Stress response in the rufous hummingbird (*Selasphorus rufus*): mechanisms of personality and social dominance

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ABSTRACT

The glucocorticoid stress response has recently been linked to characteristic, stable suites of behavior ("personality") that hold fixed through time and environmental context. To lay groundwork for future study of this personality-CORT association, the stress responses of wild-caught rufous hummingbirds (*Selasphorus rufus*) were characterized by determining the corticosterone (CORT) concentration in cloacal fluid (CF) collected noninvasively over 60 min of restraint. On the basis of previous studies of sparrows and tits, we hypothesized that social dominance would be inversely correlated both with baseline CF CORT concentration and with the response of CF CORT to restraint. After capture, restrained birds were held in the hand and fed for 45 min, during which a separate CF sample was collected over each of three 15-min periods. For the final 15 min, birds were moved to a flight cage and a fourth CF sample was collected without handling the bird; all samples were analyzed by direct RIA. In contrast to our predictions, baseline CF CORT (first 15-min sample) of all three age-sex classes did not differ. Although the predicted relation between previously published dominance status in this species (adult females > first-year males > first-year females) and CORT levels was not supported, young males tended to develop higher CF CORT concentration in response to restraint than did females of any age. Of six behaviors measured in the flight cage, one was inversely correlated with CF CORT concentrations in response to restraint: birds with higher CF CORT were significantly more restricted spatially in their exploration of the flight cage. Additionally, adult and hatch-year females tended to perch more on the front and side of the cage than hatch-year males did. In contrast to a previous study of restraint stress in captive rufous hummingbirds, the wild-caught birds in our study showed significantly increased CF CORT within 30 min of capture and no significant change thereafter, suggesting that the stress response can be quantified in the field in a shorter period of time with this noninvasive method than was previously thought.

INTRODUCTION

In non-human species, individuals may harbor a unique suite of behavioral traits that persist regardless of environmental circumstance (reviewed in Webster & Ward 2011). In great tits (*Parus major*), fixed personalities have been quantified along a continuum of boldness (Baugh et al. 2012). Bolder individuals often explore readily, adhere to set routines, fail to respond to social cues, and act aggressively. Shyer individuals tend to explore less, adapt more quickly to new circumstances, respond more sensitively to social information, and to be less aggressive.

Baseline levels of corticosterone, a glucocorticoid expressed in the hypothalamic-pituitary-adrenal axis, are essential for birds in maintaining normal metabolic processes (Baugh et al. 2012). Elevated levels of the steroid serve as a key physiological adaptation for responding to and recovering from disruptive circumstances over the course of a bird's daily activity (Baugh et al. 2012). Furthermore, corticosterone levels in birds vary naturally by time of day, reproductive state and migratory readiness (Wingfield & Farner 1978; Landys et al. 2004; Baugh et al. 2012). Baugh et al. (2012) found that the observed "bold" and "shy" personalities are closely related to this stress steroid response. In their study, "shy" syndrome birds tended to express elevated levels of corticosterone relatively quickly after exposure to a stressor, reach a high peak levels, and take a relatively long time to return to baseline. "Bold" syndrome birds exhibited a slower-onset, shorter-duration stress response and produced less corticosterone overall (Baugh et al 2012; A. Baugh, pers. comm).

Both behavioral and physiological studies are vital to further investigation of the glucocorticoid-personality relationship. One of our goals was to mark, observe, and recognize individual birds living unhindered in a natural setting in order to behaviorally characterize birds by stable 'personality' profiles. Our other goal was to perform a stress series – a procedure that provides hormonal snapshots of an organism as it responds to an acute environmental stimulus, usually a restraint of some kind (Hiebert et al. 2000b) – on wild-caught hummingbirds.

At present, we know little about how consistent the corticosterone-personality relationship is across taxa. Much of the existing published literature on behavioral syndromes and physiology is based on laboratory studies of domesticated or captive bred

individuals (Carere et al. 2003, Baugh et al. 2012, and others in captive-reared great tits (*P. major*); Kurvurs et al. 2010 in captive-reared barnacle geese (*Branta leucopsis*); reviewed in Webster & Ward 2011). In some speices of hummingbirds, we have a general understanding of age and sex class dominance hierarchies (Carpenter et al. 1993; Welch & Suarez 2008). However, no one has yet investigated the existence of fixed, behavioral personalities on an individual level in a species of this family, let alone looked for underlying hormonal mechanisms. The rufous hummingbird (*Selasphorus rufus*) serves as an ideal study organism for extending our knowledge of the corticosterone-personality mechanism and placing it in an ecological context through field research on wild, free-living individuals. As insect and nectar feeders, hummingbirds return to discrete feeding locations that reliably offer a rich food source, including both naturally occurring and cultivated flowering plants and artificial feeders filled with a simple sugar-water solution. In consequence, hummingbirds are easy to observe behaving in the wild without causing acute disturbance. Furthermore, the large volume of nectar hummingbirds consume results in the production of abundant quantities of cloacal fluid (CF), which includes both their urinary and fecal waste. The nearly clear, non-fecal component of this CF contains both corticosterone and other hormonal metabolites (Calder and Hiebert 1983). By retrieving a hummingbird's highly dilute CF non-invasively as the bird expels it, one can obtain a reliable index of circulating levels of corticosterone. Most other birds lack hummingbirds' super-fast metabolism, and so produce waste much less frequently and express hormones in their feces with less temporal precision (Hiebert et al. 2000a).

In spite of their small size (approximately 3.4 grams), rufous hummingbirds annually migrate southward from their breeding grounds in Alaska, Canada and the Pacific Northwest to overwinter in Mexico (Healy & Calder 2006). Birds first arrive in their breeding grounds in March ("Hummingbird" 2012, available online at http://www.learner.org/jnorth/humm/). Adult males are the first to leave the breeding ground on their southward migration. Females are next to follow, and by late July to early August, hatch year birds depart alone (Healy & Calder 2006). Since there is a known order of dominance in rufous hummingbirds [after hatch-year (AHY) males > AHY females > hatch-year (HY) males > HY females; Carpenter et al. 1993; Welch & Suarez 2008], we hypothesized that personality varies with age and sex class. Because *S. rufus* is commonly considered to be particularly aggressive and dominant over other hummingbird species [refer to Camfield et al. 2006 in comparison with broad-tailed hummingbirds (*S. platycercus*)], it also potentially lends itself to future comparative study on an interspecies level.

We set out to investigate whether *S. rufus* individuals can be readily characterized along the corticosterone-correlated boldness scale proposed by Baugh et al. (2012). We attempted to characterize birds' hormonal and behavioral profiles and compare them across age (during or after hatch-year) and sex. To characterize individual behavior in a natural setting, our original intent was to PIT-tag, color mark, release and repeatedly observe wild hummingbirds. RFID monitoring in particular has been shown to be a potentially effective means of keeping track of individual ruby-throated hummingbirds (*Archilochus colubris*; Brewer et al. 2011; Charette et al. unpublished data, available online at www.projetcolibris.org/Charette_et_al_PIT_tag_v4.0.pdf). However, repeated

observation of marked individual, free-living birds over time was not possible because we re-observed only one out of a total of nearly fifty marked birds at all locations. We therefore focused on our other main goal: performing a stress series on wild-caught birds to investigate differences in corticosterone levels by age and sex class. We hypothesized that since social dominance is related to boldness (Baugh et al. 2012), the stress response would be inversely proportion to dominance. We characterized stress response in wild *S. rufus* individuals captured during the mid to late breeding season, using standard procedures for evaluating an organism's glucocorticoid stress response (Wingfield & Farner 1976). In a previous study, restraint stress series were carried out on a small number of captive rufous hummingbirds in the laboratory; however, all birds were within 12 months after hatching, and research was conducted during only one season after the birds had been held in captivity for at least 6 months (Hiebert et al. 2000a). Nobody has ever done a stress series on a wild-captured hummingbird of any species.

We administered a one-hour stress series on wild *S. rufus* individuals that ended in a 15-minute behavioral observation. In this final period, we sought to quantify behaviors related to the immediate stressor, and potentially, to a bird's long-term personality syndrome. Based on previous experience with captive rufous hummingbirds, we hypothesized that a bird's willingness to explore the enclosure would be inversely proportional to CF CORT levels.

MATERIALS AND METHODS

Animals and field sites

We caught, studied and released individual rufous hummingbirds (*Selasphorus rufus*) between June 13 and July 13, 2012, at six sites on San Juan Island, Washington (48°33'04.92" N, 123°04'41.18" W). Five of the island sites were relatively close to sea level. At one of these lowland sites in particular, birds took advantage of a large floral array (including *Digitalis*, *Campanula*, *Crocosmia*, fuchsia, and other species). The sixth island site was located at slightly higher elevation (234.7 m). Our high elevation field site was in the Cascades mountain range (47°23'27.48"N, 121°24'5.90"W; 872.3 m); we visited this location in an effort to capture adult males once the southward migration had begun. Each site offered plastic hummingbird feeders filled with sugar-water solution. At all but one site, feeders had been consistently maintained for more than two consecutive years (Fig. 1).

Trapping methods

Drop-door method

We constructed a drop-door trap (61 × 61 × 76.2 cm) out of hardware cloth (1.3 cm mesh size). We covered an approximately 15 × 20 cm front entrance with plastic binder clips to shield the birds from its rough edges, and painted them red to further attract the hummingbirds to enter. To aid in hummingbird removal from the trap, we cut out a smaller opening on an adjacent side of the trap and lined it with interior and exterior flaps of plastic mesh. We hung a feeder (with bee catchers and perches removed; Perky-Pet® Products, Denver, CO) so that the plastic flower openings were situated in the middle of the cage and just above the top of the entrance. We designed the door to be

operated manually with a string. In practice, however, most of the trapped birds flew toward the top of the cage to exit and caught themselves in the cage; the door was usually left clipped open.

Drop-net method

A drop-net trap (approximately 60 cm in diameter; Lee Rogers, Patagonia, AZ) containing a feeder was also used to capture hummingbirds. A weighted, cylindrical net was held open with a string, and a basal, lightweight plastic tube ensured that any partially trapped birds could escape uninjured.

