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Noninvasive Corticosterone Treatment Rapidly Increases Activity in Gambel's White-Crowned Sparrows (*Zonotrichia leucophrys gambelii*)

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Recent evidence supports the hypothesis that corticosteroids influence behavioral changes associated with stressful events. Most investigations into this relationship focus on the long-term behavioral effects of corticosterone. Because many behavioral responses to environmental perturbations occur within minutes, we determined what rapid effects corticosterone may have on behavior. With this goal in mind, we devised and evaluated a method of corticosterone delivery which allowed us to examine immediate effects of corticosterone on behavior in a noninvasive manner. White-crowned sparrows (*Zonotrichia leucophrys gambelii*) were allowed access to mealworms (*Tenebrio molitor*) injected with corticosterone. Once ingested, the corticosterone moves across the digestive epithelium into the circulation. This method was evaluated using two vehicles: dimethyl sulfoxide and peanut oil. We tested the efficiency and consistency of corticosterone transfer into the circulation for both vehicles. Dimethyl sulfoxide gave a more efficient transfer of corticosterone. Injecting mealworms with corticosterone (carried in dimethyl sulfoxide) and feeding those worms to white-crowned sparrows increased circulating corticosterone to a discrete, repeatable level which peaked within 7 min and was cleared within 60 min. Using this method, we demonstrated that intermediate levels of corticosterone caused an increase in perch hopping in white-crowned sparrows within 15 min of hormone administration. An increase in perch hopping indicated elevated locomotor activity that is consistent with behavioral responses to natural perturbations. High levels of corticosterone did not induce this behavioral change. In light of the rapid effect of corticosterone on behavior, we propose that corticosterone was acting through a nongenomic mechanism.

Vertebrates respond to unexpected physical and social changes with a rapid elevation of circulating glucocorticoids. Glucocorticoids can have diverse effects, including mobilization of glucose through gluconeogenesis, suppression of the immune system, and alteration of behavioral patterns (Holmes and Phillips, 1976; Axelrod and Reisine, 1984; Munck *et al.*, 1984; Wingfield, 1994). These changes have been characterized in laboratory and field studies. Corticosterone (CORT, the glucocorticoid in most nonmammalian tetrapods) can decrease aggression, parental behavior, and reproductive behavior (Silverin, 1986; Wingfield and Silverin, 1986), increase foraging (Wingfield *et al.*, 1990), and increase or decrease feeding rate and locomotor activity, depending on prior food availability (Astheimer *et al.*, 1992).

The majority of these behavioral experiments were accomplished using CORT implants, which raise circulating levels of CORT to a constant level over a period of days to weeks. Using implants to modulate circulating CORT levels is convenient in that implants can be

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given to free living animals and the procedure is repeatable and does not permanently alter the organism. There are, however, several drawbacks to the method. First, implantation requires surgery, which itself increases endogenous CORT levels. Second, there is a lag period of a number of hours before implanted CORT reaches maximum levels in the circulation, so immediate effects of CORT cannot be assessed. Finally, the implant brings circulating CORT levels up to a sustained high level which does not mimic dynamic changes typical of organisms. To achieve an immediate, dynamic dose of CORT, one can inject it. But, for many animals (sparrows, for example), the injection itself is stressful and increases endogenous levels of CORT in both treatment and control groups. We sought to devise a method of corticosterone delivery which was nonstressful, mimicked natural surges of corticosterone that might be seen in the wild, and which allowed us to look at immediate effects of corticosterone on behavior.

Traditionally, it was thought that catecholamines were responsible for the immediate phase (seconds to minutes) of the ''stress response'' and that glucocorticoids did not play a role until hours or days after the onset of the stressor. This hypothesis existed primarily because glucocorticoids are steroids, and the traditional steroid receptor is intracellular. Once bound by ligand, an intracellular receptor acts as a transcription factor to regulate gene expression; the subsequent change in protein levels and / or cellular activity is not usually detectable for 30–60 min after hormone application (Wehling, 1995). However, rapid actions of glucocorticoids—and other steroids as well—have been identified in many different systems. In the newt, *Taricha granulosa*, an injection of corticosterone inhibited reproductive behavior within 8 min (Orchinik *et al.*, 1991) and decreased medullary neuron firing rates within 3 min (Rose *et al.*, 1993). These rapid effects are likely to be orchestrated through a nongenomic mechanism. In fact, in *Taricha*, a membrane binding site for corticosterone has been characterized (Orchinik *et al.*, 1991). In light of these rapid actions of glucocorticoids, the timing and mode of action of specific stress hormones during the stress response needs to be revised. In the present study, we used a new method of rapid, nonstressful corticosterone delivery to determine whether corticosterone can rapidly affect locomotor activity (i.e., perch hopping) in the white-crowned sparrow.

