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Corticosterone and Nocturnal Torpor in the Rufous Hummingbird (*Selasphorus rufus*)

Sara M. Hiebert,* Katrina G. Salvante,†,[1] Marilyn Ramenofsky,† and John C. Wingfield†

**Department of Biology, Swarthmore College, Swarthmore, Pennsylvania 19081-1390; and †Department of Zoology, University of Washington, Seattle, Washington 98195-1800*

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Three experiments were designed to investigate whether corticosterone (CORT), known to have a role in restoration of energy homeostasis, regulates nocturnal torpor, an energy conservation state used by some small mammals and birds to offset environmental challenges to energy balance. In two experiments, one during autumn migration and one during early spring molt, captive rufous hummingbirds (*Selasphorus rufus*) were fed control and dilute (85% strength) nectar on alternate days. In migratory birds, torpor occurred more frequently over all, and nectar dilution resulted in increased torpor duration and increased concentration of CORT in evening but not midday cloacal fluid (CF) samples. In molting birds, torpor occurred infrequently on both control and food dilution days, but, although there was a significant increase in evening CF CORT on food dilution days, torpor duration did not increase significantly in response and there was no correlation between torpor duration and CF CORT at either time of day. Daily CF CORT patterns showed an increase from midday to evening during migration, but the reverse pattern during the molt. In a third experiment, CORT administered in the nectar elevated the use of torpor and depressed food intake. The results of these three experiments support the hypothesis that CORT is involved in the regulation of torpor, but suggest that some feature of the CORT signal other than concentration per se may be required to fully explain seasonal changes in the relations among energy challenge, CORT, and nocturnal torpor in hummingbirds.

Key Words: corticosterone; noninvasive; daily torpor; hummingbird; energy balance; body mass; foraging behavior.

The stress response mediated by the hypothalamic–pituitary–adrenal (HPA) axis can be provoked by a variety of stressors. The common theme underlying this response is that stressors represent an unpredicted challenge to homeostasis; the stress response, orchestrated in part by the adrenal glucocorticosteroid "stress hormones," in turn promotes the reestablishment of homeostasis in the wake of this challenge (Munck *et al.*, 1984; Wingfield and Ramenofsky, 1999). Challenges to energy homeostasis, such as unusually low environmental temperatures, food shortage, and starvation, stimulate an increase in glucocorticoid secretion (Schwabl *et al.*, 1985; Wingfield, 1988; Harvey *et al.*, 1984). Glucocorticoids affect a wide variety of behavioral and physiological responses that promote positive energy balance, including increases in foraging and / or feeding (Nagra *et al.*, 1963; Astheimer *et al.*, 1992; Wingfield *et al.*, 1990; Wingfield and Ramenofsky, 1999), increases in energy storage (Siegel, 1980; Schwabl *et al.*, 1985; Wingfield and Silverin, 1986), and decreases in behaviors not crucial to immediate survival (Roche and Leshner, 1979; Wingfield and Silverin, 1986; Wingfield, 1988). Administration of glu-

[1] Current address: Department of Biological Science, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6.

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cocorticoids is known to stimulate increases or decreases in locomotor behavior (Buttemer *et al.,* 1991; Astheimer *et al.,* 1992; Breuner *et al.,* 1998; Wingfield and Ramenofsky, 1999), which might be interpreted as representing indices of increased foraging activity or movement away from an unfavorable environment, or decreased activity associated with energy conservation during a period when escape is impossible and the animal must wait out the unfavorable circumstance (Astheimer *et al.,* 1992; Wingfield *et al.,* 1998; Wingfield and Ramenofsky, 1999).

The resting phase of the daily activity cycle offers additional opportunities for energy conservation in times of energy shortage. Because glucocorticoids generally act to promote positive energy balance, it is logical to ask whether these hormones increase the use of energy-conserving resting states. In support of this hypothesis, Buttemer *et al.* (1991) found that implants containing corticosterone (CORT), the principal adrenal glucocorticoid in birds, increased nocturnal restfulness in captive white-crowned sparrows. Daily torpor and sleep are considered by some to represent opposite extremes on a continuum of hypometabolic energy-conserving states occurring during the rest phase of an animal's daily cycle (Berger and Phillips, 1995), the principal difference being that sleep is accompanied by a temperature decrease of 1–2°, whereas daily torpor may be accompanied by a decrease of up to 25° or more (Geiser and Ruf, 1995). Although there is evidence for important physiological differences between sleep and extreme hypometabolic states such as hibernation (Daan *et al.,* 1991; Trachsel *et al.,* 1991; Kilduff *et al.,* 1993), the contribution of both types of states to energy conservation is undisputed. The relation between CORT and daily torpor has, however, been largely neglected in the literature.

Daily torpor is conveniently studied in hummingbirds, which experience extreme challenges to energy homeostasis because of their very small size and energy-intensive modes of flight and foraging. In these species, daily torpor has been shown to respond very sensitively to environmental manipulation of energy supply (Hiebert, 1992), although the proximate internal signals mediating the initiation of torpor are not well understood. In addition, it has been demonstrated that torpor is used to different energetic ends in different seasons and that the role of predictors such as energy reserves in regulating the use of torpor also varies, depending on both the season and the bird's recent energetic history (reviewed in Hiebert, 1994). During migration, captive birds maintain high body mass [the increase due primarily to increases in fat reserves (Carpenter *et al.,* 1993)] but nevertheless use torpor more than at any other time of year (Hiebert, 1993). During molt, rufous hummingbirds maintain a low body mass and seldom enter torpor, which is apparently reserved for times when energy reserves are dangerously depleted (Hainsworth *et al.,* 1977; Hiebert, 1992, 1993). These seasonal differences in use of torpor cannot be attributed to environmental variables such as temperature, day length, and food availability, but rather appear to be regulated by seasonal changes in the physiological condition of the birds (Hiebert, 1993).

In the present study three experiments, using noninvasive methods for measuring and manipulating CORT, were designed to elucidate the relation between CORT and daily torpor in hummingbirds. In the first two experiments, food was diluted to shift energy balance in a negative direction, and the effects of this treatment on CORT concentrations in cloacal fluid (CF) and on daily torpor were measured. This protocol was used once in the autumn, toward the end of the period when birds are in migratory condition, and in early spring, during the annual prebasic molt. In the third experiment, the effect of exogenous CORT on daytime behavior and nighttime torpor, in the absence of changes in the energy density of the food supply, was examined. If CORT functions to promote positive energy balance, CORT concentration and use of torpor should increase in response to food dilution and, when CORT is administered in the food, it should stimulate increased use of nocturnal torpor.