Methods for marking and relocating individuals

In-hand color marking and measurement

Our original experimental design depended on a two-step process: first marking individual birds, and then observing these same individuals behaving in the wild. Although our intent was to use subcutaneously-injected RFID tags to monitor individual movements, we began with color marking to assess the overall feasibility of this plan. Birds were captured using the drop-door trap for this purpose (see previous section and Fig. 5). Seven birds were held for approximately 15 minutes, during which time they were aged and sexed, and measured for wing cord, tail and culmen length, bill grooving, and body mass (Table 1). Since we were recapturing none of the marked birds, we considered the possibility that the relatively long handling time might decrease a bird's willingness to return to a site. In response, the next four birds were only weighed and assigned to an age-sex class in order to release them within two minutes or less from the time of capture (Table 1). In both cases, we then applied a small amount of orange (enamel; Testors®, Rockford, IL) or aqua (acrylic; Art Advantage®) paint to some

combination of the crown, the back of the head, and the white tips of the outer three rectrices. After a brief drying period, birds were released by placing them on their backs in the palm of the hand. We recorded the birds' latency to fly away on their own.

Passive color marking technique

Since we were able to relocate only one out of a total of nearly 50 marked birds at any location, we considered the possibility that the length and design of both of our procedures discouraged birds from returning to a feeder site. We designed an experiment to test a new method for marking wild hummingbirds that would eliminate the need for initial capture. For inspiration, we looked to pollen-bearing flower stamens and the flower-specific pollen patterns that hummingbirds often acquire in the wild. Natural pollen often collects directly below and above the bill. Perky-Pet® four-flower feeders (with bee catchers and perches removed) were suspended from poles arranged in the middle of an open, mowed grassy field [Site 1; 48°32'52.00"N, 123° 5'14.59"W] in a circular arrangement, and marked for ease of visibility with compass directions (Fig. 2). On each feeder, three flowers were adorned with experimental "stamens" that we fabricated from cosmetic applicators and coated in non-toxic, brightly colored powder (Earth Pigments©, Cortaro, AZ; Fig. 3). For each feeder, we marked one flower with an applicator reaching up from below the feeding spout, one with an applicator coming down from above the spout, and a third with applicators in both positions. The fourth flower was left without applicators as a control. To ensure that birds did not always meet the same flower type when entering the circle, we arranged the feeders so that the control flower faced north in each case (Fig. 4). We kept track of visitation rates and feeding

durations for each flower type by feeder and orientation using the open-source JWatcher© behavioral analysis software in real time.

We also utilized this same experiment setup to test a variety of other applicator designs. In particular, we set up each feeder with two opposing unaltered flowers and two experimental flowers that we adorned with loops formed from chenille stems coated in blue non-toxic pigment. We quantified the number of visits each flower type received.

Stress series and behavioral assays

Drop-door trap behavioral observations

We designed this procedure to quantify behavioral traits that might characterize an individual, free-flying rufous hummingbird's personality syndrome. A drop-door trap was assembled with a feeder suspended in its center. To attract birds and serve as novel objects, five red, plastic feeder disks with yellow flowers were placed on the inside of the trap directly below the door. Birds were carefully observed as they approached, entered or departed from the feeder and trap area. Behaviors were recorded using JWatcher in real time. In most cases, the top of the cage was propped open to allow birds to leave the trap easily, thus enabling us to observe multiple birds in sequence without disrupting their independent behavior. In a few cases, the top of the cage was fastened closed and the birds were recovered for measuring (see Table 1), in-cage behavioral observations and eventual release (see next section). This procedure ensured that we could identify an individual bird without marking it and facilitated coordination with data from the stress series.

Stress series with in-cage behavioral observations

Two-hour assay and in-cage stress series

We set up a (75 × 52 × 47 cm) behavioral observation cage in a shaded, relatively undisturbed area to reduce external influences. We fitted the cage with two perches and two small feeders, to each of which we fastened a red plastic flower from the larger four-flower feeder model in order to entice birds to use them. We placed one feeder near the top front corner of the cage in quadrant II, and one near the bottom front corner in quadrant IV (Fig. 6). Since hummingbirds tend to fly up toward a light source, we expected the former to be used more readily and the latter to be a gauge of a bird's willingness to explore. In each case, a wooden dowel (0.3 cm in diameter) was placed so that a bird could feed while perching. A white sheet covered the top and three sides of the cage to minimize exposure to direct sunlight (Fig. 6). We video-recorded the exposed side of the cage with a Samsung (Memory F40) or Canon (Vixia HFS10) HD digital camcorder. At the onset of each trial, birds were held to the upper dowel with their abdomens touching the surface of the wood to encourage them to perch (although some birds flew before this procedure was possible). Each trial ran for approximately 2 h. Cloacal fluid (CF) was collected every 30 min from Saran Wrap®-covered boards placed on the bottom of the cage (Dow Chemical).

One-hour stress series with 15-minute behavior observation

Since the hummingbirds' mobility prevented us from reliably collecting CF samples from free-flying birds coming to feeders, we designed a new procedure to characterize the birds' hormonal and behavioral stress response. The behavior cage setup was identical to that in the two-hour assay and in-cage stress series (Fig. 6). Birds were captured with the drop-net trap and restrained in a flannel jacket fastened with an alligator clip to restrict wing movement and prevent feather damage (Fig. 7). Each bird

was weighed, and all other measurements were performed (Table 1). Birds were offered a 5:1 sugar-water solution from a syringe at approximately three-minute intervals. CF was collected in-hand over a 45-minute period (changing 0.7-ml microcentrifuge tubes every 15 minutes; Fig. 7). Birds were marked with a small amount of aqua acrylic paint (Art Advantage®) on the crown (marking pattern varied by field site). In the hope that natural variation in gorget pattern might aid in identifying re-observed birds, we photographed each bird from the front and both sides. The birds were then placed in the prepared behavior cage (Fig. 6) and videorecorded for 15 minutes. Birds were removed, weighed, fed and then released with a tonic immobility test (see description below).

Field measurements

We conducted a set of anatomical and morphological measurements to assess each bird's age, sex, body size, physiological condition, and nectar source, following the standard protocol for banding and handling birds (Pyle 1997; Allison Moran, pers. comm.). Because plasma steroid levels shift along with migratory readiness (Landys et al. 2004), we included measurements related to life history physiology. We identified birds by age as either hatch-year (HY) or after hatch-year (AHY). See Table 1 for measurement details.

CF sampling

We used two techniques to collect CF from wild-caught Rufous hummingbirds. For the in-cage method, we fastened Saran Wrap® to foam-core boards with strip magnets, using rubber gloves to keep them free of contamination. We then placed them on the bottom of the behavior cage (Fig. 6; Hiebert et al. 2000a). CF droplets found on the plastic wrap were collected with a pipettor and placed into a microcentrifuge tube.

For the in-hand method, we held birds in the hand with the uncapped mouth of a microcentrifuge tube held up to the cloaca. Within 30 min of obtaining a CF sample, we wrapped the sample tube in Saran Wrap® or sealed it in a plastic bag and placed it in a cooler with ice for temporary storage. Within less than 24 hours, all samples were moved to a 20°C freezer. Some but not all birds provided fecal samples, which were collected separately for microbial DNA analysis. We took a sugar-water sample from each field site to confirm concentration.

Tonic Immobility

To induce tonic immobility, we placed the bird on its back on a flattened palm of the hand and stood near a tree so that birds could readily find a perching location upon release. Timing began when the bird's feet had completely disengaged from any other object and were not being moved by the bird in an effort to grab an object and ended when the bird flew off of its own accord. In the two cases where birds righted themselves while remaining perched in the investigator's hand, we counted only the time before the birds sat upright as the duration of tonic immobility.

Radioimmunoassay

CF samples were assayed directly, without extraction (Hiebert et al. 2000a). Samples (15 ul each) were incubated overnight at 4° C with 150 ul distilled water, 100 ul [3H] corticosterone, and 100 ul corticosterone antibody. Dextran-coated charcoal (500 ul) 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. All CORT values were divided by specific gravity (referred to here simply as CORT) to standardize for differences in CF dilution (González-Gómez et al., in review).

Behavior video analysis

Videos from the one-hour stress series with 15-minute behavior observation were analyzed using the slow motion and sped-up commands offered by the JWatcher computer software (Table 2). Since birds produced chip calls in rapid sequence, videos were first examined while excluding this behavior. A separate run-through was subsequently carried out solely to measure chip calls, and this was included within the same data file to ensure appropriate alignment and integration.

Data analysis

One-way ANOVA was used to compare CF CORT across the four 15-minute time periods in the one-hour stress series with 15-minute behavior observation. Two-way ANOVA was used to assess the effect of (1) time, (2) age, sex, or age-sex class, and (3) interaction between time and age, sex or age-sex class on CF CORT. For behaviors observed during the final 15 minutes of the test, regression analysis was used to determine the relation between continuously quantifiable behavioral variables and CF CORT concentration and one-way ANOVA was used to compare behaviors among the age and sex classes. Data are presented as mean ± SEM, and statistical relationships were considered significant when $P < 0.05$.

RESULTS

At initiation of the study in mid-June, all age and sex classes were present. After hatch-year (AHY) males continued to display through the month of June, in declining

frequency; they became scarce to absent in most sites by early July as they headed east for their southward, high elevation migration. AHY females and hatch-year (HY) birds continued to be present throughout the remainder of the study period, although HY males were difficult to find in early to mid July. As the season progressed, we observed fewer birds at artificial feeders and more birds feeding from natural flowers.

Marking and relocating individuals

In-hand color marking and measurement

We recaptured only one bird out of the nearly fifty birds we painted over the course of the study period. None of the marked birds were ever identified in the wild, suggesting either that the birds had moved to other locations or that the color marks, while easily visible in the hand, were hard to see in free-flying birds. Although we continued to mark each bird we captured, we were forced to abandon our original goal of studying individually marked birds behaving in the wild over time.

Passive color-marking technique

The experimental setup pictorialized in Figs. 2, 3 and 4 was abandoned. Although birds were feeding from all flowers, including those with the cosmetic applicators attached, we observed only one bird that accumulated a visible quantity of pigment above and below the bill. In the modified setup with chenille stem loops, the number of visits to altered flowers (39) over a 95-minute period did not significantly differ from the total number of visits to control flowers (53) over the same time period (chi-square = 2.12, d.f. = 1, $P > 0.05$). Again, however, only one individual was marked enough by the pigment to be identifiable. We also observed that some birds handled the feeders by inserting the bill behind, rather than through, the powdered loop and thereby bypassed the marking

mechanism. Because none of the individual color-marking methods were successful, we did not attempt to track birds with RFID tags and readers, and we were also unable to use visual color marks for this purpose.