MATERIALS AND METHODS

Animals

Gambel's white-crowned sparrows were captured at Sunnyside, Washington during fall migration (September 15–October 1, 1994, 1995, and 1996). All experiments were completed within 9 months of capture. After capture, sparrows were housed in outdoor aviaries at the University of Washington for at least 2 weeks, to allow acclimation to captivity (Wingfield *et al.*, 1982). Several experiments began after January of each year; to avoid photostimulation in the outdoor aviaries, all birds used in these experiments were housed in indoor environmental chambers (2.4 m $\times$ 1.7 m $\times$ 2.2 m rooms with air, temperature, and light control) under short-day photoperiod (8L:16D) until the experiment began. Bird chow, wild bird seed mix, and water were available *ad libitum* at all times. All experiments were done between 09:00 and 14:00 to exclude changes in baseline CORT levels; basal CORT levels do not vary during daylight hours under short-day (8L:16D) or long-day (20L:4D) photoperiods (Breuner, Wingfield, and Romero, submitted for publication).

A total of 44 sparrows were used to complete this study: 23 sparrows were used to evaluate CORT blood levels after mealworm ingestion (DMSO trials, 16 sparrows; peanut oil trials, 7 sparrows) and 21 sparrows were used to evaluate the behavioral effects of CORT (control, low CORT and high CORT trials, 11 sparrows; uninjected mealworm trials, 10 sparrows). It was not possible to obtain circulating CORT levels from sparrows during the behavioral trials. Therefore, experiments evaluating circulating CORT levels and the behavioral effects of CORT were completed in separate groups of sparrows.

Mealworm Injection

To deliver CORT in a nonstressful manner, CORT solution was injected into mealworms (*Tenebrio molitor*), and those worms were fed whole to sparrows. This method of CORT delivery was evaluated using

two separate vehicles: dimethyl sulfoxide (DMSO) and peanut oil.

DMSO (Sigma, St. Louis, MO) is a powerful nonpolar solvent which readily dissolves crystalline CORT (Sigma). Peanut oil is a nonpolar solvent as well, but does not dissolve CORT directly. Crystalline CORT was first dissolved in a small amount of ethanol (EtOH), which was then mixed with peanut oil. The EtOH evaporated overnight. CORT can crystallize in peanut oil (personal observation). To counteract this problem, the peanut oil–CORT solution was sonicated before each use to ensure an even suspension of CORT throughout the solution.

Mealworms were injected with 20 µl of DMSO or peanut oil containing one of the following concentrations of CORT: (1) control, no CORT; (2) low CORT, 0.2 mg/ml CORT; and (3) high CORT, 1.0 mg/ml CORT. Hence, birds received 0, 4, and 20 µg CORT per mealworm, respectively. We injected solution into mealworms with a 50-µl Hamilton syringe using a 30-gauge ½-inch needle. Prior to injection, mealworms were placed at −20°C for 5 min to reduce movement during injection. The needle was inserted ventrally, into the posterior abdomen, between two segments. If fluid leaked from the mealworm after injection, it was not used.

We used mealworms of approximately 25 mm and 0.13 g. Mealworm size was critical because it must be small enough for the sparrow to eat without tearing apart, but big enough to hold 20 µl of injected solution. We chose to inject 20 µl for two reasons: (1) any volume higher than 20 µl was not usually retained in the mealworm; and (2) when volume was reduced to 10 µl, too much solution was retained in the needle tip for accurate dosage.

