing factor secretion during hypoglycemia. *Am J Physiol Endocrinol Metab* 263: E394–E399, 1992.
17. **De Goeij DCE, Dijkstra H, and Tilders FJH.** Chronic psychosocial stress enhances vasopressin, but not corticotropin-releasing factor, in the external zone of the median eminence of male rats: relationship to subordinate status. *Endocrinology* 131: 847–853, 1992.
18. **Dunlap KD and Schall JJ.** Hormonal alterations and reproductive inhibition in male fence lizards (*Sceloporus occidentalis*) infected with the malarial parasite *Plasmodium mexicanum*. *Physiol Zool* 68: 608–621, 1995.
19. **Fowler GS, Wingfield JC, and Boersma PD.** Hormonal and reproductive effects of low levels of petroleum fouling in megallanic penguins. *Auk* 111: 20–27, 1995.
20. **Gray JM, Yarian D, and Ramenofsky M.** Corticosterone, foraging behavior, and metabolism in dark-eyed juncos, *Junco hyemalis*. *Gen Comp Endocrinol* 79: 375–384, 1990.
21. **Hennessy MB and Levine S.** Sensitive pituitary-adrenal responsiveness to varying intensities of psychological stimulation. *Physiol Behav* 21: 295–297, 1978.
22. **Holmes WN and Phillips JG.** The adrenal cortex of birds. In: *General, Comparative, and Clinical Endocrinology of the Adrenal Cortex*, edited by Chester-Jones I and Henderson IW. London: Academic Press, 1976, p. 293–420.
23. **Levine S, Coe C, and Wiener SG.** Psychoneuroendocrinology of stress—a psychobiological perspective. In: *Psychoendocrinology*, edited by Brush FR and Levine S. San Diego: Academic, 1989, p. 341–377.
24. **Marra PP, Lampe KT, and Tedford BL.** Plasma corticosterone levels in two species of *Zonotrichia* sparrows under captive and free-living conditions. *Wilson Bull* 107: 296–305, 1995.
25. **Marti O, Gavalda A, Gomez F, and Armario A.** Direct evidence for chronic stress-induced facilitation of the adrenocorticotropin response to a novel acute stressor. *Neuroendocrinology* 60: 1–7, 1994.
26. **McDonald IR, Lee AK, Than KA, and Martin RW.** Failure of glucocorticoid feedback in males of a population of small marsupials (*Antechinus swainsonii*) during the period of mating. *J Endocrinol* 108: 63–68, 1986.
27. **Moore MC, Thompson CW, and Marler CA.** Reciprocal changes in corticosterone and testosterone levels following acute and chronic handling stress in the tree lizard, *Urosaurus ornatus*. *Gen Comp Endocrinol* 81: 217–226, 1991.
28. **Norris DO.** Endocrine disruptors of the stress axis in natural populations: how can we tell? *Am Zool* 40: 393–401, 2000.
29. **Oishi T and Lauber JK.** Photoreception in the photosexual response of quail. II. Effects of intensity and wavelength. *Am J Physiol* 225: 880–886, 1973.
30. **Peczely P and Antoni FA.** Comparative localization of neurons containing ovine corticotropin releasing factor (CRF)-like and neurophysin-like immunoreactivity in the diencephalon of the pigeon (*Columba livia domestica*). *J Comp Neurol* 228: 69–80, 1984.
31. **Remage-Healey L and Romero LM.** Corticosterone and insulin interact to regulate glucose and triglyceride levels during stress in a bird. *Am J Physiol Regul Integr Comp Physiol* 281: R994–R1003, 2001.
32. **Rich EL and Romero LM.** Daily and photoperiod variations of basal and stress-induced corticosterone concentrations in house sparrows (*Passer domesticus*). *J Comp Physiol [B]* 171: 543–547, 2001.
33. **Romero LM.** Mechanisms underlying seasonal differences in the avian stress response. In: *Avian Endocrinology*, edited by Dawson A and Chaturvedi CM. New Delhi: Narosa Publishing House, 2001, p. 373–384.
34. **Romero LM.** Physiological stress in ecology: lessons from biomedical research. *Trends Ecol Evol* 19: 249–255, 2004.