Stress series and behavioral assays

Drop-door trap behavioral observations

Since we were unable to keep track of these birds individually, we also halted efforts to quantify birds' behavior in the wild. We first attempted to use the closed-top drop-door setup as both a behavioral arena and a trap to characterize the stress response of known birds immediately and without need for additional recapture. However, fewer birds interacted with the trap than would have been necessary to obtain meaningful sample sizes. Without color marking, it was also impossible to determine whether individual birds that interacted with the trap were the same as or different from birds that we had previously recorded at the trap. Thus, the only behaviors we could reliably link to an individual were those of birds that entered the trap and were immediately captured, and determining whether behaviors were stable over time was not possible.

Two-hour assay and in-cage stress series

We subjected one AHY female to the two-hour in-cage stress series procedure. The bird did not seem to feed at all for the duration of its time in the cage, nor did it provide a CF sample. We discontinued this procedure and replaced it with the following one.

One-hour stress Series with 15-minute behavior observation

CF samples and assays

We collected samples from a total of 36 birds between July 1, 2012, and July 13, 2012. We also took baseline (0-15 minute) CF samples from two other AHY females before early release. Overall, we obtained CF samples from 19 AHY females, 10 HY males, and 10 HY females. We found that birds feeding largely on natural sources of nectar rather than at a feeder [site 4 (Fig. 1), sampled on July 13, 2012], tended to produce larger volumes of CF upon capture. Age-sex classes did not differ significantly in body mass at capture (AHY females: 3.5 ± 0.2 g; HY males: 3.5 ± 0.2 g; HY females: 3.5 ± 0.1 g). We successfully assayed for CF CORT in at least one of the four 15-minute time periods for 15 of the AHY females, 9 of the HY Males, and 7 of the HY females. Because birds did not urinate consistently in the hand or in the behavior cage, we were able to collect volumes of CF necessary for duplicate CORT determinations from all four 15-min periods for only one bird, from three 15-min periods for 8 birds, from two 15-min periods for 16 birds, and from only one 15-min period for 6 birds, for a total of 65 usable samples.

Samples were analyzed in two separate radioimmunoassays. In the second assay, several CORT samples measured higher than Bmax. For this reason, Bmax used for calculations of CORT concentration was determined by averaging the samples that exceeded the value of the Bmax (J.C.Wingfield, pers. comm.). When values were calculated in this way, there was no significant difference between CORT values obtained in the two assays ($P_{\text{Wilcoxon rank-sum}} = 0.32$).

Influence of time, age, and sex on CF CORT concentration

CF CORT values differed significantly over time during the stress series (one-way ANOVA: $F_{3,61} = 17.88$, $P < 0.0001$). CF CORT values of the 30, 45, and 60-minute

samples were significantly higher than those of the 15-minute sample (Tukey-Kramer HSD: $P \leq 0.0003$), but were not significantly different from one another (Tukey-Kramer HSD: $P \geq 0.3$; Fig. 8). CF CORT concentrations in response to stress were significantly affected by age-sex class (two-way ANOVA, $P = 0.0224$; Fig. 9), but the interaction of age-sex class and time was not significant ($P = 0.21$). When birds were separated into AHY and HY birds, age did not have a significant effect on CF CORT response (two-way ANOVA, $P = 0.81$). The interaction of age and time was also non-significant ($P = 0.22$).

Sex may be a more important factor in CF CORT in response to acute stress. In a trend approaching significance, HY males expressed higher CF CORT in response to restraint than did AHY and HY females (two-way ANOVA: $P = 0.0542$). Overall, the interaction of sex and time was not significant ($P = 0.27$). However, at 45 min HY males responded with higher CF CORT than did females of both age classes ($t = 2.254$, $DF = 16$, $P = 0.0386$). At 15, 30 and 60 min, there were no significant differences between samples from HY male and from female birds of all ages (15 min: $t = 0.075$, $DF = 22$, $P = 0.94$; 30 min: $t = 0.863$, $DF = 8$, $P = 0.41$; 60 min: $t = 0.606$, $DF = 11$, $P = 0.56$; Fig. 11).

Behaviors, age, sex, and CF CORT

Overall, perching on the front or side of the cage was significantly related to age-sex class (Kruskal-Wallis: $DF = 1$, $P = 0.0435$), with AHY and HY females tending to perch in this location more than HY males. Of the remaining four in-cage behaviors measured (chip call rate, time flying, rate of perching on the upper dowel, and total number of quadrants visited), none were significantly associated with age, sex, or age-sex class (Table 3). None of the five behaviors we measured were significantly correlated

with 60-min CF CORT (Table 4). However, there was a significant correlation between the number of cage quadrants visited and CORT when the behavior was regressed against CF CORT at 60 min or at 45-min CORT when a 60-min value was not available ($P = 0.0289$) and when it was regressed against the mean of 60- and 45-min CF CORT ($P = 0.0429$; Fig. 12). In addition, the inverse correlation between "chip" vocalizations and CF CORT approached significance either when using 60-min CF CORT supplemented with 45-min CF CORT when a 60-min value was not available ($P = 0.0733$) or when using the mean of 60- and 45-min CF CORT ($P = 0.0686$; Fig. 13). Time to arouse from tonic immobility, however, had no significant relation to age, sex, age-sex class, or any measure of CF CORT (Tables 3 and 4).

DISCUSSION

The observed significant increase in CF CORT levels over the course of the 60-minute stress series represents the first characterization of a wild-caught hummingbird's stress response. HY birds of both sexes did not exhibit a greater CF CORT response than AHY females did. In daily life, hatch-year birds encounter more novel and thus unpredictable situations than older birds do. As these birds fledge, locate dependable food sources, and compete for resources with other hatch-year birds and more experienced adults, they must be able learn and respond appropriately. We would therefore expect young birds to gain from adapting quickly and flexibly to diverse social and environmental circumstances. In great tits, Baugh et al. (2012) showed that this is a trait associated with the shy "personality" syndrome and a faster onset, higher peak glucocorticoid response. Previous studies of tits and sparrows found empirical evidence

for an inversely proportional relationship between dominance status and CORT levels (Rohwer & Wingfield 1981; Silverin et al. 1984).

Our findings do not support this hypothesized relationship between dominance, boldness, and glucocorticoid levels on in rufous hummingbirds. Hatch-year males are situated above HY females and below AHY females in the known dominance hierarchy (Carpenter et al. 1993; Welch & Suarez 2008), and we predicted that HY males would also express intermediate levels of CF CORT under stress. In our study, however, HY males expressed higher levels of CF CORT under restraint stress than did females of any age. Including AHY males (by capturing birds earlier in the breeding season before AHY males have begun to depart) and increasing sample size overall will be essential in future studies seeking to determine whether social dominance is associated with CF CORT in rufous hummingbirds.

In support of our prediction, birds with lower levels of CF CORT during the second half-hour of restraint tended to explore more within an enclosure. Previous studies have shown that in many vertebrates, relative behavioral activity exhibits an "inverted-U" shape with respect to circulating CORT levels (reviewed in Breuner & Wingfield 2000). Through artificially elevating circulating CORT in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*), Breuner & Wingfield (2000) found that intermediate circulating CORT correlated with high relative activity, while both low and high circulating CORT correlated with low relative activity. The observed inversely proportional relationship between cage exploration and restraint CF CORT levels in our study would be consistent with this finding if the lower-CORT, higher-activity hummingbirds represented the intermediate range of the stress response, while the higher-

CORT, lower-activity hummingbirds represented the high stress range. Since rate of chipping exhibited a parallel (although non-significant) inversely proportional correlation with CF CORT, it seems possible that these two behaviors are both facets of stress-induced activity.

We know that activity in the behavior cage can tell us about the behaviors of rufous hummingbirds under acute stress, but it is unclear how or whether they relate to personality traits under unstressed conditions. Future studies in the wild will be needed to determine whether these observed behavioral trends are consistent with the finding of Baugh et al. (2012) that bolder-personality birds tend to consistently explore more and express lower elevated CORT under restraint.

Although four of the observed in-cage behaviors were not significantly associated with age, sex and age-sex class, AHY and HY females perched on the front or side of the behavior cage more often than HY males did. Since HY males expressed higher peak CF CORT under restraint than did females of any age (Fig. 11), and high peak CF CORT levels correlated with reduced exploration (Fig. 12), this finding further supports the "inverted-U" association between CORT levels and relative activity.

The relatively rapid appearance of CORT in CF in this study provides important guidance for implementing the stress series in wild-caught hummingbirds. In a typical stress series conducted on a larger bird, CORT is measured in plasma by taking blood samples at 0-3 min and again at 30 min (Wingfield and Farner 1976). This method allows quantification of both baseline and stressed levels of CORT. In contrast, prior characterization of stress series in captive hummingbirds required about twice as much time to capture the same level of response, since the transfer of CORT from the

bloodstream and into cloacal fluid is thought to take about 15 minutes (Hiebert et al. 2000a). In our study, however, we quantified what seems to be peak levels of response after only thirty to forty-five minutes, matching the shorter time scale of the plasma CORT method used with most other (larger) birds (Wingfield et al. 1982). This difference may result because our wild-caught individuals had never previously experienced capture, restraint or captivity. It is also possible that our particular restraint procedure – in which we manipulated birds for an extensive series of measurements for most of the restraint period – functions as a more effective stressor than existing techniques. Knowing that wild-caught birds subjected to this procedure respond to stress in this way should enable investigators to release birds sooner after capture and potentially double the number of birds processed in the same amount of time in future field studies.

Successfully marking individual hummingbirds and observing how they behave in the wild over time will be crucial in searching for differences in "personality" syndrome on the level of individuals across and *within* age-sex class. By the time we initiated our study, most birds had finished nesting activities and some were beginning to prepare for migration (none of the adult females caught were in egg-laying condition). We expect that the post-nesting condition of the birds increased overall mobility and accounts for our low success in observing marked birds after release. We considered the possibility that the handling process itself discouraged birds from returning to capture sights. This seems unlikely, however, since birds handled for 90-seconds were recaptured at the same low rate as those handled for 10 minutes or more. Furthermore, in other studies, birds were recaptured readily despite being subjected to color-marking or PIT-tagging

procedures exceeding five minutes in length (A. Calder & S. Hiebert pers. comm.; Brewer et al. 2011; Charette et al. unpublished data, available online at www.projetcolibris.org/Charette_et_al_PIT_tag_v4.0.pdf). Conducting behavioral field studies earlier in the breeding season, when birds exhibit higher site fidelity, will be necessary.

