

Noninvasive Methods for Measuring and Manipulating Corticosterone in Hummingbirds

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The adrenocortical response to stress has been shown to be important in energy management of vertebrates. Although hummingbirds (Trochilidae) are useful models for studying energy balance, they are not amenable to traditional methods of studying hormones. In this study we report noninvasive methods for measuring and manipulating corticosterone (CORT), the principal stress glucocorticoid in birds. CORT was measured in cloacal fluid (CF) collected from unrestrained rufous hummingbirds (*Selasphorus rufus*). We demonstrate that CF CORT can be measured by radioimmunoassay without extraction. CF creatinine, when used as a reference measure for CF CORT, corrects for changes in hydration state. As in other birds, CORT in both plasma and CF rose in response to capture and handling stress and decreased after the termination of that stress, except that changes in CF concentration were delayed with respect to changes in plasma. When CORT, complexed with cyclodextrin to improve solubility, was added to artificial nectar, CF CORT concentrations changed in a predictable, dose-dependent fashion. Measuring CORT in CF is advantageous because it allows frequent and repeated sampling without itself provoking a detectable stress response and because baseline samples need not be obtained within the very short time between the onset of a stressor and the appearance of CORT in the plasma, as is true for blood

sampling. Administration of exogenous CORT in the food offers a noninvasive, nonstressful, temporally sensitive method for experimentally manipulating hormone levels in an avian model that has already been used extensively for studies of energetics. © 2000 Academic Press

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Hummingbirds have been the subjects of many physiological and behavioral investigations, including studies of foraging, territoriality, migration, population dynamics, flight mechanics, metabolic fuel mobilization, and nocturnal torpor. A common theme for many of these studies is the regulation of energy balance (see Hainsworth, 1981). Hummingbirds are valuable subjects for addressing such questions because they exist at the lower limits of body size for endotherms; in addition, many live in cool climates and some, such as the rufous hummingbird (*Selasphorus rufus*), incur the additional energetic cost of annual long-distance migrations. Thus, hummingbirds exemplify August Krogh's observation that particular groups of animals are often especially useful for investigating particular biological problems (Krebs, 1975).

Hummingbirds have evolved profound and easily measured responses to the problem of energy shortage, such as daily torpor, and are therefore potentially valuable subjects for studies of the hormonal regula-

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tion of energy management. The primary disadvantage of this avian group for endocrine studies, however, is the same feature that renders them especially suitable for studies of energy regulation: small body size. Endocrine studies are typically carried out by measuring plasma hormone concentrations, but the small samples that can be obtained from hummingbirds are far smaller than those required for most hormone analyses and prohibitive for repeated-sampling protocols. Corticosterone (CORT), the predominant glucocorticoid in birds, is known to be important in the response to unpredicted energy shortfall (Harvey *et al.*, 1984; Dallman *et al.*, 1994; Wingfield *et al.*, 1997, 1998). However, concentrations of this hormone in the blood increase in response to blood sampling itself. In fact, the elevation of corticosteroid induced by capture has been used as a standard protocol for probing the ability of the hypothalamic–pituitary–adrenal axis to respond to environmental stressors (e.g., see Wingfield *et al.*, 1992; Wingfield, 1994). In the interest of making use of the hummingbird model for studies involving the role of the adrenocortical stress response in energy management, we have developed noninvasive methods by which endogenous CORT can be measured at frequent (≤ 30 -min) intervals and by which exogenous CORT can be administered.

The flower nectar (and sometimes tree sap; Southwick and Southwick, 1980) on which wild hummingbirds feed is primarily a dilute solution of simple sugars (Baker and Baker, 1975), the consumption of which typically results in a water excess (Calder and Hiebert, 1983). Consequently, hummingbirds urinate frequently in volumes relatively large for their body size. In addition to waste products and water, urine contains hormones (and/or their metabolites) in concentrations that reflect the presence of the hormone in plasma. Thus, urine concentrations rise and fall with plasma concentrations, the primary differences being that (1) the appearance of the hormone in the urine is delayed with respect to its appearance in the plasma and (2) peaks in urinary hormone concentrations tend to last longer and have lower amplitude than those in the plasma. However, some of these effects should be offset by the short digestive transit times (Hainsworth, 1974) and rapid metabolism of hummingbirds, which should in turn result in rapid clearance rates of blood-borne components. The collection of studies reported here demonstrates that CORT concentration can be

measured in the cloacal fluid (CF) of hummingbirds, which consists of combined renal and alimentary wastes, and that this concentration is biologically meaningful.

MATERIALS AND METHODS

General Methods

Animals. Juvenile male rufous hummingbirds were captured in July 1997 at a lowland site near Hood Canal, Jefferson County, Washington (elevation ~ 100 m) and at a montane site near Mt. Baker, Skagit County, Washington (elevation ~ 600 m). Birds were housed in individual wire-mesh cages ($76 \times 53 \times 48$ cm) kept within walk-in environmental chambers. Cages were covered on three sides with absorbent plastic-backed laboratory bench cover to prevent birds from seeing each other. These covers also reduced evaporation of CF from the surface of the collection boards by reducing the otherwise high rates of convection in the environmental chamber. Birds were kept on a simulated natural photoperiod designed to approximate photoperiodic conditions as experienced in nature by rufous hummingbirds allowed to undergo their normal annual migrations between wintering grounds in Mexico and breeding grounds in the Pacific Northwest of the United States (Phillips, 1975). Day length was programmed digitally, with lights-on at 0800 h and the lights-off time changed in 10-min increments to yield the calendar-appropriate day length. Ambient temperature was 20° during lights-on and 5° during lights-off. In the environmental chambers, changes between these two temperatures were programmed to begin at lights-on and lights-off and were completed within 10 min. As in previous studies with rufous hummingbirds (e.g., Hiebert, 1988, 1990, 1991, 1992), these two temperatures were selected to provide daytime and nighttime temperatures typical in the habitats where these birds are found throughout the year. Birds were fed a nutritionally complete artificial nectar (Nekton USA, Inc., Tarpon Springs, FL).