Circulating Plasma Titers after Ingestion

To enter the circulation, the hormone must cross the gut epithelium. Therefore, circulating levels of CORT could not be predicted directly from CORT concentration in solution. To determine circulating levels of CORT after mealworm ingestion for each vehicle used, we fed injected mealworms to sparrows and collected blood samples over the next hour (DMSO treatments: 0, 7, 15, 22, 30, and 60 min; peanut oil treatments: 0, 7, 15, and 30 min). The DMSO and peanut oil experiments were completed on separate groups of birds; all birds were housed under similar conditions, and all were photosensitive.

Sparrows were housed in individual cages in environmental chambers. They were kept on short days (8L:16D) with 4–10 birds per chamber. Birds were housed under these conditions for at least 2 weeks before the experiment began to allow them to acclimate to chamber conditions. Prior to the experiments, uninjected mealworms were provided every day or every other day, to help train the birds to eat the mealworms. During the experiment, mealworms were only given as part of the experimental trial. Once the experiment began, two to three trials were completed per week; individual birds were tested three or four times per 2-week period. In each trial, individuals received only one treatment and only had blood taken once. Thirty minutes before the trial started, the injected mealworm was hidden in a covered dish inside the cage. The mealworm became accessible when we pulled a string attached to the lid of the dish from outside the chamber. The environmental chambers were equipped with one-way mirrors, which allowed us to mark when the mealworm was fully ingested. Blood was collected only if the mealworm was fully ingested within 1 min of the sparrow initially grabbing the mealworm. After the allotted time, we collected a blood sample from a wing vein into heparinized microcapillary tubes after puncture with a 26-gauge needle. This method allows the bird access to the mealworm (and hormone) without exposure to the experimenter, which could potentially affect endogenous CORT. The process of entering the chamber and obtaining a blood sample significantly elevates endogenous CORT levels. Therefore, we obtained blood samples from every experimental bird within 3 min of entering the chamber, before endogenous CORT levels increased (Wingfield *et al.*, 1982).

Radioimmunoassay. We determined plasma levels of CORT by radioimmunoassay after extraction of 10- to 20-µl samples in 4 ml of redistilled dichloromethane. Recoveries after extraction were 75–95% (measured for each sample independently and adjustments to the final assayed concentration made accordingly). Interassay variation was less than 12%. The assay procedure has been described in detail by Wingfield *et al.* (1992).

Statistics. A one-way repeated measures ANOVA was used to look for treatment effects. In these two experiments (the DMSO and peanut oil trials) each bird received multiple treatments, at multiple times. If all times and treatments were analyzed all together, there would be two repeated factors: treatment and time. This model is too complex for a standard repeated measures ANOVA. Therefore, only a subset of the timepoints were analyzed, each with a separate repeated measures ANOVA.

The DMSO trials utilized 16 sparrows. Only 11 birds received all three treatments (control, low CORT, and high CORT) at the 7-min timepoint; only 8 birds received all three treatments at the 60-min timepoint. Therefore, one-way repeated measures ANOVAs were performed with $n = 11$ and $n = 7$ for the 7- and 60-min timepoints, respectively. The peanut oil trials utilized seven sparrows. All seven birds received each treatment at each timepoint. A one-way repeated measures ANOVA was used to compare treatments at the 7-min timepoint. If a significant treatment effect was found, Tukey's HSD post hoc analyses were used to identify differences between treatments. The Bonferroni correction was used to adjust P values, when multiple comparisons were made.

Rapid Effects of Corticosterone on Behavior

We measured perch hopping in response to elevated levels of circulating corticosterone. DMSO delivered CORT more efficiently than peanut oil, so we used DMSO as the vehicle in these experiments. Perches were attached to microswitches; hops were recorded in LabView (National Instruments Corp.).

For the behavioral trials, sparrows were housed in isolation boxes (0.5 m $\times$ 0.33 m $\times$ 0.5 m) under long-day photoperiods (20L:4D). Sparrows were allowed to acclimate to the new housing conditions for 2 weeks before the trials began. Eleven sparrows were fed three mealworm treatments (control, low CORT, and high CORT). Nine sparrows received all three treatments; two sparrows only received control and low CORT treatments.

We recorded perch hopping before and after mealworm ingestion, to account for daily variation in behavior. Thirty minutes after a mealworm was placed in a covered dish inside the cage, we recorded 15 min of baseline perch hopping activity. From outside the isolation box, we then removed the lid on the mealworm dish. After the sparrow had eaten the entire mealworm, we recorded perch hopping for the subsequent hour.