Further future studies might seek to apply the same techniques used here to illuminate the hormonal underpinnings not only of intraspecific behavioral differences, as outlined above, but also of *inter*specific interactions and social dominance structures. In the Pacific Northwest, the Anna's hummingbird (*Calypte anna*) is a non-migratory resident that is typically less dominant than *S. rufus* in competition for food and breeding territory during the breeding season (a trend shown for Anna's hummingbird in general; see Stiles et al. 1971; González-Gómez et al., in review). We expect that rufous hummingbirds may, on average, exhibit lower peak CORT in response to restraint stress in comparison with that of Anna's hummingbirds. Such a study could not only help us to understand the glucocorticoid stress response as a potential mechanism of individual "personality" and behavioral interactions within a species, but could be integral to our understanding of ecological interactions between species.

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LITERATURE CITED

Baugh, A.T., S.V. Schaper, M. Hau, J.F. Cockrem, P. de Goede, and K. van Oers. 2012. Corticosterone responses differ between lines of great tits (*Parus major*) selected for divergent personalities. *General and Comparative Endocrinology* 175:488-495.

Breuner, C.W. and J.C. Wingfield. 2000. Rapid behavioral response to corticosterone varies with photoperiod and dose. *Hormones and behavior* 37:23-30.

Brewer, L.W., C.A. Redmond, J.M. Stafford and G.E. Hatch. 2011. Marking Ruby-throated Hummingbirds with radio frequency identification tags. *Journal of Wildlife Management* 75:1664-1667.

Calder III, William A. and S.M. Hiebert. 1983. Nectar feeding, diuresis, and electrolyte replacement of hummingbirds. *Physiological Zoology* 56(3):325-334.

Carpenter, L.F., M.A. Hixon, R.W. Russell., D.C. Paton, and E.J. Temeles. 1993. Interference asymmetries among age-sex classes of rufous hummingbirds during migratory stopovers. *Behavioral Ecology and Sociobiology* 33:297-304.

Carere, C., T.G.G. Groothuis, E. Möstl, S. Daan, and J.M. Koolhaas. 2003. Fecal corticosteroids in a territorial bird selected for different personalities: daily rhythm and the response to social stress. *Hormones and Behavior* 43:540-548.

Charette Y., F. Rousseu, M.J. Mazerolle and M. Belisle. Unpublished. Tracking hummingbird foraging movements and patch-use in the wild with passive integrated transponders. Available online at: www.projetcolibris.org/Charette_et_al_PIT_tag_v4.0.pdf. Accessed July 2012.

González-Gómez, P.L., W.S. Blakeslee, S.M. Hiebert, and J.C. Wingfield. In review. Aggression, body condition and seasonal changes in sex-steroids in four hummingbird species.

Healy, Susan and William A. Calder. 2006. Rufous Hummingbird (*Selasphorus rufus*), The Birds of North America Online (A. Poole, Ed.). Ithaca: Cornell Lab of Ornithology. Retrieved in July 2012 from the Birds of North America Online: http://bna.birds.cornell.edu/bna/species/053/articles/introduction

Hiebert, S.M, M. Ramenofsky, K. Salvante, J.C. Wingfield, and C.L. Gass. 2000a. Noninvasive methods for measuring and manipulating corticosterone in hummingbirds. *General and Comparative Endocrinology* 120:235-247.

Hiebert, S.M, K. Salvante, M. Ramenofsky, and J.C. Wingfield. 2000b. Corticosterone and nocturnal torpor in the rufous hummingbird (*Selasphorus rufus*). *General and Comparative Endocrinology* 120:220-234.

"Hummingbird." 2012. Retrieved in July 2012 from *Journey North*: http://www.learner.org/jnorth/humm/

Kurvers, R. H. J. M., K. van Oers, B.A. Nolet, R.M. Jonker, S. E. van Wieren, H.H.T. Prins and R.C. Ydenberg. 2010. Personality predicts the use of social information. *Ecology Letters* 13:829-837.

Landys, M.M., J.C. Wingfield and M. Ramenofsky. 2004. Plasma corticosterone increases during migratory restlessness in the captive white-crowned sparrow *Zonotrichia leucophrys gambelli*. *Hormones and Behavior* 46:574-581.

Rohwer, S. and J.C. Wingfield. 1981. A field study of social dominance, plasma levels of luteinizing hormone and steroid hormones in wintering harris' sparrows. *Z. Tierpsychol* 57:173-183.

Silverin, B., P. Viebke, and J. Westin. 1984. Plasma levels of luteinizing hormone and steroid hormones in free-living winter groups of willow tits (*Parus montanus*). Hormones and Behavior 18:367-379.

Stiles, F.G. 1971. Time, energy, and territoriality of the Anna's Hummingbird (*Calypte anna*). *Science* 173(3999):818-821.

Webster, M.M. and A.J.W. Ward. 2011. Personality and social context. *Biological Review* 86:759-773.

Wingfield, J.C. and Farner, D.S. 1976. Avian endocrinology: field investigations and methods. *The Condor* 78(4):570-573.

Wingfield, J.C. and Farner, D.S. 1978. The endocrinology of a natural breeding population of the white-crowned sparrow (*Zonotrichia leucophrys pugetensis*). *Physiological Zoology* 51(2):188-205.

Wingfield, J.C., J.P Smith, and D.S. Farner. 1982. Endocrine responses of white-crowned sparrows to environmental stress. *The Condor* 84(4):399-409.

Welch, Jr., K.C. and R.K. Suarez. 2008. Altitude and temperature effects on the energetic cost of hover-feeding in migratory rufous hummingbirds, *Selasphorus rufus*. *Canadian Journal of Zoology* 86:161-169.

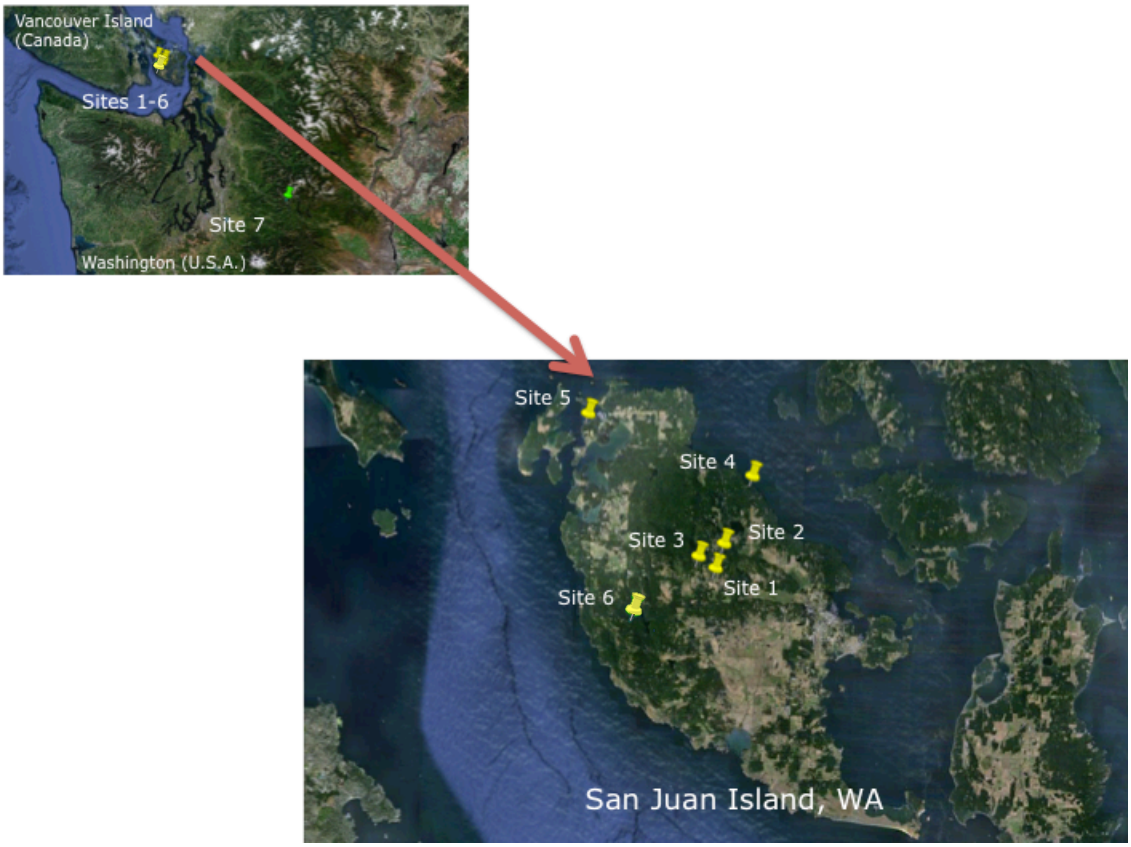

Fig. 1: Locations of field sites used in this study. Six were on San Juan Island, WA (48°33'04.92" N, 123°04'41.18" W), and one (Site 7, in green) was in the Cascades mountain range on the mainland (47°23'27.48"N, 121°24'5.90"W; 872.3 m in elevation). The rest were between sea level and approx. 240 m in elevation. At Site 4, birds accessed a particularly rich floral array. Site 1 was the only location where artificial hummingbird feeders had not been installed in previous years.

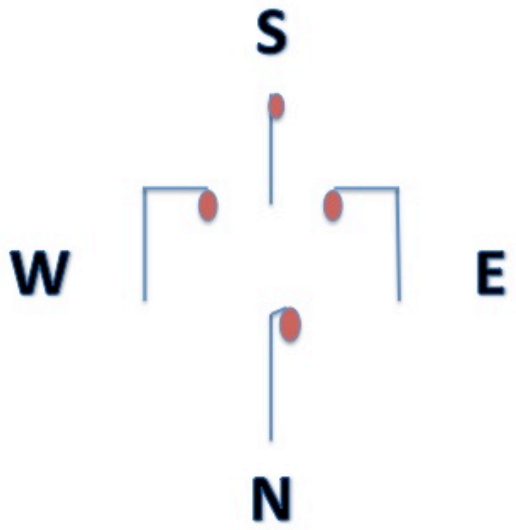

Fig. 2: Frontward, eye-level perspective schematic of feeding stations used in the passive color marking technique setup. Four-flower Perky-Pet© feeders (with the four flower arrangements shown in Fig. 3) were suspended approximately six feet above ground, arranged equidistant from one another, and marked by compass direction for ease of recording observations in JWatcher.