35. **Romero LM.** Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *Gen Comp Endocrinol* 128: 1–24, 2002.
36. **Romero LM, and Remage-Healey L.** Daily and seasonal variation in response to stress in captive starlings (*Sturnus vulgaris*): corticosterone. *Gen Comp Endocrinol* 119: 52–59, 2000.
37. **Romero LM and Sapolsky RM.** Patterns of ACTH secretagog secretion in response to psychological stimuli. *J Neuroendocrinol* 8: 243–258, 1996.
38. **Romero LM, Soma KK, and Wingfield JC.** Hypothalamic-pituitary-adrenal axis changes allow seasonal modulation of corticosterone in a bird. *Am J Physiol Regul Integr Comp Physiol* 274: R1338–R1344, 1998.
39. **Romero LM and Wikelski M.** Corticosterone levels predict survival probabilities of Galápagos marine iguanas during El Niño events. *Proc Natl Acad Sci USA* 98: 7366–7370, 2001.
40. **Romero LM and Wingfield JC.** Alterations in hypothalamic-pituitary-adrenal function associated with captivity in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*). *Comp Biochem Physiol* 122B: 13–20, 1999.
41. **Romero LM and Wingfield JC.** Seasonal changes in adrenal sensitivity alter corticosterone levels in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*). *Comp Biochem Physiol* 119C: 31–36, 1998.
42. **Rosner W.** The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. *Endocr Rev* 11: 80–91, 1990.
43. **Sapolsky R, Brooke S, and Stein-Behrens B.** Methodologic issues in studying glucocorticoid-induced damage to neurons. *J Neurosci Methods* 58: 1–15, 1995.
44. **Sapolsky RM and Altmann J.** Incidence of hypercortisolism and dexamethasone resistance increases with age among wild baboons. *Biol Psychiatry* 30: 1008–1016, 1991.
45. **Sapolsky RM, Romero LM, and Munck AU.** How do glucocorticoids influence stress-responses? Integrating permissive, suppressive, stimulatory, and adaptive actions. *Endocr Rev* 21: 55–89, 2000.
46. **Selye H.** The general adaptation syndrome and the diseases of adaptation. *J Clin Endocrinol* 6: 117–230, 1946.
47. **Westerhof I, Lumeij JT, Mol JA, van den Brom WE, and Rijnberk A.** In vivo studies on the effects of ovine corticotrophin-releasing hormone, arginine vasotocin, arginine vasopressin, and haloperidol on adrenocortical function in the racing pigeon (*Columba livia domestica*). *Gen Comp Endocrinol* 88: 76–82, 1992.
48. **Westerhof I, Van den Brom WE, Mol JA, Lumeij JT, and Rijnberk A.** Sensitivity of the hypothalamic-pituitary-adrenal system of pigeons (*Columba livia domestica*) to suppression by dexamethasone, cortisol, and prednisolone. *Avian Dis* 38: 435–445, 1994.
49. **Wingfield JC, Hunt K, Breuner C, Dunlap K, Fowler GS, Freed L, and Lepson J.** Environmental stress, field endocrinology, and conservation biology. In: *Behavioral Approaches to Conservation in the Wild*, edited by Clemmons JR and Buchholz R. Cambridge, UK: Cambridge University Press, 1997, p. 95–131.
50. **Wingfield JC and Romero LM.** Adrenocortical responses to stress and their modulation in free-living vertebrates. In: *Handbook of Physiology; Section 7: The Endocrine System; Volume IV: Coping with the Environment: Neural and Endocrine Mechanisms*, edited by McEwen BS and Goodman HM. New York: Oxford University, 2001, p. 211–234.
51. **Wingfield JC and Silverin B.** Effects of corticosterone on territorial behavior of free-living male song sparrows *Melospiza melodia*. *Horm Behav* 20: 405–417, 1986.
52. **Wingfield JC, Smith JP, and Farner DS.** Endocrine responses of white-crowned sparrows to environmental stress. *Condor* 84: 399–409, 1982.
53. **Wingfield JC, Vleck CM, and Moore MC.** Seasonal changes of the adrenocortical response to stress in birds of the Sonoran Desert. *J Exp Zool* 264: 419–428, 1992.