To determine if mealworm ingestion itself affects perch hopping, we fed uninjected mealworms to a separate group of sparrows, following the protocol for behavioral trials outlined above.

Statistics. To estimate the effect of CORT on perch hopping, we computed the ratio of the number of hops after mealworm ingestion to the number of hops before mealworm ingestion ("relative activity"). We separated the hour of perch hopping recorded after mealworm ingestion into four 15-min segments. The total hops from each segment (f) was then divided by the total hops from the initial 15-min segment recorded before mealworm ingestion (i): (f/i). The calculation f/i was used instead of percentage change, $((f - i)/i) \times 100$, because transformation of the data was not possible after the percentage change calculation.

To complete statistical analysis, we normalized the data through log transformation ($\log((f/i) + 1)$) and used a one-way repeated measures ANOVA to detect differences between treatments during the first 15-min segment. Only one of the four time segments was analyzed because of the limitation of the repeated measures ANOVA; in the behavioral trials, as in the last set of experiments, both time and treatment are repeated. Therefore, only one of the four time segments was analyzed. The uninjected mealworm trials were performed on a separate set of sparrows and so were not included in the repeated measures analysis.

RESULTS

Circulating Plasma Titers after Ingestion

In each treatment, the control mealworms contained only the vehicle (DMSO or peanut oil); the low CORT mealworms contained 4 µg CORT, and the high CORT mealworms contained 20 µg CORT.

DMSO treatments. Circulating CORT remained at baseline levels after ingestion of a control mealworm (Fig. 1a). The highest levels of CORT in each treatment were detected at 7 min after mealworm ingestion (low CORT = 25 ng/ml, high CORT = 98 ng/ml). There