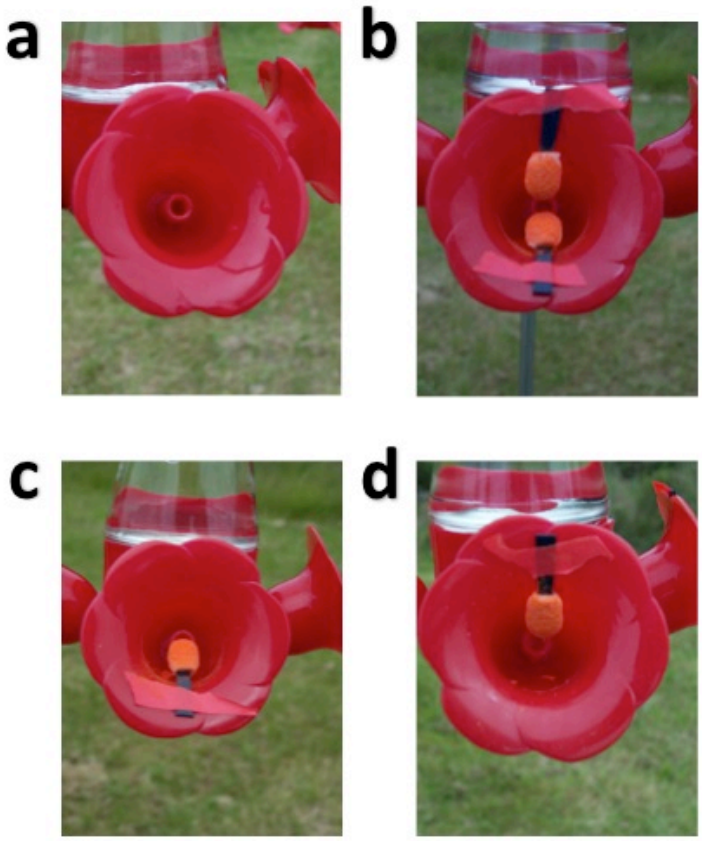

Fig. 3: The four Perky-Pet© plastic flower types on each feeder used in the passive color marking setup: a, control; b, bottom and top applicators; c, bottom applicator only;, d, top applicator only. Faux "stamens" that we fabricated out of cosmetic applicators were coated in non-toxic, brightly colored orange powder.

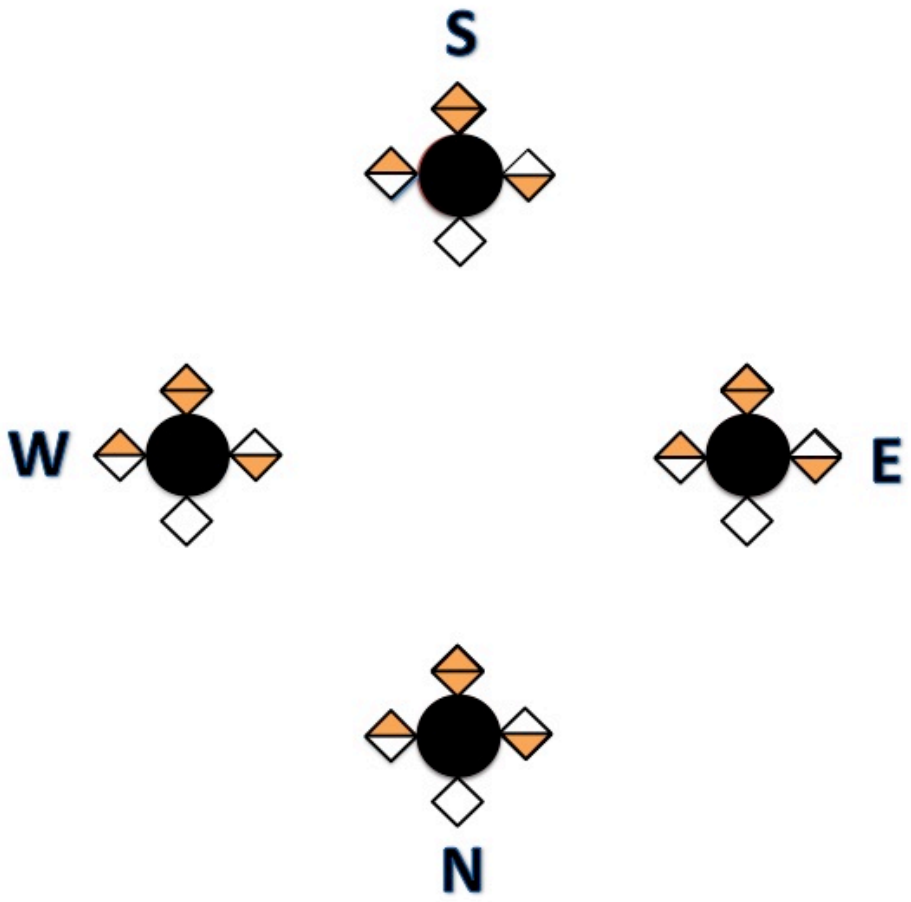

Fig. 4: Top view of the passive color marking technique setup. Perky-Pet© feeders (represented by solid black circles) were arranged so that the undecorated control flower (white diamond) faced north in each direction, so that birds approaching the array from different sides would first see different flower types. For the experimental flowers, we marked one flower with an applicator reaching up from below (diamond with the bottom half colored orange), one with an applicator reaching down from above (diamond with the top half colored orange), and a third with both applicators (fully orange diamond) (see Fig. 3).

Fig. 5: Setup for drop-door trap behavioral observations. A Perky-Pet® feeder was suspended in its center so that its four flowers were just above level with the top of the entrance. Five red, plastic feeder discs were placed on the inside of the trap right below the door to help attract birds to the cage and provide an additional set of novel objects to interact with. Birds were carefully observed as they approached, entered or departed from the feeder and trap area. The top of the cage was usually left open (as in this photo) to allow birds to leave the cage on their own. In some cases, the top was fastened closed and the birds were recovered for measuring, in-cage behavioral observations and eventual release.

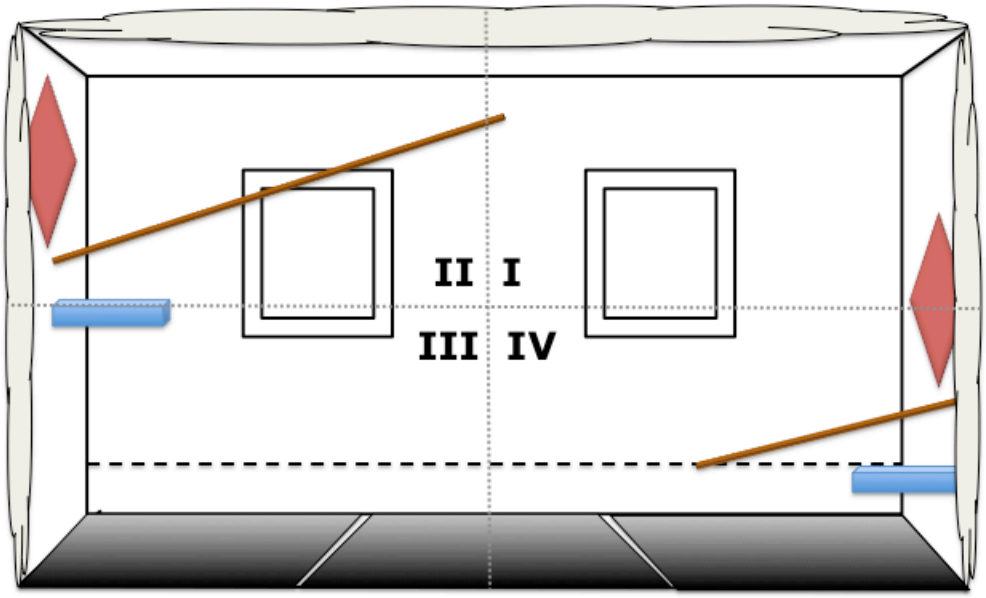

Fig. 6: Behavior cage setup (75 × 52 × 47 cm). Two "Little Beginner" Perky-Pet® feeders (red diamonds) – with large red plastic flowers attached were situated at either end of the cage. Each feeder was situated near a 0.3 cm wooden dowel to serve as a perch (brown lines). Two mesh-lined openings in the back allowed for the investigator to reach in and out of the cage when introducing or removing a bird. All sides were covered by a white sheet except the front, at which we pointed an unmanned video camera for the duration of behavioral observations (15 min). Three foam-core boards covered with Saran Wrap© were placed on the floor of the cage to catch voided cloacal fluid (CF), and a hinged flap on the back wall (dotted line) enabled easy access for board removal. We positioned shop cloth-covered drip catchers below each feeder (blue prisms) to prevent dripping sugar water from contaminating CF samples collected on the foam core boards. For analysis, we visually divided the cage into four quadrants (I, II, III, and IV; marked with fine gray dotted line).

Fig. 7: In-hand cloacal fluid (CF) collection technique. We fastened the bird into a flannel jacket using an alligator clip so that both its head and lower body were exposed for easy measuring and observational accessibility. An open 0.7-ml microcentrifuge tube was held directly up to its cloaca to collect CF, which was later assayed for corticosterone.