Cloacal fluid collection. CF samples were collected on lightweight foam-core boards covered with plastic wrap (Saran Wrap; Dow Chemical), which were inserted through a flap on the bottom of the cage

and placed on the floor of the cage, covering its entire surface. After 30–60 min, boards were removed and droplets of CF were aspirated from the surface of the boards with a micropipetter, expelled into a microcentrifuge tube, placed immediately on ice, and moved to a -20° freezer when all samples had been collected. During aspiration, care was taken not to include fecal masses in the CF sample. All droplets from a single bird during one collection period were pooled into one microcentrifuge tube. Total volume of CF obtained in this manner typically ranged from 50 to 1200 μ l for a 60-min collection. Although care was taken to locate feeders and perches so that most of the CF voided would land on the collection boards, the volume of CF recovered was only an approximation of total CF produced because it was possible for voided CF to land on the sides of the cage. In addition, some of the droplets of urine dried before the sample was collected. The amount of drying depended on the size of the droplet and the duration of exposure to air before the sample was collected. At the end of each collection, plastic wrap was removed from the boards and discarded. Boards and plastic wrap were handled with rubber gloves to prevent contamination of the samples.

Blood sample collection. A small blood sample was drawn into heparinized capillary tubes from the wing vein within 3 min of capture from the home cage. Samples were then immediately centrifuged at 2000 rpm for 5 min. Separated plasma was taken directly to radioimmunoassay.

In late April, when the birds had recently completed the annual molt, blood samples were successfully obtained by alar venipuncture as described above. In mid June, we attempted to repeat this procedure on three additional birds. We were unable, however, to obtain blood samples from the wing vein in these birds because of an apparent change in the mechanical properties of the skin that caused the skin to seal immediately at the site of puncture.

Radioimmunoassay (RIA)

Plasma. After centrifugation, plasma samples (1–2 μ l) were brought to 300 μ l by adding phosphate-buffered saline with 3% gelatin (PBSg). After incubation overnight with [3 H]corticosterone (approximately 10,000 cpm, New England Nuclear), 200 μ l were trans-

ferred to one tube for assay and the remaining 100 μ l were transferred to a second tube for recovery determination, using the methods described in detail by Wingfield *et al.* (1992).

Cloacal fluid. CF samples were introduced directly into the radioimmunoassay immediately after thawing. Techniques for the CF RIA described below are modifications of the methods of Wingfield *et al.* (1992). Samples (50 μ l each) in duplicate were incubated overnight at 4° with 150 μ l PBSg, 100 μ l [3 H]corticosterone, and 100 μ l corticosterone antibody (B21-42; Endocrine Sciences, Tarzana, CA). In each assay, water blanks and charcoal-stripped CF blanks were analyzed to test for contamination. Additional tubes were run in every assay with 1000 pg of standard that was incubated either with charcoal-stripped CF or PBSg to assess accuracy of the assay. Dextran-coated charcoal (500 μ l) 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) and read in a Beckman LS 3500 scintillation counter. In initial validation tests, parallelism of dilution curves with the standard curve was determined by comparing slopes of the two curves according to the method of Zar (1984).

Creatinine assay. Creatinine concentration was determined with reagents from Sigma Kit 555A and a slightly modified procedure for use with CF in 96-well microtiter plates (Falcon). Because preliminary tests showed no significant difference between wells in the center and periphery of the 96-well plate, all wells on each plate were used. Each plate contained 20 μ l of creatinine standard in quadruplicate at concentrations of 0, 0.5, 1.0, and 3.0 mg/dl. Each of the remaining wells contained 20 μ l of CF sample, which were assayed in duplicate. CF samples were pipetted into wells immediately after thawing. To each well was added 190 μ l of alkaline picrate solution, mixed according to kit instructions. The plate was placed on a shaker for 2–2.5 h while color developed, and the absorbance was then read at 490 nm. Twelve microliters of acid reagent (diluted with distilled water to a 56% solution) was then added to each well, and the plate was returned to the shaker. Absorbance was

then read every 10 min until absorbance had stabilized. In some cases, absorbance then began to increase again as a result of an increase in turbidity, due presumably to the presence of varying concentrations of uric acid (Eldon Braun, pers. comm.). Because the time course of these reactions varied from sample to sample, it was necessary to track each duplicate pair of wells individually. By varying the concentration of the acid solution or the volume of the sample, the clearing reaction and subsequent development of turbidity could be accelerated or decelerated; however, as long as the reaction was slow enough to allow the development of an absorbance plateau after clearing and before the onset of turbidity, the numerical outcome of the assay was unchanged. Creatinine concentration in most undiluted samples ranged between 0 and 1 mg/dl. Standard curves were linear in this range with R^2 usually greater than 0.996. Samples with optical density due to creatinine <0.005 absorbance units (corresponding to a creatinine concentration of approximately 100 ng/dl) were not used; samples in this range accounted for $<1\%$ of the samples (4/524) assayed in this study. Samples with creatinine >1000 ng/dl constituted 20/524 or 4% of the samples assayed.