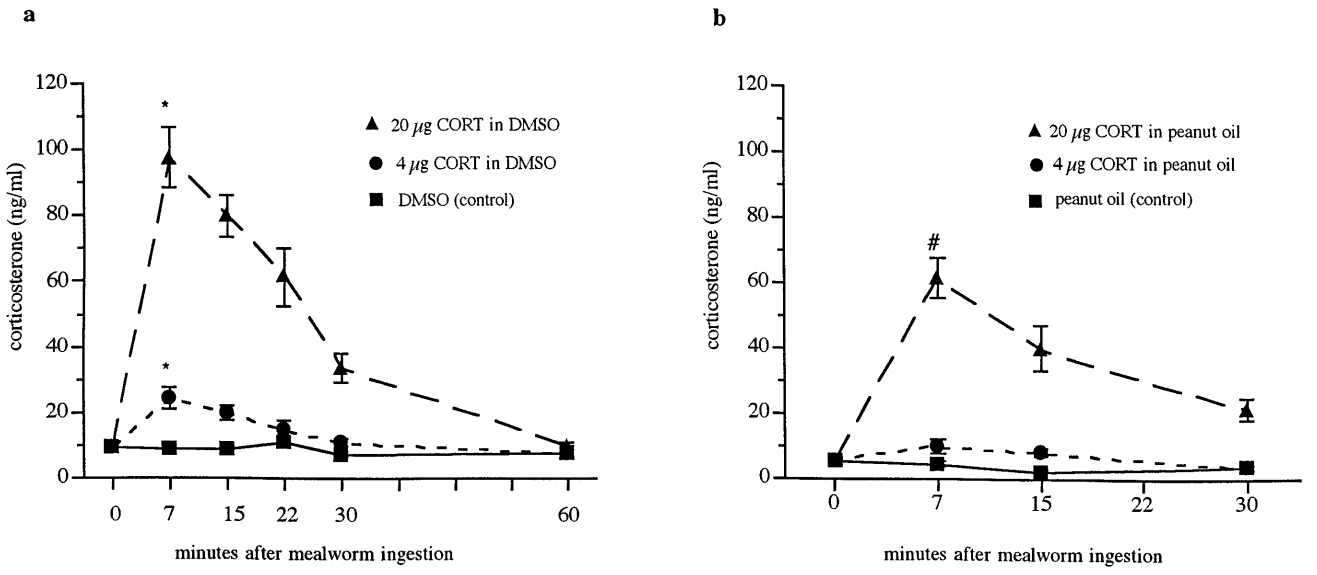

FIG. 1. Circulating levels of corticosterone after mealworm ingestion. Figures show means ± SE. (a) Corticosterone delivered in DMSO. $n \geq 12$ for each point. Both treatments were significantly different from controls and each other by 7 min $^*P < 0.0001$. (b) Corticosterone delivered in peanut oil. $n = 7$. High CORT treatment was significantly different from low CORT and control treatments by 7 min after mealworm ingestion $\#P < 0.0001$.

was a significant treatment effect at 7 min (repeated measures ANOVA, $F = 175$, $P < 0.0001$, Bonferroni critical value = 0.025); high and low CORT treatments were significantly different from controls and from each other at 7 min after mealworm ingestion (Tukey's HSD post hoc: $q_{(0.05,30,4)} = 3.845$). There was no significant treatment effect at 60 min (repeated measures ANOVA: $F = 1.559$, $P > 0.05$).

Peanut oil treatments. Circulating CORT remained at baseline levels after ingestion of a control mealworm (Fig. 1b). The highest levels of CORT in each treatment were detected at 7 min after mealworm ingestion (low CORT = 10 ng/ml, high CORT = 60 ng/ml). High CORT treatment was significantly different from low CORT and control treatments by 7 min after mealworm ingestion (repeated measures ANOVA: $F = 76.78$, $P < 0.0001$; Tukey's post hoc test: $q_{(0.05,12,3)} = 3.773$). Low CORT treatment did not vary significantly from controls.

Rapid Effects of Corticosterone on Behavior

Ingestion of an uninjected mealworm or a DMSO-injected control mealworm had no effect on perch hopping. Perch hopping activity was significantly greater during the 15 min following ingestion of a low CORT mealworm than following ingestion of a control mealworm (repeated measures ANOVA: $F = 4.968$; $P < 0.05$; see Fig. 2a). However, perch hopping during the 15 min after ingestion of a high CORT mealworm did not differ significantly from controls (repeated measures ANOVA, $F = 0.079$, $P = 0.79$, see Fig. 2b).

DISCUSSION

Noninvasive Administration of Corticosterone

We have devised a method of CORT delivery which is noninvasive, mimics natural surges of CORT induced by environmental perturbations, and allows us to examine immediate effects of CORT on behavior without the complications of capture and injection stress. The efficiency of CORT delivery was evaluated using two vehicles: DMSO and peanut oil. There were benefits and drawbacks to each vehicle. DMSO was the most efficient vehicle, transferring the highest level of CORT into the bloodstream. It is also a very powerful solvent, so the dose is very reliable. DMSO is poten-