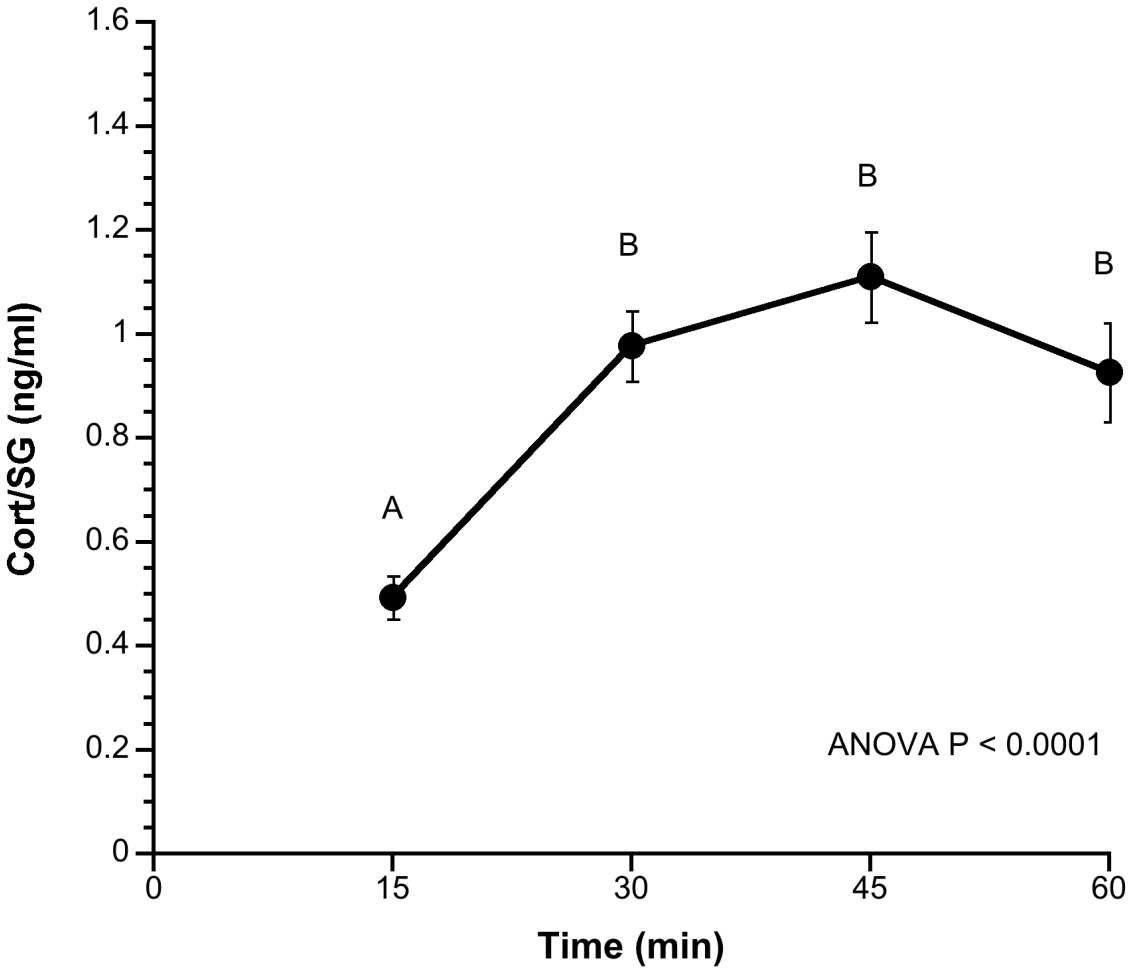

Fig. 8: Change in CF CORT of 31 rufous hummingbirds (*Selasphorus rufus*) over 45 minutes of hand-held restraint and a final 15 minutes of in-cage behavioral observation. CF CORT at 30, 45, and 60 min was significantly higher than that at 15 min (Tukey-Kramer HSD: $p \leq 0.0003$ in all cases), but were not significantly different from one another (Tukey-Kramer HSD: $p \geq 0.3$ in all cases; Fig. 8). Error bars represent standard error of the mean.

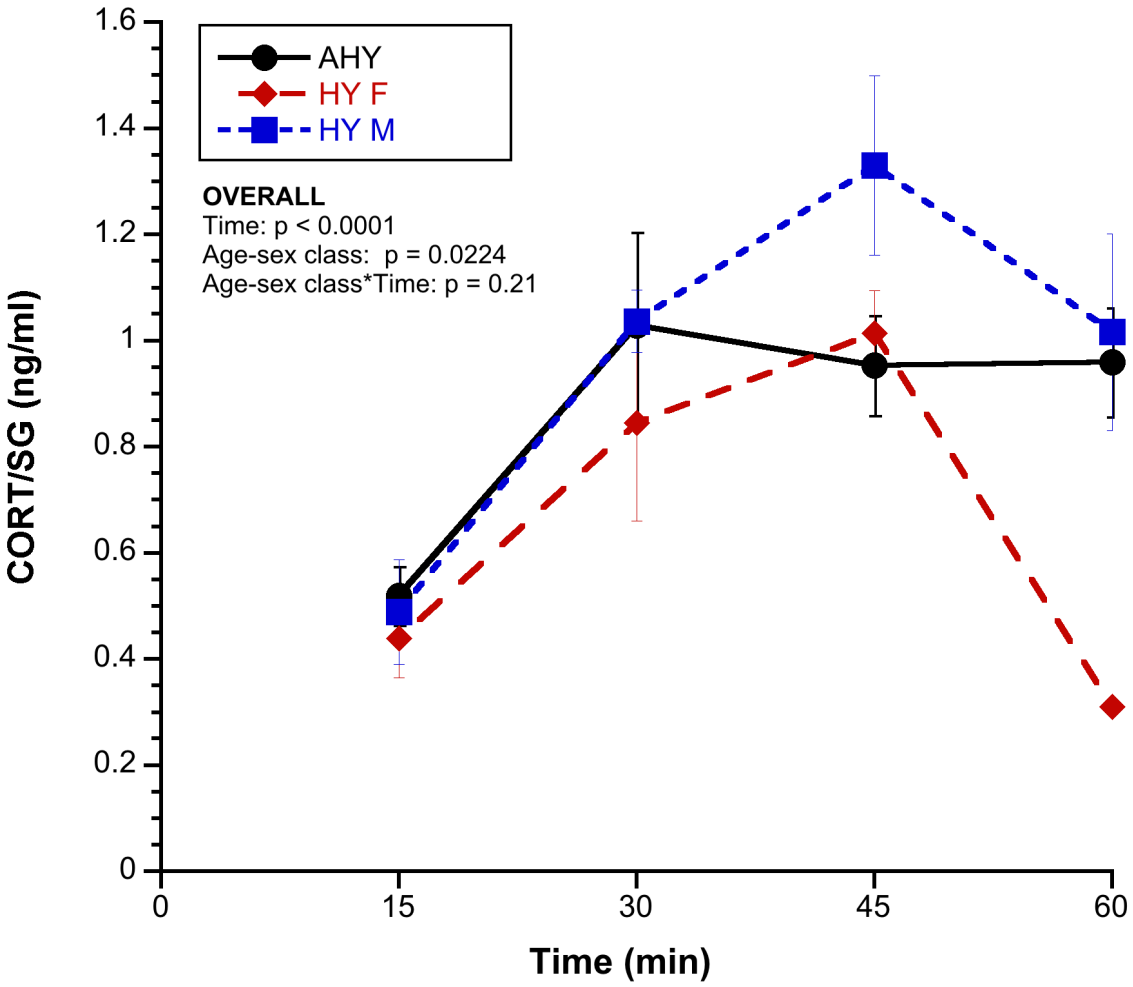

Fig. 9: Change in CF CORT of rufous hummingbirds (*Selasphorus rufus*) over 45 min of hand-held restraint followed by a 15-min in-cage behavioral observation, grouped by age-sex class [after hatch year (AHY) female, hatch year (HY) female, and HY male birds). Overall CF CORT response to restraint stress differed by age-sex class. The interaction of age-sex class and time was not significant. Error bars represent standard error of the mean (point without error bar represents a single sample).

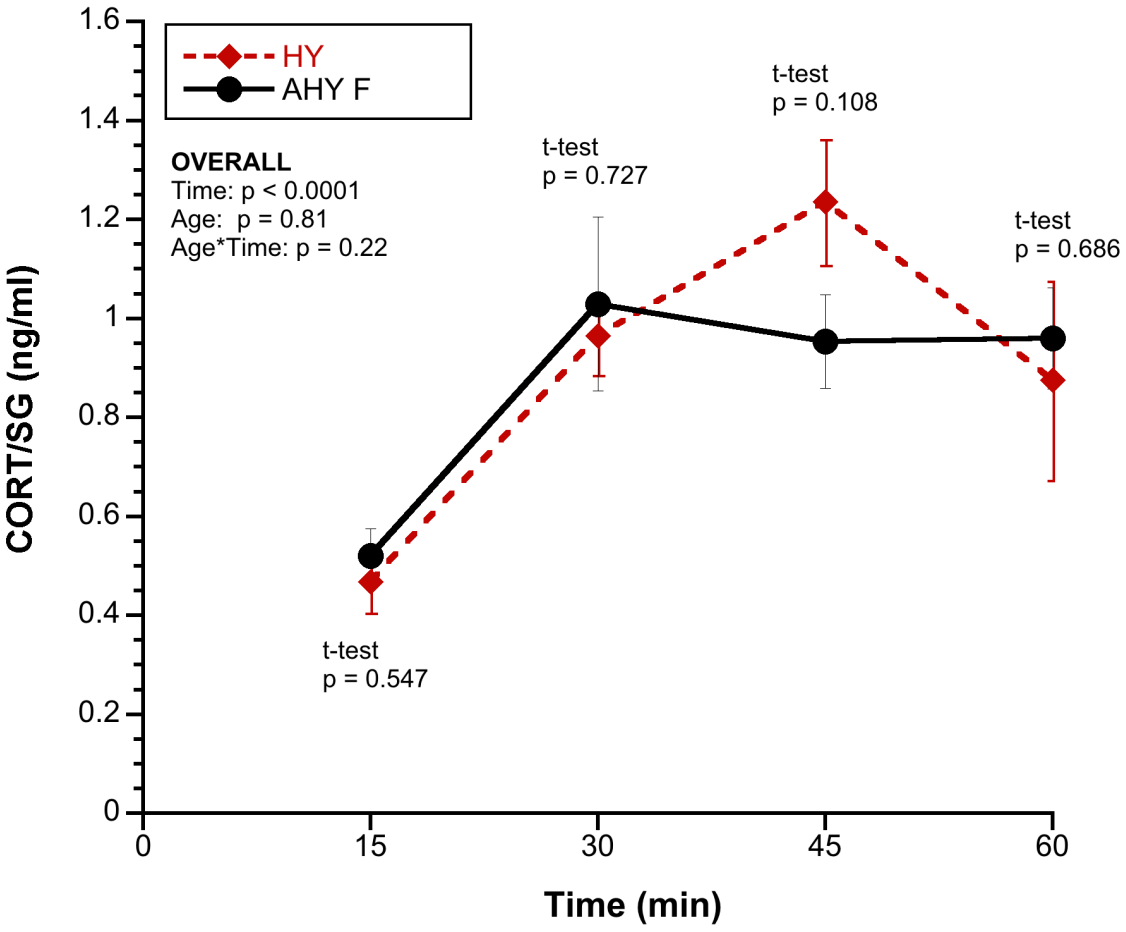

Fig. 10: Change in CF CORT of rufous hummingbirds (*Selasphorus rufus*) grouped by age [after hatch year (AHY) females vs. hatch year (HY) males and females] during 45 min of hand-held restraint followed by a 15-min in-cage behavioral observation. CF CORT changed significantly over time, but AHY and HY birds did not differ significantly in their CF CORT response and there was no interaction between age and time. While HY birds tended to have higher CF CORT than AHY females at 45 min, this difference was not significant. Error bars represent standard error of the mean.