Osmolality. Total CF osmolality was measured in duplicate or triplicate with a Wescor 5200 vapor pressure osmometer, using 10 μ l of sample for each determination.

Experiment 1: Osmolality and Creatinine as Reference Measures

In plasma, total osmolality remains relatively constant and hormone concentrations can therefore be used directly. In urine, however, the absolute concentration of hormone would be expected to vary with the hydration state of the bird. For this reason, it is essential to express hormone concentration with reference to the concentration of some other component of the CF whose concentration changes with CF osmolality in the same proportion as the concentration of the hormone in question. Here we compare two reference measures for hormone concentration in CF—total osmolality and creatinine (Cr).

CF samples were collected for a separate study designed to investigate the effect of moderate reduction in net energy intake on CORT concentrations and use

of nocturnal torpor (Hiebert *et al.*, 2000). After 5 days of *ad lib* feeding on nectar at normal (control) concentration, birds were given an unlimited supply of diluted nectar (85% of normal concentration) on days 6, 8, and 10. On days 7, 9, and 11 through 15, birds resumed *ad lib* feeding on nectar at control concentration. CF was collected over a period of 1 h, once at midday (beginning 5 h after lights on) and again in the evening (beginning 10 h after lights on). Total day length was approximately 11.5 h. After collection, samples were frozen at -20° until they were assayed for CORT, osmolality, and creatinine.

Mean CF CORT for days 6, 8, and 10 was compared with mean CF CORT for days 7, 9, and 11 in both midday and evening samples with the Wilcoxon signed-rank test because of significant heterogeneity of variance in the groups being compared. Wilcoxon signed-rank tests were also used to compare midday and evening CF CORT within food dilution or control days. Multiple regression analysis was used to determine whether there was a significant relation between CF CORT and torpor duration on the following night, using a total of six data points for each variable from each bird (corresponding to the 3 food dilution days, 6, 8, and 10, and the 3 control solution days that immediately followed them, 7, 9, and 11). The model that we used controlled for both body mass and individual bird, thus making it possible to use multiple data points from each bird. The results of these statistical analyses are reported for uncorrected CF CORT, as well as for creatinine- and osmolality-referenced CF CORT values, to compare the qualitative results provided by these two methods of referencing CF CORT. Results were considered statistically significant when $P < 0.05$.

Experiment 2: Stability of Cloacal Fluid Samples

To determine whether it is necessary to freeze CF samples immediately after collection, we pooled CF collected during a 60-min period from eight birds, mixed the pooled sample, and aspirated 200 μ l into each of eight 500- μ l microcentrifuge tubes. The tubes were immediately capped but allowed to sit at room temperature (approximately 22°) for 0 min, 15 min, 30 min, 1 h, 2 h, 6 h, 12 h, and 24 h, respectively, before being placed on ice and taken immediately to a 20°

freezer for storage. Samples were then assayed for CORT and creatinine.

Experiment 3: Stress Series

The secretion of glucocorticoids in response to an acute stressor has been used extensively as a tool for probing the effects of environmental variables, seasonal physiological state, experience, or sex on the responsiveness of the hypothalamic–pituitary–adrenal axis to stress (e.g., Wingfield *et al.*, 1992, 1998). The conventional protocol consists of catching the animal, taking a blood sample within 2–3 min (before plasma glucocorticoids begin to rise in response to capture), restraining the animal for 30–60 min, obtaining one or two additional blood samples during the period of restraint, and finally releasing the animal. To demonstrate the comparability of our CF sampling method with the standard blood sampling protocol for stress series, we first used a protocol in which both plasma and CF were sampled. In a second experiment, we devised a modified method of restraint to allow the birds to continue feeding and producing CF throughout the protocol.

Plasma and CF collection, with standard restraint.

In late April 1998, three birds were selected to begin the procedure at approximately 1030 h. Each bird was captured from its home cage, and a blood sample was obtained within 3 min. The bird was then held in a cloth bag for 30 min, during which time it did not feed. A second blood sample was then obtained and the bird rereleased into its home cage. CF was collected at 30, 90, and 150 min after the end of the restraint period, as well as at 1030 h the following day to serve as a baseline for comparison. A paired *t* test was used to compare CORT in plasma samples at the beginning and end of the restraint period (T0 and T30) and to compare CORT in CF samples at T0 and T90 (at other time points there were insufficient data to perform a repeated-measures ANOVA).

CF collection only, modified restraint. The protocol began sometime between 0830 and 1100 h with two consecutive 30-min collections of CF from an unrestrained bird in its home cage; these samples provided baseline CORT concentrations in CF. The bird was then captured and restrained in a flannel jacket. The jacket was designed to allow free movement of the head but not the wings; in addition, a hole was cut in

the underside of the jacket to permit collection of voided CF in a plastic weighing dish suspended below the bird. Birds in flannel jackets were suspended in front of a feeder containing artificial nectar so that the birds could continue to feed while restrained. To ensure that birds fed during the restraint procedure, an experimenter inserted the restrained bird's beak into the feeder spout every 10 min. After 60 min of restraint, the birds were again released into the home cage. CF samples were then collected from the unrestrained bird every 30 min for the next 2 h. Repeated-measures ANOVA (rANOVA) was used to assess the overall effect of time on CF CORT, and individual paired comparisons were made between baseline (T0) and samples taken later (T30, T60, T90, T120, T150) with paired *t*-test and alpha adjusted downward to $0.05/5 = 0.01$ to account for the use of multiple tests (Bonferroni correction).