MATERIALS AND METHODS

Animal Capture and Maintenance

In July 1997, male rufous hummingbirds (*Selasphorus rufus*) were captured as juveniles near Hood Canal (Jefferson County) and on the slopes of Mt. Baker (Skagit County) in Washington, United States. Birds were held in captivity at the University of Washington

until June 1998, when they were released. Birds were housed in individual cages (76 × 53 × 48 cm), hereafter referred to as working cages, which were kept in walk-in environmental chambers that cycled daily between 20° (day) and 5° (night). Six birds were kept in each chamber, but were prevented from seeing each other by absorptive laboratory bench covering material that lined the sides and back of each cage. Changes in temperature were programmed to begin at lights-on or lights-off, and were completed within 10 min. Photoregime was designed to simulate changes in day length experienced by free-living birds, which migrate annually between wintering grounds in Mexico and breeding grounds ranging from Oregon to Alaska (Phillips, 1975), except that an extended period of LD 12:12 h lasting several weeks occurred around the autumnal and vernal equinoxes to permit experiments conducted at those times to take place in a constant photoperiod.

Food Consumption

Birds were fed a commercially available artificial nectar containing a complete mixture of nutrients, vitamins, and minerals (Nektar Plus, Nekton USA, or Quiko Nektar). The placement of feeders in each cage was such that hummingbirds had to hover to feed. Total daily food consumption (wet mass) was determined by weighing feeders containing artificial nectar at the beginning and end of each day. Dry mass of daily food consumption was computed to determine the relative energy content of the food consumed. Powdered Nektar Plus consists of 93.6% carbohydrates (mainly sugars), 2.0% protein, and 4.4% vitamins, minerals, negligible lipid, and nonmetabolizable components (Hiebert, 1992). Thus, most of the dry mass is comprised of energy-yielding carbohydrates. Reconstituted Nektar Plus at normal concentration consists of approximately 11% sucrose equivalents.

Torpor and Body Mass

For noninvasive measurement of torpor, each bird was weighed at lights-off on a top-loading electronic balance and placed in a smaller cage (18 × 28 × 13 cm) kept in the same environmental chamber as the working cages. Rufous hummingbirds normally do not fly at night, even when given adequate illumination and opportunity to do so, and even during autumnal and vernal migration times; thus, remaining on a perch throughout the night is normal for these birds (Hiebert, 1993). Each of these small cages contained a single, 2-cm perch onto which a fine thermocouple had been mounted. Temperatures from up to eight perch thermocouples could be recorded simultaneously on a Leeds and Northrup Speedomax 250 chart recorder. Contact between the bird's abdomen and the sensor provided surface body temperature, which has previously been shown to reflect accurately the large changes in body temperature that accompany entry into and arousal from torpor (Hiebert, 1993). At lights-on, birds were again weighed and returned to their working cages.

Cloacal Fluid Collection

CF was collected and analyzed according to methods of Hiebert *et al.* (2000). Briefly, foam-core boards covered with Saran Wrap (Dow Chemical) were placed on the floor of each cage. After 1 h, the boards were removed. Droplets of CF (but not feces) were then aspirated with a micropipettor, transferred to microcentrifuge tubes, placed immediately on ice, weighed, and frozen at −20° until assayed for CORT and creatinine (Cr) concentration.

Radioimmunoassay for Corticosterone

CF samples were introduced into the assay immediately after thawing and immediately refrozen for further analysis. Samples (50 μl each) in duplicate were incubated overnight at 4° with 150 μl PBSg, 100 μl [^{3}H]CORT, and 100 μl CORT antibody (B21-42; Endocrine Sciences, Tarzana, CA). In each assay, water blanks were analyzed to test for contamination. Additional tubes were run in every assay with 1000 pg of standard that was incubated with either charcoal-stripped CF or PBSg to assess accuracy of the assay. Dextran-coated charcoal (500 μl) was added to assay tubes following incubation to separate bound from free components. Samples were centrifuged at 2000 rpm for 10 min at 4° in a Beckmann TJ-6 refrigerated centrifuge. The resulting supernatant (containing CORT bound to antibody) was decanted into scintillation vials with 4.5 ml of scintillation fluid (Ultima Gold; Packard), which were read in a Beckman LS

3500 scintillation counter. CVs for these assays were 9.9 and 12.5%, respectively, and 6.4% for a CF pool (Hiebert *et al.*, 2000).

Creatinine Assay

Creatinine concentration in CF was determined using Sigma Kit 555-A and the modified method described in Hiebert *et al.* (2000). Cr concentration was used as a reference measure, and all CORT concentrations are expressed in units of ng CORT/mg Cr.

Video Analysis of Behavior

All six birds in each environmental chamber were videotaped simultaneously during each of the 1-h midday and evening CF collection periods. As described above, birds could hear but not see each other during videotaping. Observers who did not know which treatment that each bird had received recorded the duration of each bout of flying or feeding. All birds hovered while feeding, so that all feeding bouts were also flying bouts; unless explicitly stated, however, these two types of flying bouts are considered separately. From these data, total time spent flying and feeding, total number of feeding and flying bouts, and average duration of flying and feeding bouts were calculated for each bird during each hour of recording.

Experiment 1: Effect of Food Dilution during Autumn Migration

Experiment 1 took place from 24 October to 7 November 1997. Captive juvenile male rufous hummingbirds are usually in migratory condition from late August through November (Hiebert, 1993). Body mass before the experiment began (4.42–5.06 g) and the higher incidence of spontaneous nocturnal torpor (occurring in the absence of any experimental manipulation) were further verification of the birds' migratory status (Hiebert, 1993). Hence, these birds are referred to as "migratory birds" throughout this report. Day length of the simulated natural photoperiod decreased from 11 h 40 min on 24 October to 11 h 10 min on 7 November by changing lights-off time in 5-min increments. All birds used in this experiment ($N = 6$) had experienced CF collection and nighttime torpor measurement in a previous study.

Daily schedule. At lights-on (0900 PST), each bird was given freshly made artificial nectar. CF collecting boards were inserted into cages at 1415 h and removed at 1515 h (midday sample). CF collecting boards were again inserted into cages during the hour preceding the final 45 min of the day (evening samples; time varied as day length decreased). At 45 min before lights-off, both food and CF collecting boards were removed from the cage. Because clearing of the gut takes approximately 45 min, removing the food at this time ensures that body mass at lights-off reflects the amount of energy stored on the body and is negligibly affected by the mass of gut contents (Hiebert, 1992). At lights-off, each bird was weighed and placed in the night cage for monitoring torpor. At lights-on the following morning, each bird was reweighed and returned to its working cage.

Experimental timeline. On all days of the experiment, food consumption, body mass, and nocturnal torpor were monitored. During the first 4 days of the experiment, designed to habituate the birds to the daily schedule of the experiment, birds were fed artificial nectar at normal concentration. CF collection boards were inserted into and removed from cages at the appropriate time for the midday and evening collections, but CF was not collected. For the remainder of the experiment, CF was collected each day at midday and again in the evening. On days 5, 7, 9, and 11–14 of the experiment, birds were fed artificial nectar at normal concentration. On days 6, 8, and 10, birds were fed artificial nectar diluted to 85% of normal concentration, a concentration that is known to affect energy balance in this species but in the face of which the birds are able to adjust energy expenditures so that they do not exceed energy intake (Hiebert, 1991). Otherwise, treatment on days 6, 8, and 10 was identical to those on the remaining days of the experiment.