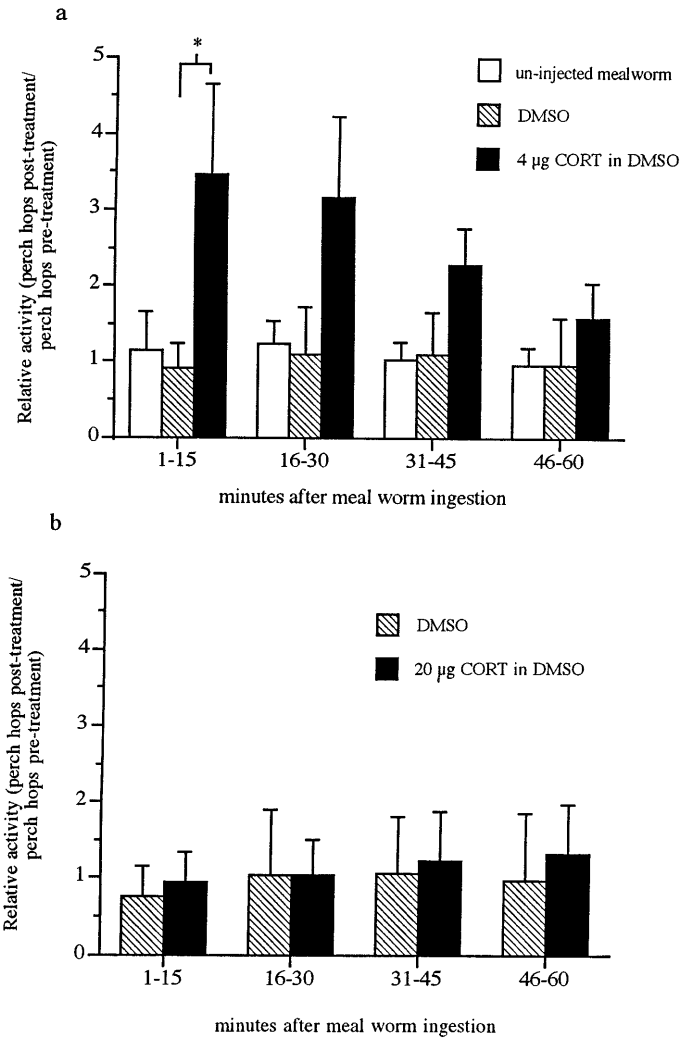

FIG. 2. Relative activity after mealworm ingestion. The hour after mealworm ingestion was divided into four segments. The total number of hops from each segment was then divided by the total number of hops from the initial 15-min segment recorded before mealworm ingestion, to correct for variations in daily behavior (1 = no change in activity). Figures show means ± SE. (a) comparison of uninjected ($n = 10$), control, and low CORT mealworms ($n = 11$; $^*P < 0.05$). The uninjected mealworm trials were performed on a separate group of birds and therefore were not included in the statistical analysis. They are included here for visual comparison only. (b) Comparison of control and high CORT mealworms ($n = 9$).

tially an irritant, but did not have an adverse effect on perch hopping in this experiment. An alternative vehicle is peanut oil, which is potentially less reactive than DMSO, but did not transfer as much CORT into the bloodstream. Peanut oil did not dissolve CORT as well as DMSO. Because CORT formed crystals in the peanut oil solution (personal observation), the dose would not be as reliable without sonication of the solution prior to use.

Another method for noninvasive CORT administration was recently created by Dr. R. Knapp (Knapp and Moore, 1997). In this method, surgery and injection are avoided by taping a patch of CORT-soaked bandage to the animal, which delivers CORT over a period of 24 h. While extremely useful in field studies, this method is inappropriate for evaluating rapid effects of CORT because hormone administration requires handling of the animal, which increases endogenous CORT secretion.

The use of mealworms to achieve noninvasive,

nonstressful steroid administration is widely applicable in the field of behavioral and physiological endocrinology. It can be applied to study the immediate effects of CORT on various aspects of the stress response, such as metabolic rate and gluconeogenesis, as well as the variety of behavioral responses associated with an increase in CORT. It may be used in many classes of vertebrates, although the rate of CORT absorption into the bloodstream will need to be tested for each species, especially when different food vehicles are used. It may also be used in the laboratory and field, potentially with a variety of steroids.

Rapid Effects of Corticosterone

Intermediate levels of CORT increased perch hopping, whereas high levels of CORT did not. This suggests an inverted-U relationship of CORT level and response, which has been demonstrated in other systems: the effect of CORT on hippocampal firing rate (Diamond *et al.*, 1992), serotonin levels, and passive–avoidance behavior (Kovacs *et al.*, 1977). To establish a true dose–response curve between perch hopping and CORT, however, more doses are needed.

There is another possible explanation for the lack of response seen in the high CORT treatment. In response to capture and handling, free-living white-crowned sparrows experienced CORT levels similar to those in the high CORT treatment (~100 ng/ml, see Wingfield *et al.*, 1983; Astheimer *et al.*, 1994). Peak levels recorded in the laboratory in response to capture and handling only reached ~37 ng/ml (Breuner, Wingfield, and Romero, unpublished). This raises the possibility that the high CORT dose was pharmacological for captive sparrows, and the lack of behavioral response may not be physiologically relevant. Alternatively, it is possible that highly elevated levels of CORT trigger different responses than slightly elevated levels (Wingfield *et al.*, 1997; 1998). In a free living animal, intermediate levels of CORT may increase movement (increasing foraging or searching for more suitable habitat), whereas higher levels may restrict movement.