Experiment 4: Noninvasive CORT Supplementation

Although steroid hormones are normally insoluble in water, they can be made soluble by complexing them with nontoxic cyclodextrins (Pitha and Pitha, 1985). Cyclodextrins thus afford the potential for administering steroids in water or water-based food. To determine whether hummingbirds would ingest cyclodextrins delivered in this way and to establish a relation between dose and CF CORT output, hummingbirds were fed artificial nectar containing different concentrations of cyclodextrin-complexed CORT.

Adult hummingbirds ($N = 3$) were each given artificial nectar containing 2-hydroxypropyl- β -cyclodextrin (Research Biochemicals International, Natick, MA) complexed with CORT (HBC–CORT). HBC–CORT was stored under refrigeration for 5 days or less as a stock solution containing 1.92×10^{-3} M CORT. This stock solution was added to artificial nectar to produce four batches of nectar, containing CORT in concentrations of 0 M, 2.87×10^{-6} M, 5.73×10^{-6} M, and 8.60×10^{-6} M respectively. HBC (not complexed with CORT) was added to all but the 8.60×10^{-6} M HBC–CORT solution so that all solutions contained the same total concentration of HBC as a control. Each bird received each of the solutions for 2 h, but the order of presentation was randomized for each bird. Trial 1 lasted from 0900 to 1100 h on May 20, Trial 2 from 1400 to 1600 h on May 20, and Trials 3 and 4 were

TABLE 1
Comparison of CF Radioimmunoassay Results for Samples with and without Extraction in Dichloromethane

Tube contents	No extraction	Extraction	<i>P</i> value
Water blank + 1000 pg CORT	978 ± 34 (4)	866 ± 45 (3)	0.10
Water blank +500 pg CORT	572 ± 77 (4)	472 ± 82 (3)	0.46
Charcoal-stripped CF + 1000 pg CORT	785 ± 24 (4)	889 ± 9 (3)	0.05
Charcoal-stripped CF + 500 pg CORT	426 ± 29 (4)	580 ± 190 (3)	0.28

Note. Data are shown as mean pg ± SE (N).

scheduled on May 22 at the same times of day as Trials 1 and 2. CF samples were collected for 60 min before each trial, every 30 min during the trial, and every 60 min between trials.

Repeated-measures ANOVA was used to determine overall effects of HBC-CORT treatment for each of three doses over seven time points ($df = 6$). rANOVA was also used to determine the effect of HBC-CORT dose on amount of food consumed during the testing period, using data from each of three birds at each of four doses ($df = 3$).

RESULTS

CF CORT Radioimmunoassay

The antibody cross-reacted sufficiently well with CF CORT, as a serial dilution curve for CF with PBSg was parallel to the standard curve in the same concentration range (i.e., slopes of the two lines were not significantly different, $P > 0.5$). The curve itself showed sensitivity to the least detectable value of 16 pg of CORT standard.

Initial studies (four assays) were run to determine the most salient methods for handling CF CORT. The difference between the 1000 and the 500 pg spiked samples caused by unknown interference is greater for the extracted samples (27%) than for the unextracted samples (10%). Although extraction with distilled dichloromethane followed by measurement of percentage recovery of steroid is commonly performed with plasma samples, results using this technique with CF were no more accurate than without (Table 1). Blanks for the assays indicated little contamination and ranged between 84 and 100% binding.

Accuracy of the assay was assessed by measuring CORT in PBSg and stripped CF spiked with 500 or 1000 pg standard, as well as in a CF pool collected from both captive and free-living birds, frozen in 100- μ l aliquots to be used in later assays. Mean and SE for eight assays were 1098 ± 45 for the PBSg samples and 640 ± 25 for stripped CF. CV for these assays were 9.9 and 12.5%, respectively, and 6.4% for a CF pool.

Experiment 1: Osmolality vs Creatinine as a Reference Measure

Uncorrected CORT values for CF over the course of the experiment at both midday and in the evening showed no discernible pattern in relation to food dilution (Figs. 1a and 1c). When CF CORT was expressed as either ng CORT/mOsm or as ng CORT/ng Cr, however, CORT generally followed the pattern of increasing on days when food was diluted (Figs. 1b and 1d). This pattern was evident in both the midday and the evening samples but was more pronounced in the evening; however, only in evening samples of creatinine-referenced measures were the increases in CF CORT due to food dilution statistically significant (statistical comparisons shown in Table 2).

Creatinine-referenced, but not osmolality-referenced, CF CORT showed a significant increase from midday to evening on control days and a trend toward an increase from midday to evening on food dilution days; uncorrected CF CORT from midday and evening samples showed a significant difference, but in the opposite direction from creatinine-referenced samples (Table 2). Multiple regression analysis revealed a significant negative relation between torpor duration and CF CORT concentration at midday in uncorrected CORT measures and a negative relation approaching significance in the two referenced mea-