Statistical analysis. The effects of food dilution on wet and dry mass of food intake, evening body mass, daytime mass gain, torpor duration, and CF CORT at midday and in the evening were determined by averaging each variable over days 6, 8, and 10 (food dilution days) and over days 7, 9, and 11 (control solution days) for each bird. Days 7, 9, and 11 were chosen for comparison because each of these days is preceded by a food dilution day. Averages for food dilution days were compared with averages for control solution days with a paired t test for variables

with normal distributions and nonheterogeneous variances and with a Wilcoxon signed-rank test for variables that did not meet the assumptions of the parametric *t* test. Based on data from previous studies (e.g., Hiebert, 1991), food dilution should result in increased use of torpor; thus a one-tailed analysis was used for this variable. Multiple regression analysis was used to determine whether there was a correlation between CF CORT and torpor duration, using a total of six data points for each variable from each bird (corresponding to the three food dilution days, 6, 8, and 10, and the three control solution days that immediately followed them, 7, 9, and 11). The model that we used controlled for both body mass and bird individual, thus making it possible to include multiple data points from each bird.

Experiment 2: Effect of Food Dilution during Molt

Experiment 2 took place from 19 March to 2 April, during which time day length of the simulated natural photoperiod increased from 11 h 55 min to 12 h 45 min in 5-min increments. All birds in this study ($N = 8$) were molting, and all birds had experienced CF collection and nighttime torpor measurement in previous studies. Because of asynchrony among individual birds and because of the relatively small numbers of captive birds, it was not possible to choose eight birds that were both molting simultaneously and not represented in Experiment 1. Three birds participated in both experiments, but all birds in both experiments had had similar exposure to the standard CF collection protocol before participating in Experiments 1 and 2.

Daily schedule of this experiment was the same as that of Experiment 1, except that afternoon CF collections began at 1415 h to compensate for the difference in average day length between Experiments 1 and 2. The experimental timeline was also the same as that in Experiment 1, except that the experimental timeline began 1 day later for half of the birds ($N = 4$), so that on any food dilution day, half of the birds were receiving diluted nectar and the other half were receiving the control solution, to avoid possible confounds with day of treatment.

Statistical analysis. Statistical analysis was performed as for Experiment 1. In addition, two-sample *t* tests (for data meeting the assumptions of parametric tests) and Mann–Whitney *U* tests (MWU; for data that did not meet these assumptions) were used to compare results from Experiment 1 and Experiment 2. Because three birds are represented in both experiments, the data do not meet the assumptions of independence normally required by the MWU. However, use of two-sample tests should yield conservative estimates because the power of repeated measures is not utilized.

Experiment 3: Effect of Corticosterone Treatment

To make possible the delivery of CORT, a lipid-soluble hormone, in water-based artificial nectar, CORT was added to the nectar as a complex with 2-hydroxypropyl-β-cyclodextrin (HBC–CORT; Research Biochemicals International). Cyclodextrins are digestible, water-soluble carbohydrates containing hydrophobic inner regions where lipid-soluble molecules may be inserted (Pitha and Pitha, 1985). When cyclodextrin-complexed hormones are ingested, the carbohydrate is digested and the hormone is released so that it can be taken up across digestive tract epithelium (Pitha *et al.*, 1986; Hiebert *et al.*, 2000). Because hormones are active at very low concentrations, the small amounts of cyclodextrin needed as a vehicle for CORT have a negligible effect on the energy content of nectar.

Experiment 3 took place on 4–5 June ($N = 8$ birds) and on 6–7 June ($N = 8$ birds), for a total sample size of 16 birds. Daylength on all days was 15 h and 45 min, with lights-on at 0800 PDT. In each 2-day segment, each bird received artificial nectar containing 2.74 μM cyclodextrin-complexed CORT from lights-on to lights-off on one day and, as a control, an equivalent concentration of 2-hydroxypropyl-β-cyclodextrin not complexed with CORT (HBC; Research Biochemicals International) from lights-on to lights-off on the other day (Hiebert *et al.*, 2000). In each group of 8 birds tested together, 4 birds received the HBC–CORT treatment on the first day, and the other 4 birds received the control treatment first. The order of treatment for each bird was chosen in such a way as to balance (1) the number of birds receiving each treatment within each walk-in environmental chamber and (2) the distribution of body masses in the two treatment–order groups.

On each day of the experiment, total daily food consumption, body mass at lights-on and lights-off,

and torpor during the following night were measured. CF was collected for 1 h in the middle of the day (1415 to 1515 h) and for 1 h just before lights-off (time changed as day length changed; see above). Nine of the 16 birds were videotaped for behavioral analysis. In contrast to Experiments 1 and 2, food was not removed from the cage before lights-off, both because food removal itself may induce a stress response and because removing food would remove the exogenous source of CORT.

Statistical analysis. The effects of treatment on midday and evening CF CORT, food consumption, daytime mass gain, and torpor duration were determined with a paired *t* test ($df = 15$). Because of significant heterogeneity of variance in behavioral response measures, the paired nonparametric Wilcoxon signed-rank test was used to determine the effect of CORT on behavior.

RESULTS

Experiment 1: Effect of Food Dilution during Autumn Migration

Habituation. Evening body mass decreased significantly from day 1 to day 4 of the habituation period (paired *t* test, $P = 0.02$), but daytime mass gain (the difference between dawn and dusk body masses) increased significantly in the same period (paired *t* test, $P = 0.003$) (Fig. 1A). Total daily food consumption (measured as either wet or dry mass consumed) showed the greatest increase between day 1 and day 2, after which it tended to decrease; however, there was still a significant increase in food consumption from day 1 to day 4 (paired *t* test, $P = 0.005$) (Fig. 1A). Torpor duration increased over the habituation period in those birds that did not use torpor on day 1 ($N = 4$), but, overall, the difference between day 1 and day 4 was not statistically significant (paired *t* test, $P = 0.26$) (Fig. 1A). Taken as a whole, the changes reported here suggest that the experimental protocol requires some energetic adjustment on the part of the birds and that a habituation period lasting several days is needed before energy balance stabilizes.

Food consumption. Total daily food consumption (wet mass) was significantly higher on food dilution days (days 6, 8, and 10) than on control days (days 7, 9, and 11) (paired *t* test, $P = 0.0005$) (Fig. 1A). However, there was no significant increase in total energy intake (dry mass of food) on food dilution days (paired *t* test, $P = 0.09$) (Fig. 1A). Because birds must hover to feed, increased wet mass food consumption requires the expenditure of additional energy, in this case for only a marginal increase in total energy intake. The result is that on food dilution days, there was a decrease in net energy gain in comparison with days on which birds fed on control nectar.