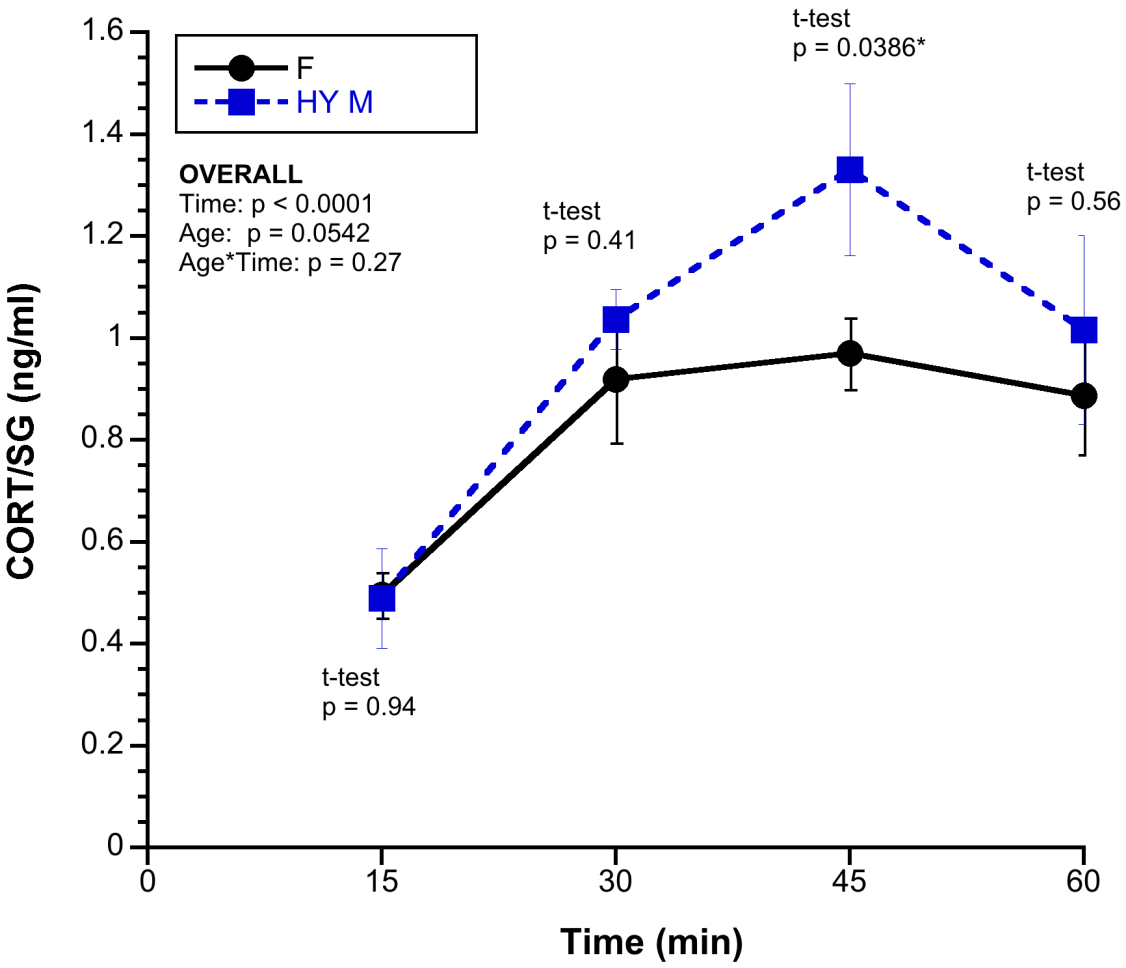

Fig. 11: Change in CF CORT of rufous hummingbirds (*Selasphorus rufus*), grouped by sex [after hatch year (AHY) and hatch year (HY) females vs. HY males) during 45 min of hand-held restraint followed by a 15-min in-cage behavioral observation. CF CORT changed significantly over time. In a trend approaching significance, HY males had higher CF CORT in response to restraint than did AHY and HY females. At 45 min, HY males responded with higher CF CORT than did females of both age classes, but the interaction of sex and time was not significant. At 15, 30 and 60 min, there were no significant differences between samples from HY male and from female birds of all ages. Error bars represent standard error of the mean.

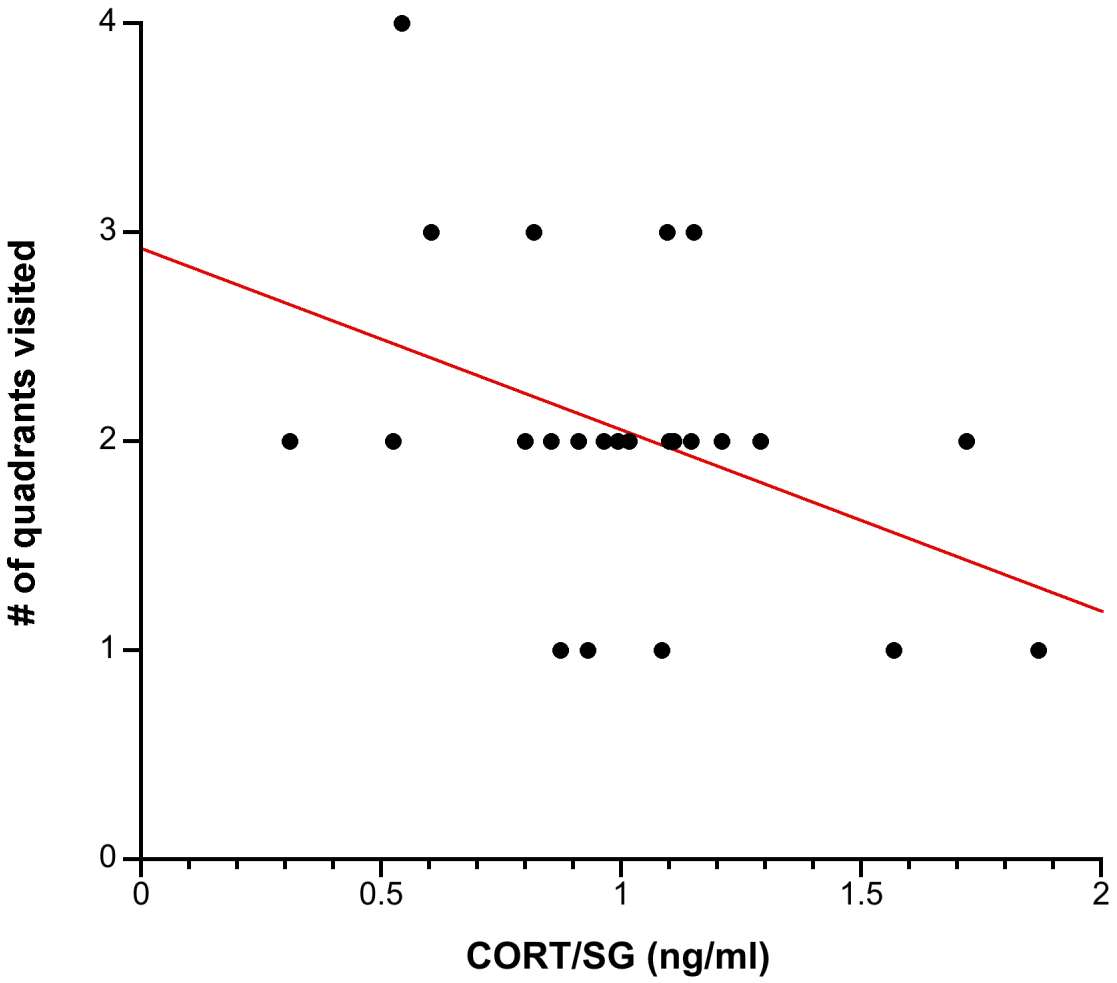

Fig. 12: Number of quadrants visited in relation to CF CORT (mean of the 45 and 60-min measurements; see column 3 of Table 4) during a 15-min in-cage behavioral observation that followed 45 min of hand-held restraint. Extent of exploration was inversely proportional to CF CORT concentration (d.f. = 23; $R^2 = 0.17$; P = 0.0429).

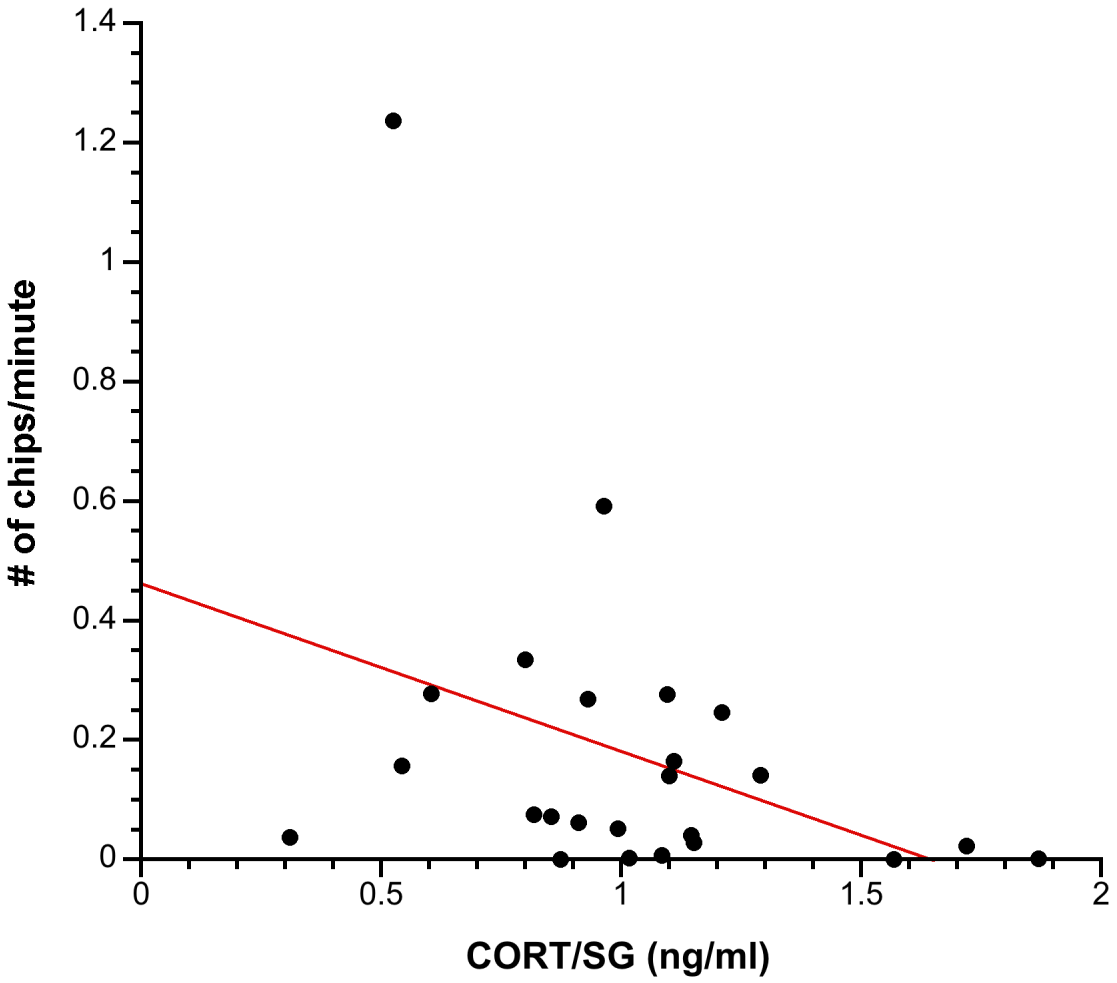

Fig. 12: Rate of chip vocalizations per minute in relation to CF CORT (mean of the 45 and 60-min measurements; see column 3 of Table 4) during a 15-min in-cage behavioral observation that followed 45 min of hand-held restraint. A non-significant trend presents rate of chipping as inversely proportional to CF CORT concentration (d.f. = 23; $R^2 = 0.14$; P = 0.0686).