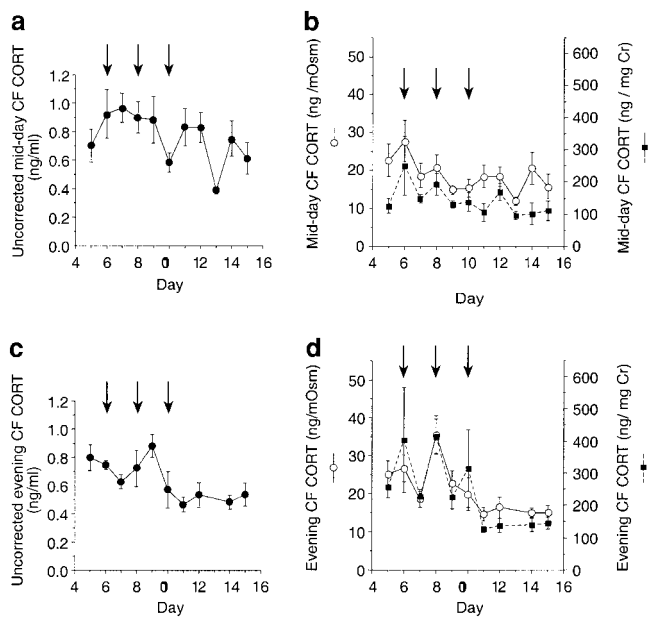


FIG. 1. Corticosterone (CORT) concentrations in cloacal fluid (CF) collected at midday (a and b) and in the evening (c and d). Arrows show days on which birds were given diluted food to decrease energy reward relative to effort expended. Samples are identical in a and b and in c and d, but CORT is expressed as uncorrected concentration (a and c) or relative to total CF osmolality or creatinine (Cr) concentration (b and d) for comparison. Vertical bars represent \pm SE.

tures of CF CORT. In evening samples, only creatinine-referenced CORT showed a significant positive relation between CF CORT and torpor duration.

TABLE 2
Comparison of Creatinine-Referenced, Osmolality-Referenced, and Uncorrected Measures of Cloacal Fluid Corticosterone (CF CORT)

Comparison	P values		
	Uncorrected CORT (ng/ml)	Osmolality referenced (pg CORT/mOsm)	Creatinine referenced (ng CORT/mg Cr)
CF CORT, food dilution days vs control days			
Midday samples	0.25	0.46	0.29
Evening samples	0.07 (FD > C)	0.43	0.046 (FD > C)
CF CORT, midday vs evening samples			
Food dilution days	0.34	0.25	0.07 (M < E)
Control days	0.03 (M > E)	0.25	0.03 (M < E)
Multiple regression, CORT vs torpor			
Midday	0.009 (-)	0.10 (-)	0.08 (-)
Evening	0.43	0.25	0.043 (+)

Note. CF CORT was compared on days when food was diluted (shifting energy balance in the negative direction and therefore predicted to increase CF CORT and torpor the following night) and on days when birds were fed normal (control-strength) nectar (predicted not to increase CF CORT or nocturnal torpor). CF CORT on food dilution days vs control days was compared with Wilcoxon signed-rank test. The direction of the effect (e.g., food dilution (FD) > control (C), midday (M) > evening (E), or \pm for regression effects) is shown in parentheses following the P value for any effects where $P \leq 0.10$ (boldface type).

During control feeding on days 11–15, both referenced and unreferenced measures of CORT were low and constant in evening samples (Fig. 1d), but in mid-day samples there was considerable variability both between and within referenced and unreferenced measures of CF CORT (Fig. 1b).

Experiment 2: Cloacal Fluid Sample Stability

Uncorrected CORT concentration in CF samples increased as a function of time at room temperature, up to at least 12 h (Fig. 3b). There was no difference between the 12- and 24-h samples. Creatinine concentrations, in contrast, remained relatively low for the first 6 h at room temperature but increased thereafter (Fig. 3b). The combined effect was that CORT/Cr remained relatively constant for the first hour after collection, rose to its maximum at 6 h, and then decreased in the 12- and 24-h samples (Fig. 2a).

Experiment 3: Stress Series

Standard restraint, plasma and CF collection. Plasma CORT increased from 11 ± 3 ng/ml immediately after capture to 47 ± 9 ng/ml after 30 min of restraint ($P = 0.028$; Fig. 3a). Because the birds did not feed during the restraint period, no CF samples could be obtained until 90 min after restraint began. In ad-

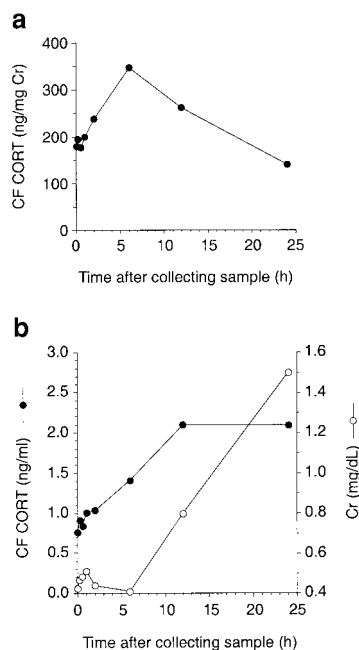


FIG. 2. (a) Corticosterone (CORT) concentration relative to creatinine (Cr), in aliquots of a pooled cloacal fluid (CF) sample held at room temperature for 0–24 h before freezing at -20° . (b) Uncorrected CORT and Cr concentrations in the same aliquots as shown in (a). Samples were collected on plastic wrap-covered boards over a period of 60 min before being aspirated and transferred to the pool at $T = 0$ h.

dition, although all birds recovered from the experiment by the following day, they were noticeably hypoactive once returned to the cage after restraint and blood sampling and fed very little during the next few hours, resulting in small CF samples after the restraint period. Only one bird provided a CF sample at each time point, but samples from all birds conformed to the pattern shown by this bird. CF CORT concentration was significantly higher than baseline in the first CF sample taken after restraint ended ($P = 0.007$) and declined thereafter to return to approximate baseline levels by 90 min following the end of restraint (Fig. 3a).