Body mass. Evening body mass decreased from day 1 through day 7 and then remained relatively constant until the end of the experiment on day 15 (Fig. 1A). There was no significant difference between evening body mass on food dilution days and on control days ($P = 0.16$). Daytime mass gain on food dilution days was significantly lower than that on control days (paired *t* test, $P = 0.009$), reflecting the decrease in net energy gained during feeding on dilute food (Fig. 1A).

CF corticosterone. In CF samples taken at midday, CORT begins to show a pattern of elevation on food dilution days, alternating with depression on control food days immediately following food dilution days; the difference, however, is not statistically significant (Wilcoxon, $P = 0.29$) (Figs. 1A and 3). In evening CF samples, this pattern is more pronounced and the difference between CORT on food dilution and CORT on control days is significant (Wilcoxon, $P = 0.046$) (Figs. 1A and 3). In addition, CORT was significantly higher in evening samples than in midday samples on control days (Wilcoxon, $P = 0.03$) but not on food dilution days (Wilcoxon, $P = 0.075$) (Fig. 3).

Torpor. Duration of nocturnal torpor increased significantly on nights following food dilution days, in comparison with nights following control days (paired *t* test, $P_{\text{one-tailed}} = 0.031$) (Fig. 1A). Multiple regression showed that torpor and evening CF CORT had a significantly positive relation ($P = 0.043$). In midday samples, however, there was no significant relation between CF CORT and torpor duration ($P = 0.08$).

Experiment 2: Effect of Food Dilution during Molt

Habituation. Although mean body mass decreased from day 1 to day 4 of habituation as in

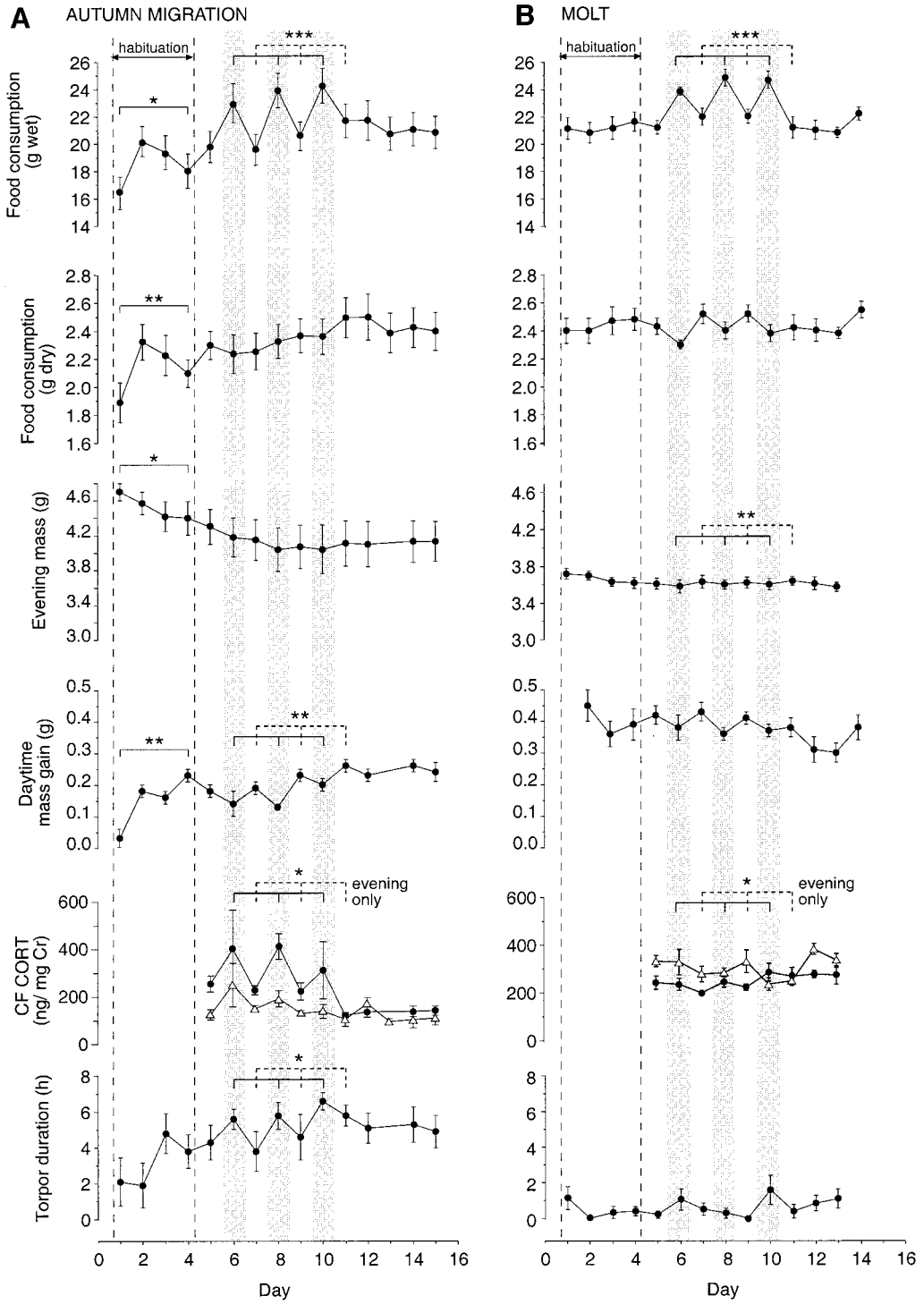

FIG. 1. Daily food consumption, expressed as both total wet mass and total dry mass (an index of energy content), evening mass, daytime mass gain, corticosterone (CORT) concentration in cloacal fluid (CF) in midday (open triangles) and evening (filled circles) samples, and torpor duration during two food dilution experiments conducted (A) during autumn migration (Experiment 1, $N = 6$ birds) and (B) during molt (Experiment 2, $N = 8$ birds). Days 1–4 of each experiment (between vertical dashed lines) were used for habituating the birds to the feeding and CF collection protocol. Food was diluted to 85% of control concentration on days 6, 8, and 10 of each experiment (vertical gray bars); on all other days, birds received an unlimited amount of artificial nectar at control concentration. Horizontal brackets show comparisons with statistically significant differences; pairs of three-pronged brackets represent comparisons between the average on days 6, 8, and 10 and the average on days 7, 9, and 11. *$P < 0.05$; **$P \leq 0.005$; ***$P \leq 0.0005$. For CF CORT, comparisons were statistically significant only for evening samples (filled circles). Vertical bar around each point represents SE; where error bars are not visible, they are contained within the diameter of the symbol.