Studies using CORT implants also indicate a role for CORT in locomotor activity. CORT implants increase locomotor activity in captive white-crowned sparrows (Astheimer *et al.*, 1992), although only under conditions of food restriction. This increase is thought to represent escape activity, as related to irruptive behavior in the wild.

We have demonstrated that corticosterone caused a rapid increase in perch hopping in white-crowned sparrows within 15 min of hormone administration. This rapid increase in perch hopping raises the question of what mechanism mediated CORT's effects on behavior. The intracellular glucocorticoid (Type II) receptor was probably not responsible for mediating this behavioral change. Once this hormone–receptor complex changes transcription levels, a change in protein levels and/or cellular activity is not usually detectable for 30 to 60 min after application of the hormone (Wehling, 1995). We have demonstrated a rapid increase in perch hopping in the sparrow which paralleled the rapid increase of CORT in the blood, suggesting that, in this case, CORT may be acting through a nongenomic mechanism.

One possibility is that CORT acted through a neuronal membrane glucocorticoid receptor. Several lines of evidence suggest that corticosteroid membrane receptors may be present in other animals. First, many systems show rapid responses to corticosteroids. For example, CORT treatment increases locomotor response to novel environments in the rat within 7.5 min (Sandi *et al.*, 1996), inhibits reproductive behavior in the newt within 8 min (Orchinik *et al.*, 1991), and increases attack behavior in the rat after 10 min (Haller *et al.*, 1997). Second, specific membrane binding sites for CORT have been identified in many systems (Koch *et al.*, 1978; Towle and Sze, 1983; Orchinik *et al.*, 1991; Trueba *et al.*, 1991; Allera and Wildt, 1992). Finally, a neuronal membrane receptor for CORT has been well characterized in the newt (Orchinik *et al.*, 1991).

Involvement of a membrane receptor in the "stress response pathway" may have another interesting consequence. If an animal is experiencing an unpredictable event in the environment, such as low food availability or a severe storm, one may predict that the behavioral response should occur while the stressful event persists and end when the stressor is over. It appears that changes in perch hopping due to increased CORT only occur while the hormone is elevated. This direct temporal relationship between hormone change and behavioral response cannot be mediated through a genomic mechanism. Intracellular glucocorticoid receptors would not effect behavioral

changes immediately upon CORT elevation, and they would continue to have effects after circulating levels of CORT have returned to baseline. Hence, a membrane mechanism for CORT would allow for behavioral and physiological responses to stress to occur concurrently with the stressful event.

The behavioral experiments occurred under long-day photoperiods (20L:4D), whereas the CORT titer experiments occurred under short-day photoperiods (8L:16D). This difference in photoperiod would be important if clearance rates of CORT varied under the different photoperiods (the level of circulating CORT after mealworm ingestion would be different under long-day and short-day conditions). There are several lines of evidence which support equal clearance rates between long- and short-day photoperiods under laboratory conditions. First, when mealworms are injected with 14 μg CORT and fed to both long-day and short-day sparrows, circulating levels of CORT reached similar levels within 7 min in both groups (preliminary experiment, data not shown). Second, laboratory experiments measured basal and stress-induced levels of CORT under long-day and short-day photoperiods in white-crowned sparrows. There was no significant difference in basal CORT levels, rate of CORT increase, maximum CORT levels, or CORT decline between short- and long-day photoperiods (Breuner, Wingfield and Romero, submitted for publication). Third, preliminary experiments indicate CBG levels are similar under the two photoperiods in captive white-crowned sparrows (Breuner and Wingfield, unpublished data).

In conclusion, we have demonstrated the effectiveness of using CORT-injected mealworms as a noninvasive means of rapidly increasing blood CORT levels. We also report that CORT causes a rapid increase in perch hopping in photostimulated white-crowned sparrows within 15 min of hormone administration. Many long-term (hours to days) effects of CORT have been studied in passerines, but this is the first quantification of a rapid behavioral response. Due to this rapid increase in perch hopping in the sparrow, we propose that CORT is acting through a nongenomic mechanism—possibly through a membrane glucocorticoid receptor. A nongenomic mechanism of action for CORT may allow organisms to redirect behavior and physiology toward survival in a much shorter time frame than had previously been supposed.

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