Modified restraint, CF collection only. CORT profiles of birds beginning the protocol at different times during the morning did not differ in baseline concentrations or in amplitude of response. Therefore, the results of all birds ($N = 11$) were combined (Fig. 3b). Overall, there was a significant effect of time after restraint on CF CORT (rANOVA, $P = 0.0001$). Mean CORT concentrations remained at

baseline (243 ± 46 ng CORT/mg Cr at $T = 0$) in the first 30-min sample after the beginning of restraint ($T30$, $P = 0.38$), began to increase at $T60$ ($P = 0.08$), were significantly higher than baseline at $T90$ ($P = 0.006$), began to return to baseline at $T120$ ($P = 0.06$), and returned to baseline levels (199 ± 21 ng CORT/mg Cr) by $T150$ ($P = 0.55$). Peak CF CORT concentrations obtained in this protocol were approximately half that of peak CF CORT concentrations obtained in the protocol using standard restraint with both plasma and CF collection (Fig. 3).

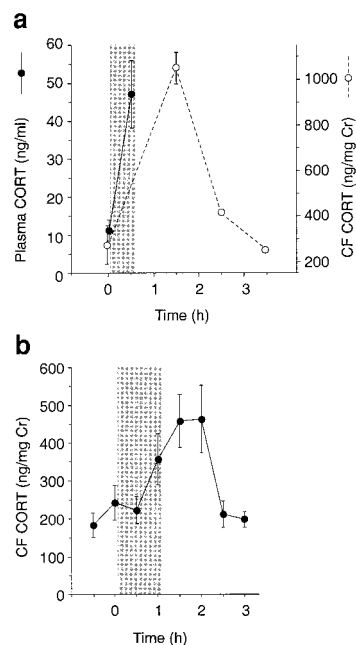


FIG. 3. Corticosterone (CORT) profiles in plasma and cloacal fluid (CF) before, during, and after restraint. (a) Birds ($N = 3$) were captured, bled at time 0, held in a cloth bag where they could not feed for 30 min (restraint period shown by gray bar), and released into the home cage where CF samples were collected. For CF concentrations, each point represents pooled fluid collected on the floor of the cage for the previous hour. Vertical bars represent SE; at $T = 2.5$ h, the SE bar is not shown because this point represents a single individual; in other cases error bars, if not visible, are contained within the diameter of the symbol. Baseline CF concentration shown at $T = 0$ was obtained on the following day at the same time of day. (b) Birds ($N = 11$) were captured at $T = 0$, restrained but allowed to feed for 60 min (restraint period shown by gray bar), and released back into the home cage at $T = 1$ h. Each point represents pooled fluid collected on the floor of the cage for the previous 30 min. Vertical bars represent \pm SE.

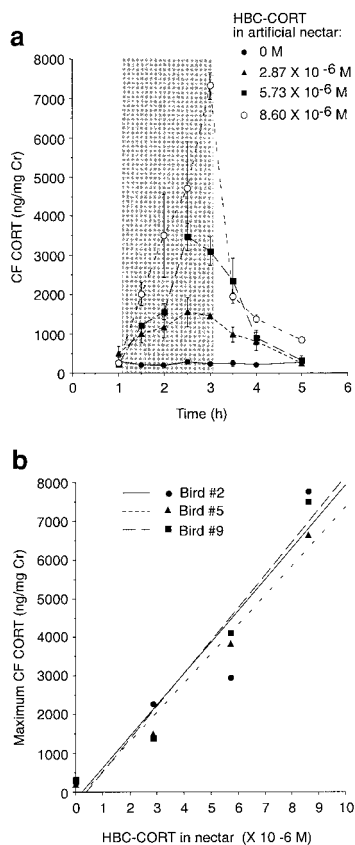


FIG. 4. (a) Corticosterone (CORT) in cloacal fluid (CF) before, during, and after a 2-h period (shown by gray bar) during which cyclodextrin-complexed CORT (HBC-CORT) in one of four doses was given to the birds dissolved in artificial nectar. Total concentration of cyclodextrin was the same in all treatments. Points at 1 and 5 h represent CF samples collected over the previous 60 min; all other points represent CF samples collected over the previous 30 min. $N = 3$ birds, each of which received each of the four treatments, but in randomized orders. (b) Maximum concentration of CF CORT developed by each bird during the treatment described in (a), usually occurring at $T = 2.5$ or 3 h. For the 0 dose treatment, for which no clear maximum developed, we used the $T = 2.5$ or 3 h sample, depending on the typical pattern of each bird, to control for the effects of the treatment and sampling protocol. Regression lines are shown for each individual. Bird 2: $R^2 = 0.89$, $P = 0.058$; Bird 5: $R^2 = 0.98$, $P = 0.012$; Bird 9: $R^2 = 0.96$, $P = 0.022$.

Experiment 4: Dietary Corticosterone Supplementation

CORT concentrations in CF remained at baseline when birds were fed the 0 M solution (rANOVA, $df = 6$, $P = 0.6$; Fig. 4a). In trials in which the birds fed on higher concentrations of HBC-CORT (2.87×10^{-6} M,

5.73×10^{-6} M, and 8.6×10^{-6} M), CORT in CF rose within the first 30 min after birds began feeding on artificial nectar containing HBC-CORT and began to decrease within 30 min after birds resumed feeding on normal artificial nectar (rANOVA: $P = 0.04$ and $P < 0.0001$, respectively). CF CORT returned to baseline levels within 2 h except at the highest concentration of HBC-CORT; in these cases CORT clearance was incomplete at the end of the measurement period. Peak concentrations of CF CORT had an approximately linear relation to concentration in the food (Fig. 4b) and were similar among birds, even though food consumption during the 2-h testing period varied among these individuals from 1.55 to 3.28 g. There was no relation between dose of HBC-CORT and amount of food consumed during the testing period (rANOVA, $df = 3$, $P = 0.3$).