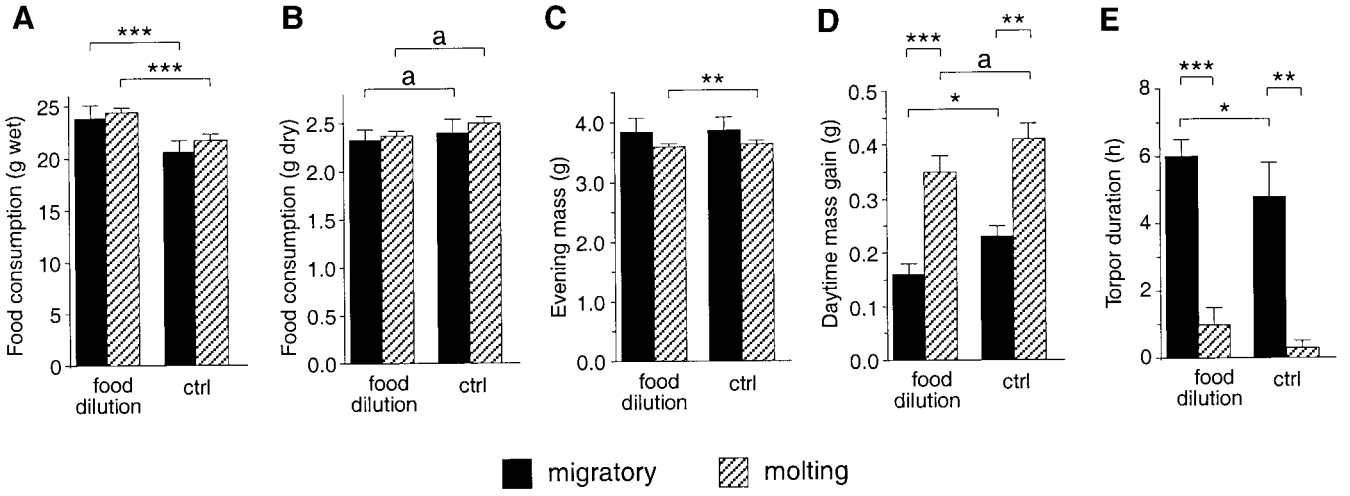

FIG. 2. Comparisons between treatments (food dilution or control) and between birds in different seasonal physiological states (migratory, $N = 6$; or molting, $N = 8$) of five response measures: food consumption (A, wet mass, a measure of total nectar intake, and B, dry mass, a measure of energy intake); C, evening body mass; D, body mass gain from lights-on to lights-off; and E, torpor duration during the immediately following night. Each bar represents the grand mean (+ 1 SE) of mean values for each bird of either the 3 food dilution days or the 3 control days with which the food dilution days alternated. $^{a}0.10 > P > 0.05$; $*P < 0.05$, $**P < 0.005$; $***P < 0.0005$.

migratory birds, this difference was not statistically significant in molting birds (paired *t* test, $P = 0.25$) (Fig. 1B). Whereas daytime mass gain and daily food consumption increased significantly during habituation in migratory birds, neither changed significantly during the habituation period in molting birds (paired *t* test, $P > 0.2$ in both cases) (Fig. 1B). As in migratory birds, torpor duration did not change significantly over the habituation period (paired *t* test, $P = 0.5$) (Fig. 1B). A habituation period may thus be more important for this protocol during migration than during molt.

Food consumption. As in migratory birds, total daily food consumption (wet mass) was significantly higher on food dilution days than on control days (paired *t* test, $P < 0.0001$), and there was a tendency toward decreased dry mass food consumption (a measure of gross energy intake) on food dilution days, but this difference was not significant (paired *t* test, $P = 0.068$) (Fig. 1B). Net energy gain from feeding should therefore have been reduced on food dilution days because birds must hover longer to obtain the same or less energy. Amount of food consumed, expressed either as wet mass or as dry mass (energy content), was not significantly different between migratory and molting birds on either food dilution or control days (paired *t* test, $P > 0.3$ in all cases) (Fig. 2).

Body mass. Although mean difference between evening mass on food dilution days and evening mass on control days was small, paired analysis showed that evening mass was significantly lower on food dilution days than on control days in molting birds (paired *t* test, $P = 0.0048$) (Figs. 1B and 2). Consistent with this finding, mean daytime mass gain was lower on food dilution days than on control days (Figs. 1B and 2), but this difference was not significant (paired *t* test, $P = 0.08$).

Comparisons between migratory and molting birds showed that variance in evening mass was significantly greater in migrating than in molting birds on both food dilution and control days (*F* test, $P = 0.01$ in both cases), but that there was no significant difference in evening mass between seasons on either food dilution or control days (MWU, $P > 0.60$ in both cases) (Fig. 2). Daytime mass gain, however, was significantly greater in molting than in migratory birds, on both control and food dilution days (two-sample *t* test, $P < 0.004$ in both cases) (Fig. 2).

Corticosterone. Midday CF CORT in molting birds shows no discernible pattern relative to treatment, and, as in migratory birds, there is no significant difference between food dilution and control days (paired *t* test, $P = 0.9$) (Figs. 1B and 3). Evening samples are also like those from migratory birds,

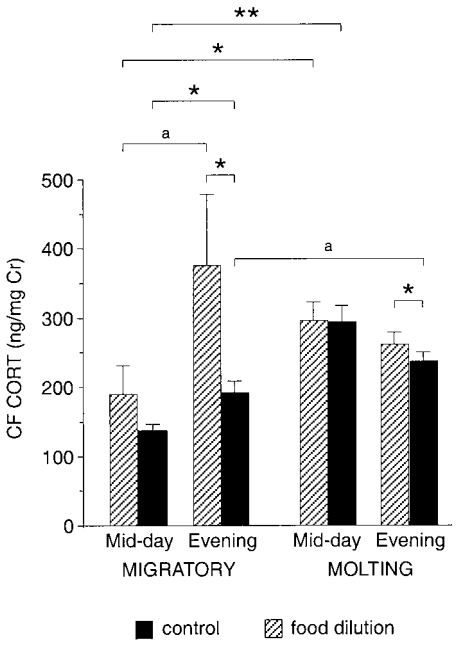

FIG. 3. Concentration of corticosterone (CORT) in cloacal fluid (CF) samples collected at midday and in the evening in food dilution experiments conducted on hummingbirds during autumn migratory period ($N = 6$) and annual prebasic molt ($N = 8$). Bars represent means for 3 food dilution days and each of the 3 subsequent control days. Only those comparisons that were statistically significant are shown with horizontal bars ($^{a}P = 0.054$; $^{*}P < 0.05$; $^{**}P < 0.005$).

showing a significant elevation in CORT on food dilution days relative to control days (paired t test, $P = 0.04$) (Figs. 1 and 3).