Table 1: Measurements used in studies where anatomical data were collected:

Measurement or characteristic	Units, scale or character state	Experiments used in[1]	Further details
Date		S_1, S_2, $CM_{c/p}$, DD	
Time	h:min	S_1, S_2, $CM_{c/p}$, DD	
Location	GPS coordinates and site #	S_1, S_2, $CM_{c/p}$, DD	See Fig. 1.
Weather	Temperature = ˚F; wind speed = mph; qualitative notes	S1, S2, CMc/p, DD	Temperature data from weather.com; wind speed from weatherunderground.com
Age	After hatch year (AHY) or hatch year (HY)	S1, S2, CMc/p, DD	Determined primarily by presence of bill grooving. and confirmed by rectrix color pattern (Pyle 1997); the existence of buffy feather margins was also noted
Sex	Male or female	S1, S2, CMc/p, DD	Estimated by gorget and back coloration, and confirmed by rectrix pattern (Pyle 1997)
Left wing cord	mm	S1, S2, CMc, DD	Measured according to Pyle 1997, unflattened
Tail length	mm	S1, S2, CMc, DD	Measured according to Pyle 1997
Length of exposed culmen	mm	S1, S2, CMc, DD	Measured according to Pyle 1997
Bill grooving	Present or absent	S1, S2, CMc/p, DD	Hatch year birds were identified by the presence of bill grooving (Pyle 1997)
Length of bill grooving	mm	S1, S2	The proportional extent of grooved versus smooth bill surface served as a potential further method for aging a hatch-year birds on a relative scale (A. Moran, pers. comm.)

Measurement or Characteristic	Units, Scale or Character State	Experiments Used In	Further Details
Length of bill grooving	mm	S1, S2	The proportional extent of grooved versus smooth bill surface served as a potential further method for aging a young bird on a relative scale (A. Moran, pers. Comm.)
Gorget feathers	Total number colored orange/red; large or small	S1, S2, CMc/p, DD	
% Gorget	Percentage of total gorget area with orange/red color	S1, S2	
Initial mass	g	S1, S2, CMc/p, DD	Body mass taken at capture
Final mass	g	S1, S2, DD	Body mass taken directly before the release procedure
Furcular fat score	0, TR, 1, 2, or 3	S1, S2, CMc, DD	Measure of fat on the furculum; 0 = no fat, TR = trace amnt, 1-3 = from superficial coat to bulging
Right tarsus length	mm; measured left-right and anterior-posterior	S1, S2, CMc, DD	Measured using a caliper held in either orientation
Color mark	Gave new mark to unmarked bird or recovered marked bird; paint color/type; design pattern; on crown, back of the head, or left/right rectrices	S1, S2, CMc, DD	
Pollen	Present or absent	S1, S2	Observed to characterize a bird's food source
Pollen location	Above or below bill	S1, S2	
CP Breed	For AHY females; evidence of brood patch absent or present	S1, S2	To see if adult females had recently been or were currently in an egg-laying state

Measurement or Characteristic	Units, Scale or Character State	Experiments Used In	Further Details
CP Breed	For AHY females; evidence of brood patch absent or present	S_1, S_2	To see if adult females had recently been or were currently in an egg-laying state
Parasite type	Parasites present on gorget region only: tubular white, round white and/or louse	S_1, S_2	We used a knitting needle to gently push aside a bird's throat feathers to reveal parasites; lice were removed and discarded
Parasite number	Total amount of each type present	S_1, S_2	
Back color	For AHY Males: green or rufous	S_1, S_2	Although most AHY male birds of this species have rufous back feathers, some birds have been observed with green feathers on this region (Pyle 1997)
Time to release (informal)	min:sec	$CM_{c/p}$, DD	Birds were placed on their backs and timed to depart, without a formal procedure
Time to release (with tonic immobility procedure)	min:sec	S_1, S_2	See section on *Tonic immobility* for details

[1]S_1: One-hour stress series with 15-minute behavior observation
S_2: Two-hour assay and in-cage stress series
CM_c: Complete in-hand color marking and measurement (with all measurements taken)
CM_p: Partial in-hand color marking and measurement (with limited measurements taken)
DD_s: Closed drop-door trap behavioral observations

Table 2: Scoring used during 15 min of in-cage behavioral observations following 45 min of hand-held restraint in rufous hummingbirds (*Selasphorus rufus*).

Event or Behavior	Code Letter[1]	Description and Purpose
Introducing procedure begins or ends	u	Marks when investigator first inserts bird into cage for release, and after the investigator has released the bird and repositioned the sheet in back of the cage
Investigator attempts to position bird on upper dowel	y	Marks when bird first held directly to the upper dowel and encouraged to perch
Initially flies right away (no contact with dowel)	e	Bird flies directly out of investigator's hand before attempted placement on the dowel
Initially flies right away (contact with dowel)	o	Bird flies directly out of investigator's hand after being positioned adjacent to and making contacting with dowel
Initially sits on dowel	i	Bird initially sits on dowel when placed by investigator
Disturbance by investigator	b	Usually applied when investigator makes effort to retrieve bird during initial release
Other disturbance	m	Other noise, object or animal (specific to case) appears to cause response by subject
Quadrant I	;	Bird positioned in upper right quadrant of cage
Quadrant II	a	Bird positioned in upper left quadrant of cage
Quadrant III	v	Bird positioned in lower left quadrant of cage
Quadrant IV	n	Bird positioned in lower right quadrant of cage
Moving left	4	Bird moves left from the viewer's perspective (usually applied when perched on object)
Moving right	6	Bird moves right from the

		viewer's perspective (usually applied when perched on object)
Perched on front	d	Bird clings to front of cage; when noted, also applies to birds clinging to top, sides or back
Flying in front	f	Bird flies at/around the exposed front of the cage
Flying in front with head stuck through	s	Bird flies toward the exposed front of the cage while inserting head through an opening
Perched on front with head stuck through	q	Bird clings to front of cage while inserting head through an opening
Flying through or around inside or back of cage	l	Bird turns away from exposed front to explore or move toward other discrete location
Explores top feeder	-	Approaches top feeder or takes apparent interest in it from a distance
Explores bottom feeder	[	Approaches bottom feeder or takes apparent interest in it from a distance
***Perched on object**	S [modifier]	Lands and perches on the object in question
Perched on bottom dowel	]	Lands and perches on bottom dowel
Perched on top dowel	p	Lands and perches on top dowel
Preens	g	Actively preening while perched; obvious bout ends with reiteration of perching location
Head or tail movement	k	Bird looks around or moves tail while perched (aside from or in addition to a bout of preening)
Wing shuffle	w	Shuffles wings while perched (aside from or in addition to a bout of preening)
Seems to feed from top feeder	x	Appears to drink sugar water from top feeder

Seems to feed from bottom feeder	z	Appears to drink sugar water from bottom feeder
***Confirmed**	C [modifier]	Confirmation of apparent event or process
***While hovering**	h [modifier]	Hovering while exploring or feeding from an object
***While perched**	P [modifier]	Perched while feeding from an object
Chip call	j	Bird makes audible "chip" call (does not apply to other vocalizations)
Removing procedure begins or ends	r	Marks when investigator first visible in camera field or clearly audible and after the investigator has finally secured the bird in-hand

[1]Behaviors indicate discrete events or processes. Modifiers (asterisked and labeled) describe a particular state or condition of a behavior.

Table 3. Behavior scores compared by age, sex, and age-sex class during 15-min in-cage observations following 45 min of hand-held restraint in rufous hummingbirds (*Selasphorus rufus*).

Behavior	By age (AHY vs HY)	By sex (F vs M)	By age-sex class (AHYF, HYF, HYM)
Chip calls/minute[a]	p = 0.1238	p = 0.3213	p = 0.3023
Percent time flying[b]	p = 0.3554	p = 0.4288	p = 0.6216
Times perching on upper dowel/minute[a]	p = 0.2364	p = 0.4213	p = 0.4891
Times perching on front or side/minute[a]	p = 0.1312	p = 0.0435*	p = 0.1175
Total number of quadrants visited[a]	p = 0.7355	p = 0.4484	p = 0.4904
Duration of tonic immobility[a]	p = 0.8112	p = 0.6846	p = 0.7881

[a]Kruskal-Wallis
[b]One-way ANOVA

Table 4: Regressions of behavior score during 15-min in-cage observations (following 45 min of hand-held restraint) against CF CORT in rufous hummingbirds (*Selasphorus rufus*).

Behavior	Using 60-min CORT	Using 60-min *or* 45-min CORT	Using *mean* of 60-min and 45-min CORT
Chip calls/minute	p = 0.1917	p = 0.0733	p = 0.0686
Percent time flying	p = 0.2990	p = 0.1872	p = 0.2043
Times perching on upper dowel/minute	p = 0.8699	p = 0.9117	p = 0.7724
Times perching on front or side/minute	p = 0.4992	p = 0.3933	p = 0.3890
Total number of quadrants visited	p = 0.1481	p = 0.0289*	p = 0.0429*
Duration of tonic immobility	p = 0.4282	p = 0.4740	p = 0.3483