DISCUSSION

CF CORT Radioimmunoassay Validations and Criteria

Parallelism of standard and dilution curves demonstrate that our radioimmunoassay can be used to determine concentration of CORT in hummingbird CF. Extraction with dichloromethane can be omitted because final concentration was not changed by this step. Comparisons between water blanks and charcoal-stripped urine to which known amounts of CORT have been added suggest that there is some competitive binding in CF samples but the coefficients of variation fall within the acceptable range ($\leq 12.5\%$), thus demonstrating the reliability of this method. In addition, the low interassay CV (6.4%) obtained for the CF pool increased our confidence in the repeatability of our assay result.

Osmolality and Creatinine as Reference Measures

When expressed relative to creatinine, the pattern of CORT in the CF of birds that have consumed artificial nectars of different dilutions follows the expected pattern of being elevated on days when food is diluted. Osmolality-referenced CORT does not show these patterns as clearly, perhaps because CORT can influence

renal reabsorption of osmotically active components of the urine (Brown, 1992; Jamison and Gehrig, 1992; Stanton and Giebisch, 1992). As expected, uncorrected CF CORT values show no such patterns and are unsuitable for addressing the aim of the experiment. In addition, subsequent studies in which we have manipulated CORT experimentally at different times of day (S. M. Hiebert, unpublished data) further support the use of creatinine-referenced CORT, by indicating that birds that receive exogenous CORT for only the first half of the day show less torpor than birds that receive CORT only in the second half of the day or all day. This pattern is consistent with significant correlations between evening CF CORT and torpor, but not between midday CF CORT and torpor.

The ideal reference substance should be filtered, but neither absorbed nor secreted, by the kidney. Creatinine is commonly used for this purpose in mammalian studies, since it meets these criteria under most conditions. Creatinine excretion, a result of the normal turnover of muscle tissue, is nearly constant in mammals unless muscle is being metabolized, as would be the case in an animal that was undergoing extraordinarily heavy exercise (such as migration) or experiencing starvation (Levinsky and Lieberthal, 1992; Carpenter *et al.*, 1993). Total osmolality of CF, in contrast, is a function of both water and ion content. Water content of the urine is regulated initially by the kidney but may be further reduced by reabsorption in the hindgut, where the urine mixes with digestive wastes before being voided (Braun and Dantzler, 1997; Karasov and Hume, 1997). Neither of these processes should alter the concentration of CORT relative to osmolality. However, selective absorption of solutes, which may occur in either the kidney or the hindgut, is a potential source of error when total osmolality is used as a reference measure.

The use of creatinine as a reference measure in birds and some mammals is somewhat controversial (Levinsky and Lieberthal, 1992), because there is some evidence for tubular secretion of creatinine (Sturkie, 1954) and because plasma creatinine concentrations may change in response to stress and reduced food intake. There is some evidence that creatinine excretion in birds increases in response to reduced food intake or other kinds of stress. Work *et al.* (1999) report that translocation in two species of passerines that, like hummingbirds, are nectarivorous and insectivorous,

results in hypercreatinemia. In addition, the muscle protein breakdown that occurs in response to food shortage and/or high glucocorticoid levels (Gray *et al.*, 1990) would tend to increase plasma creatinine concentrations. However, the implications of this finding strengthen rather than weaken the interpretation that CORT concentration in CF rises when net energy intake is decreased: if creatinine in CF also rises slightly in response to the stress of food dilution, the effect would be to reduce the ratio of CORT/Cr and thus to blunt the apparent effect of this stressor on creatinine-referenced CORT concentration. Thus, increases in CF concentration of creatinine-referenced CORT in response to stress might be considered conservative measures, or possible underestimates, of the actual response.

The ability to control for changes in the hydration state of the birds when measuring hormones in CF is particularly important for studies of hummingbirds in which energy supply is manipulated, because a hummingbird's principal energy supply is also its principal source of water. Both of the most commonly used methods for regulating energy supply, changing the concentration of the artificial nectar or removing the nectar for short periods of time, can also change the bird's water balance. The present study provides evidence that creatinine can be used as a reference measure to control for the effects of osmoregulation on CF composition. The use of osmolality as a reference measure is similar in function to the practice of freeze-drying samples, a method commonly used to prepare fecal samples of birds and mammals for hormone analysis (e.g., Goymann *et al.*, 1999: hyenas (*Crocuta crocuta*); Monfort *et al.*, 1998: wild dogs (*Lycaon pictus*); Wasser *et al.*, 1997: spotted owls (*Strix occidentalis caurina*); Whitten *et al.*, 1997: chimpanzee (*Pan troglodytes*); Palme and Möstl, 1997: domestic sheep (*Ovis aries*); Brown *et al.*, 1994: felids). Freeze-drying effectively eliminates the variable water content of the sample as a potential source of variation in hormone concentration, but, according to our results, is less useful than creatinine for analysis of hummingbird CF.

Cloacal Fluid Sample Stability

Samples frozen within 1 h of collection (i.e., up to 2 h after being voided by the bird) maintained a relatively stable CORT/creatinine ratio. Because micro-

centrifuge tubes were capped, and because the amount of evaporation from the tubes could not account either for the changes in concentration or for the relative changes in concentration between CORT and creatinine, we suggest that some other components of CF, perhaps bacterial enzymes, are changing the apparent concentrations of these two components over time at room temperature. Freezing the samples within 1 h of collection, however, minimizes such effects.