There are, however, some differences in hormone concentrations in the two seasons. Midday CF CORT was higher in molting than in migratory birds, on both food dilution and control days (two-sample t test, $P = 0.04$ and MWU, $P = 0.0002$, respectively) (Fig. 3). In evening samples, there is no seasonal difference in CF CORT on food dilution days (MWU, $P = 0.4$), but on control days the higher mean values in molting birds than in migratory birds approach significance (two-sample t test, $P = 0.054$) (Fig. 3). The result is that in migratory birds, CF CORT tends to increase from midday to evening, whereas during the molt, CF CORT decreases from midday to evening, and the magnitude and direction of these changes are significantly different between seasons both on food dilution days (two-sample t test, $P = 0.039$) and on control days (two-sample t test, $P = 0.0008$) (data not shown).

Torpor. In molting birds, there was no significant difference in mean torpor duration on food dilution and control days (paired t test, $P = 0.16$) (Figs. 1B and 2). Multiple regression showed no significant relation between torpor and CF CORT concentration, at either midday or in the evening ($P > 0.6$ in both cases).

In general, torpor occurred much less frequently in molting birds (23 of 104 bird-nights = 22%) than in migratory birds (65 of 78 bird-nights = 83%) (Figs. 1 and 2). During molt, one bird did not enter torpor on any night of the study. As a result, mean torpor bout duration was significantly greater in migratory birds than in molting birds, on both food dilution and control days (two-sample t test, $P < 0.0001$ and MWU, $P = 0.002$, respectively) (Figs. 1 and 2).

Experiment 3: Effect of Corticosterone Treatment

Corticosterone. When birds were fed artificial nectar containing cyclodextrin-complexed CORT, they had significantly higher CF CORT than when they were fed the control solution containing only cyclodextrin, in both midday and evening samples (paired t test, $P < 0.0001$ in both cases) (Fig. 4). On days when birds were given CORT in their food, mean CF CORT was higher in evening samples than in midday samples, but this difference was not significant (paired t test, $P = 0.10$) (Fig. 4). There was a significant day effect on CF CORT at midday in response to CORT treatment: birds receiving CORT treatment on the second day had significantly higher CF CORT than those receiving CORT on the first day (two-sample t test, $P = 0.026$), suggesting that there might be additional "stress" on the second day of the experiment (Fig. 5). This difference had disappeared, however, in the evening samples (two-sample t test, $P = 0.78$) (Fig. 5). Because CORT was higher in birds receiving CORT on the second day of the study, higher CORT on the second day could not be attributed to exogenous CORT remaining in the birds from the previous day's treatment.

Although we expected birds that consumed more food to consume more dietary CORT and therefore to have higher CF CORT, there was no significant correlation between total daily food consumption and CF CORT in either midday or evening samples ($P > 0.20$ in both cases) (data not shown).

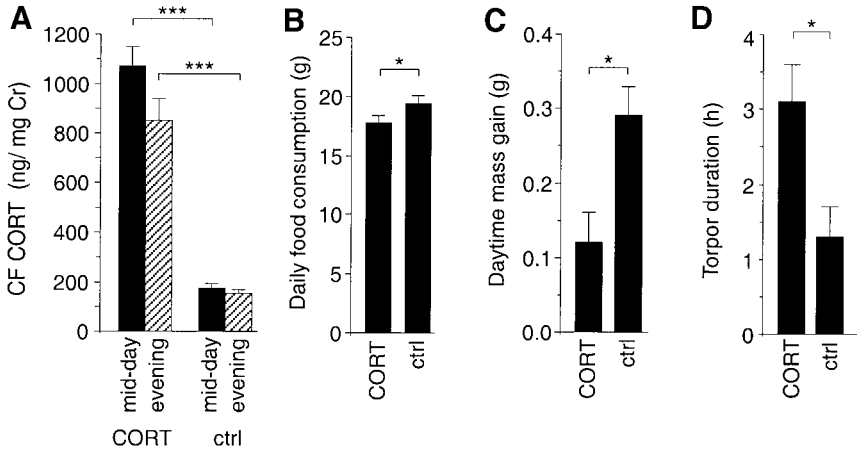

FIG. 4. Effects of oral treatment with cyclodextrin-complexed corticosterone (CORT) on concentration of CORT in cloacal fluid (CF) of hummingbirds ($N = 16$) at midday and in the evening, daily food consumption, daytime mass gain, and duration of torpor the following night, as compared with effects of treatment with control nectar (*$P < 0.05$; ***$P < 0.0005$).

Food consumption. When feeding on CORT-supplemented nectar, birds consumed significantly less food than when feeding on control nectar (paired *t* test, $P = 0.037$) (Fig. 4). There was no significant effect of treatment order on food consumption, in response to either CORT or control treatments (two-sample *t* test, $P > 0.4$ in both cases).

Body mass. In congruence with food consumption data, mass gain from lights-on to lights-off was significantly lower on days when birds consumed CORT-containing nectar than when they consumed control nectar (paired *t* test, $P = 0.014$) (Fig. 4).

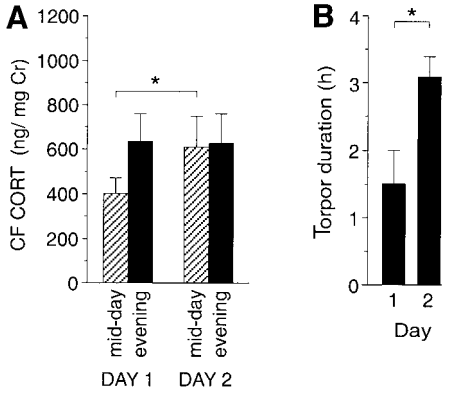

FIG. 5. Day effects of oral treatment with cyclodextrin-complexed corticosterone (CORT) on concentration of CORT in cloacal fluid (CF) of hummingbirds ($N = 16$) at midday and in the evening and on torpor duration the following night (*$P < 0.05$).

Torpor. Torpor duration was significantly higher following days when birds were fed CORT-containing nectar than when they were fed control nectar (paired *t* test, $P = 0.014$) (Fig. 4). In addition, there was a significant day effect: mean torpor duration was significantly higher on the second day of the experiment than on the first day (paired *t* test, $P = 0.018$) (Fig. 5). This overall day effect was due not to a difference in the effect of CORT treatment on day 1 and day 2 of the experiment (two-sample *t* test, $P = 0.54$), but on a significantly greater tendency for birds receiving control nectar to enter torpor if they received the control treatment on day 2 (two-sample *t* test, $P = 0.0004$) (Fig. 5).