Stress Series

Stress series with and without blood sampling demonstrate a response similar to that expected (Wingfield *et al.*, 1992, 1998): capture and restraint induces transient release of CORT, which appears first in plasma and later in CF. Although delayed with respect to the plasma peak, the CF peak is easily visible in samples pooled over 30 min and disappears within 90 min of the termination of the stressor. The fact that the CF CORT response matches closely the response previously observed in the plasma of captured and restrained birds indicates that the assay that we used produces biologically relevant results.

Our results demonstrate a potential advantage to using this method for assessing baseline CORT concentrations. Baseline blood samples must be obtained within 3 min of capture, because CORT rises rapidly in the blood once a stressor is applied. The stringency of this requirement makes collecting samples difficult, especially in the field. The delay in the CF peak, however, decreases the need for obtaining samples within this very short time period. The results presented here suggest that CF concentrations of CORT remain at baseline appreciably longer, for up to 30 min. However, because each CF sample represents a 30-min pool, it is possible that CF CORT may already have begun to rise toward the end of the 30 min but that this rise is obscured by averaging the concentration over the entire 30 min period. Nevertheless, samples obtained within the first 15 min after capture are likely to represent baseline hormone concentrations.

Birds responded to the bleeding and CF collection protocol with CF CORT concentrations approximately twice as high as those in birds that were subjected to the CF collection protocol only. Bleeding by alar venipuncture appears to be extremely stressful to these

birds, which remained quiescent for many hours after the procedure had ended and sometimes did not resume normal activity levels until the following day. The modified restraint protocol, in contrast, stimulates a measurable but smaller increase in CF CORT without obvious effects on behavior after the termination of restraint. Standardizing the method of restraint is therefore essential if one wishes to compare the adrenocortical stress response in different individuals, different populations, or within individuals at different stages of the annual cycle.

Dietary Corticosterone Supplementation

The appearance of CORT in CF in response to HBC-CORT consumption indicates that HBC-CORT dissolves in artificial nectar, is ingested by birds in quantities proportional to its concentration in the artificial nectar, and is detectable with the CORT radioimmunoassay. The hummingbirds in this study did not appear to have any aversion to feeding on artificial nectar supplemented with either HBC or HBC-CORT. The fact that CF CORT remained at baseline concentrations both before the presentation of the experimentally supplemented diet and during feeding on the diet containing HBC only indicates that neither HBC by itself nor the act of inserting and removing CF collection boards had a measurable effect on CORT in these birds, which had been in captivity for 10 months at the time of this study and had been exposed to CF collection protocols throughout that period.

In contrast with the results of the stress series, CF CORT in this experiment had already begun to increase in the first 30-min sample after feeding on HBC-CORT. In the stress series, an increase was first observed in the sample collected 60 min after the application of the stressor. This difference may be explained by one or both of the following factors. First, concentrations of CF CORT produced in the HBC-CORT supplementation protocol (approximately 1500–8000 ng CORT/mg Cr) were much higher than those in either of the stress series (approximately 600–1100 ng CORT/mg Cr.). Average CORT concentration over the whole 30-min period could thus show an increase over baseline concentrations even if the actual increase in the concentration of voided CF did not begin until near the end of this period. Thus, this

result does not negate the contention that, in natural situations in which only native CORT is being sampled, CF samples containing baseline concentrations of CORT can be collected longer after capture than can baseline blood samples, possibly as long as 30 min later. A second possibility is that some of the HBC-complexed CORT was not absorbed, but passed directly through the digestive tract and was eliminated without first passing through the bloodstream or kidneys. Consistent with this interpretation, previous studies comparing buccal and intestinal absorption of cyclodextrin-complexed steroids suggest that intestinal absorption is not as complete as buccal absorption (Pitha *et al.*, 1986). This effect would be more likely to occur at high HBC-CORT concentrations and could explain the faster than usual appearance of CORT in CF. Our data cannot distinguish directly between these possibilities, but experiments in which HBC-CORT affects both behavior and thermal physiology in rufous hummingbirds demonstrate that at least some of the HBC-bound CORT is absorbed by the birds (Hiebert *et al.*, 2000). In addition, Breuner *et al.* (1998) have demonstrated that orally administered CORT is absorbed across the gut, is circulated in the plasma, and has rapid effects on locomotor behavior. Although the CORT used in their study was not complexed with HBC, the result confirms the ability of CORT to cross the avian intestinal wall.

In summary, a noninvasive protocol for sampling stress hormones is beneficial because it reduces or eliminates the confounding factor of stress induced by the sampling method itself. However, experiments for elucidating the roles of these hormones in regulating physiological and behavioral states also require the ability to manipulate the hormones in the absence of the stressor. Although most methods for manipulating hormone levels require injections or implantation of subcutaneous pumps or capsules, the ideal protocol would be noninvasive as well, for the same reasons that noninvasive techniques for sampling hormones might be preferred. The advantages of using our technique are that CORT can be manipulated without handling the birds and with tight temporal resolution in patterns that better mimic responses to transient stressors (Breuner *et al.*, 1998). We are currently using this technique in the laboratory to investigate the timing of elevated CORT during the daily cycle and its importance in regulating energy balance. Investiga-

tion of energy regulation by endocrine signals is novel in this avian model, which has already shown itself to be extremely useful in testing functional relations among behavior, physiology, and energy balance.

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