Behavior. In general, birds were more active at midday than in the evening. When all birds in both treatment groups were considered together, total flying time (Wilcoxon, $P = 0.006$) and number of flying bouts (Wilcoxon, $P = 0.015$) were significantly greater at midday, but feeding bout duration (Wilcoxon, $P = 0.0045$) was significantly greater in the evening. Qualitatively similar results were obtained when treatment groups were considered separately, but only the feeding bout durations in control birds were significantly different between midday and evening samples (paired *t* test, $P = 0.03$) (Fig. 6). The effects of hormone treatment, however, were less clearly defined. Median values for all behavioral measures (total flight time, total feeding time, number of

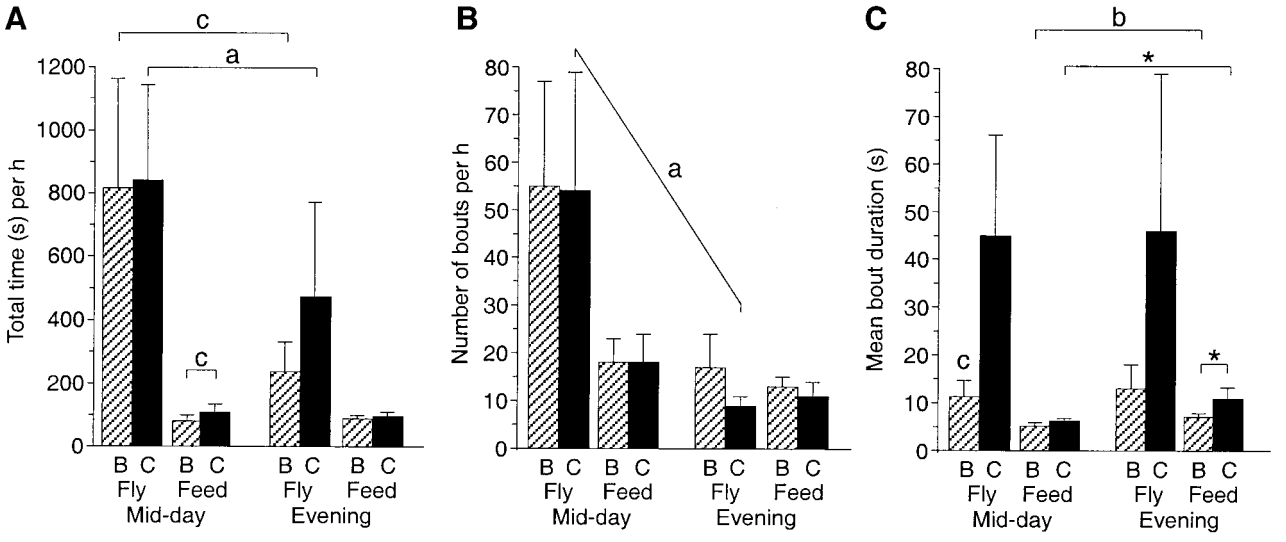

FIG. 6. Effects of oral treatment with cyclodextrin-complexed corticosterone, B, on behaviors displayed by hummingbirds ($N = 9$) over a 1-h period at midday or in the last hour before lights-off, as compared with effects of control, C, nectar. Behavioral measures were (A) total time spent flying without feeding (FLY) or hovering to feed (FEED), (B) number of bouts (each bout begins when bird leaves the perch and ends when it perches again) of FLYing or FEEDing, and (C) mean duration of FLY or FEED bouts (*$P < 0.05$; [a]$P = 0.0506$; [b]$P = 0.0587$; [c]$P = 0.0663$).

flying bouts, number of feeding bouts, average duration of flying bouts, and average duration of feeding bouts) were depressed in CORT-treated birds relative to controls in both midday and evening recording periods, but only in the case of evening feeding bout duration (Wilcoxon, $P = 0.03$) was this difference significant. Increased total feeding time at midday relative to total feeding time in the evening approached significance ($P = 0.066$), but for all other variables and times, $P > 0.10$ (Fig. 6).

DISCUSSION

Food Dilution and CORT in Migratory and Molting Birds

In the first experiment, conducted during the time of the autumnal southward migration, food dilution was demonstrated to shift energy balance in the negative direction. As predicted, CF CORT concentrations increased in response, and the duration of torpor was positively correlated with increases in evening CF CORT. These data are consistent with the hypothesis that CORT can act as a signal that responds to the energy status of the bird and causes an increase in the duration of nocturnal torpor.

The response of birds tested with the same protocol during the molt indicates that there are differences, both in the response of CORT to energy manipulation and in the apparent ability of CORT to stimulate the use of torpor, related to the seasonal physiological state of the birds. Although migratory birds had higher body masses than molting birds at the beginning of the habituation period (as in Hiebert, 1993), body masses on food dilution and intervening control days were not significantly different in the two seasons. In both seasons, energy balance was shifted negatively by diluting the food, and total food consumption, calculated either as total mass of wet food consumed or as energy content, was not different in the two seasons. Despite these similarities, the effect of food dilution in reducing daytime mass gain was significantly greater in migratory birds than in molting birds, an effect which may result from differences in daytime activity (not measured in these experiments). It could therefore be argued that daytime mass gain, rather than the evening mass of the bird, is the important factor in predicting nighttime torpor use. This interpretation is attractive because it postulates that

the birds are somehow integrating information about their recent energetic history rather than relying simply on a report of current energy status. Previous experiments with this species strongly suggest that current energy status, by itself, is not an accurate predictor of torpor, at least in some seasons (Hiebert, 1991).

How does CORT fit into such a hypothesis? In migratory birds, food dilution increases evening but not midday CF CORT, CORT tends to increase from midday to evening on all days, torpor occurs more frequently, and evening CORT concentrations are positively correlated with the duration of torpor. It is possible that molting birds could also have a significant positive relation between CORT concentration and torpor duration, but that the manipulation that we used was not sufficiently stringent to demonstrate a measurable response. Previous experiments in our laboratory have demonstrated that torpor can be elicited reliably at any time of year, as long as food is sufficiently restricted (Hiebert, 1990, 1991, 1992). Because molting birds appear to avoid the use of torpor whenever possible (Hiebert, 1991, 1992) and because they can compensate for decreases in energy intake in other ways, molting birds tend to require more food restriction to elicit torpor than do birds at other times of year.

In molting birds, food dilution also increases evening CORT but not midday CORT, but CORT levels tend to decrease from midday to evening, torpor occurs less frequently, and evening CORT levels are not significantly correlated with the duration of torpor. Furthermore, CORT levels at both times of day during the molt tend to be equal to or higher than their counterparts in autumn. These findings demonstrate that evening CORT per se cannot be the major predictor of torpor duration. At the very least, a seasonal difference in sensitivity to CORT would be needed to explain how higher CORT concentrations can result in lower torpor use during the molt than in the autumn. A second possibility is that nocturnal torpor is predicted by the change in CORT concentration during the preceding day. The daily increase in CORT during autumn and daily decrease in CORT during the molt are consistent with such a hypothesis. A third possibility is that other signals modify the seasonal response to CORT. We are currently conducting studies in which CORT is manipulated exogenously to address these questions.

Exogenous CORT and Torpor

In the third experiment, administration of CORT in the diet increased the use of daily torpor in the absence of food dilution, supporting the hypothesis that the propensity to use nocturnal torpor is regulated at least partially by CORT in hummingbirds. In addition, there was evidence that CORT also increases feeding behavior. Paradoxically, however, artificially administered CORT had other effects on these birds that were inconsistent with the prediction that CORT should promote positive energy balance. Exogenous CORT decreased total daytime mass gain, which has been shown in this and other studies to increase the use of torpor in hummingbirds (Hainsworth *et al.*, 1977; Hiebert, 1991, 1992). These decreases in daytime mass gain could be due to a variety of factors, including decreased feeding and/or increased flying time and increased catabolic activity as protein stores are broken down to provide precursors for gluconeogenesis (e.g., Gray *et al.*, 1990). Thus, the results of this experiment do not distinguish between the possibility that CORT directly stimulated the use of nocturnal torpor and the possibility that CORT stimulated the use of nocturnal torpor indirectly through decreases in energy reserves (measured as body mass).

This paradox—that CORT should decrease daily accumulations of energy reserves—might be explained by the relatively high concentrations of CF CORT resulting from exogenous hormone treatment. The highest endogenous CORT concentrations measured so far in hummingbird CF in our laboratory have come from studies in which birds were restrained for 1 h, so that the transient increase in CORT concentrations could be measured (Hiebert *et al.*, 2000). Maximum CF CORT during these studies ranged from 315 to 811 ng CORT/ng Cr (mean = 543 ng CORT/ng Cr), whereas the CF from birds given exogenous CORT in the present study had somewhat higher CORT concentrations, ranging from 352 to 1511 ng CORT/ng Cr (Hiebert *et al.*, 2000). It is possible that CF CORT concentrations exceeding 800 to 900 ng CORT/ng Cr are outside the physiological range, suppressing food intake because of an overdose effect rather than a normal physiological response. Because

intestinal absorption of cyclodextrin-complexed steroids may not be as effective as absorption across buccal epithelium (Pitha *et al.*, 1986), it is also possible that some of the exogenous CORT measured in the CF was never absorbed; if this is the case, CF CORT concentrations measured in HBC-CORT-treated birds should be considered overestimates of the dose that each bird actually experienced.

In a study examining the dose-dependent effects of CORT on perch-hopping behavior in white-crowned sparrows (*Zonotrichia leucophrys*), Breuner *et al.* (1998) found that high doses of CORT induced only low levels of activity, whereas low doses induced significantly higher levels of activity. Similar effects of high and low doses of CORT have been reported for mammals (Dallman *et al.*, 1993). A possible adaptive explanation for these findings is that under mildly stressful conditions it may benefit the bird to increase its activity, including foraging effort, but that under very poor environmental conditions the bird may increase its chance of survival by conserving as much energy as possible until conditions improve (Wingfield and Ramenofsky, 1999). Breuner *et al.*'s (1998) study demonstrates that suppressed activity may be a normal, physiological component of the adrenocortical stress response in birds.

One important way in which Breuner *et al.*'s (1998) study and the present study differ is that Breuner's treatments were single, acute administrations of CORT in a single mealworm, whereas in our study CORT was administered continually throughout the day. Because it is known that acute and chronic elevations in CORT may have different effects even though peak concentrations are similar, it is possible that the two experiments provided the birds with different kinds of CORT signals (Wingfield and Ramenofsky, 1999). However, we consider this interpretation unlikely because most chronic effects require several days to develop (Wingfield and Ramenofsky, 1999).

The principal advantage of administering exogenous CORT in the diet is that dosing does not itself provoke a stress response. A potential complication of this method is that food consumption may vary over time as a consequence of food (and hormone) consumption during the previous time interval. If, as in Breuner *et al.*'s (1998) experiment, feeding is nonlinearly related to dose, a situation could arise in which a bird begins to feed, is stimulated to increase food intake by the low hormone dose ingested, then is stimulated by the resulting high hormone dose to feed less, and so on, with the ultimate consequence that hormone concentrations fluctuate cyclically throughout the day. Such an effect could explain the counterintuitive result in the present study that CF CORT concentration is not significantly correlated with total daily food consumption. If the hormone consumption cycles of individual birds are out of phase with one another, a single sampling period may isolate different phases of the cycle for different birds, thus obscuring any overall relation between food consumption and hormone concentration.

Relation between CORT and Torpor in Birds and Mammals

Although the effect of CORT on torpor has been previously considered, studies have focused primarily on seasonal patterns of adrenal glucocorticosteroids in hibernating mammals and the relation of those patterns to the coming hibernation season (Saboureau and Boissin, 1983; Shivatcheva *et al.*, 1988; Armitage, 1991; Boswell *et al.*, 1994). With a few exceptions (e.g., Fowler, 1988), the findings of these studies generally demonstrate that plasma corticosteroid concentrations are lowest around the time when animals emerge from their hibernacula in spring, thereafter increasing to their peak values just before the next hibernation season begins. Although daily torpor and seasonal hibernation are considered by many to represent homologous physiological states, there are substantial differences in the energy ecology of animals that must prepare for months of hibernation in comparison with those that can make use of brief periods of torpor during each 24-h cycle. Daily heterotherms may require torpor regulation that differs not only in degree (i.e., is more sensitive to immediate energy status) but also in kind (incorporates additional or different signals). Thus, previous studies on adrenocortical responses in relation to hibernation do not obviate the need for studying these responses in relation to daily torpor.

Previous studies have demonstrated that hormones other than glucocorticoids have potent effects on the ability of rodents to enter daily or seasonal torpor. High concentrations of testosterone and prolactin ap-

pear to be incompatible with daily or seasonal torpor (Darrow *et al.*, 1988; Lee *et al.*, 1990; Ouarour *et al.*, 1991; Ruby *et al.*, 1993) in many small mammals. Although rufous hummingbirds retain the ability to enter torpor at any time during the annual cycle, there is substantial evidence both from the present and from previous studies (Hiebert, 1991, 1993, 1994) that the propensity for entering torpor varies seasonally. The present study suggests that seasonal changes in the HPA axis are at least partly responsible for seasonal differences in the use of torpor, but reproductive hormones may play an important role as well. A substantial literature documents the antagonistic interactions between the adrenocortical stress response and reproduction (Wingfield *et al.*, 1998; Wingfield and Ramenofsky, 1999; Sapolsky *et al.*, 2000; Tilbrook *et al.*, 2000), suggesting that effects of corticosterone on torpor might be at least partially mediated by its antagonistic effects on the activity of testosterone, which ordinarily suppresses torpor. Current studies are aimed at testing this hypothesis.

Although evidence from the present study suggests that CORT is involved in regulating nocturnal torpor, the question remains: Can CORT alone predict the duration of torpor throughout the annual cycle, or is seasonal variation in the use of torpor due to the interaction of CORT with other signals or changes in receptor number or affinity? If the former, some higher-order feature of CORT, such as the direction and/or rate of change in CORT during the preceding hours, is likely to be more important than CORT concentration per se. Alternatively, CORT may encode different information in different seasons, or the responsiveness of the torpor control system to CORT may be modulated by other season-specific factors. Further experiments are planned to answer these questions.

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