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Handling and restraint induce a significant increase in plasma corticosterone in Hispaniolan Amazon parrots (*Amazona ventralis*)

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OBJECTIVE
To measure baseline plasma corticosterone levels in Hispaniolan Amazon parrots (*Amazona ventralis*) and assess the effects of handling and restraint on corticosterone levels over 1 hour, reflective of what parrots might experience during veterinary care.

ANIMALS
10 male and 12 female Hispaniolan Amazon parrots.

PROCEDURES
Each parrot was removed from its cage and wrapped in a towel for restraint similar to that performed in a clinical setting. An initial baseline blood sample was collected in < 3 minutes upon entrance into the parrot room, after which blood samples were taken every 15 minutes for 1 hour (a total of 5 blood samples). An enzyme-linked immunoassay was validated for Hispaniolan Amazon parrots and used to determine concentrations of plasma corticosterone.

RESULTS
On average, parrots showed a significant increase in corticosterone between baseline samples and all subsequent postrestraint time points (average baseline corticosterone ± SD: 0.51 ± 0.65 ng/mL). Females, on average, displayed significantly higher corticosterone levels than males after 30, 45, and 60 minutes of restraint (P = .016, P = .0099, and P = .015, respectively). Birds with feather-destructive behavior did not have significantly higher corticosterone levels than birds without the condition (P = .38).

CLINICAL RELEVANCE
Understanding the physiological stress response in companion psittacine birds during routine handling will allow clinicians to better evaluate how this may affect the patient's condition and diagnostic test results. Assessing how corticosterone correlates to behavioral conditions such as feather-destructive behavior will provide clinicians with the potential to develop treatment options.

At baseline levels, glucocorticoid hormones primarily have metabolic functions that include managing energy intake, storage, and mobilization.[1] However, glucocorticoids also serve another important role: they help animals physiologically manage stressful situations. In response to stressful stimuli, the adrenal glands are stimulated to increase glucocorticoid secretion.[2] In birds, the primary glucocorticoid is corticosterone.[2] Acute elevation of corticosterone leads to physiologic responses, including mobilization of glucose and the enhancement of certain immune components.[2,3] The corticosterone stress response also helps to redirect the body's resources away from activities that are temporarily nonessential (eg, reproduction) to those that are needed for immediate survival.[4] Thus, acute elevations of corticosterone are an adaptive response that helps an animal survive a stressful event. However, long-term elevations of corticosterone can result in many different adverse physiologic effects (eg, reproductive suppression, immunocompromised condition, muscle wasting).[4]

To date, the most common method used to assess stress responses in birds is to measure

corticosterone.[5] Measuring this hormone has been shown to be an effective method for understanding aspects of avian biology such as reproductive success and immune responses. One study[6] conducted in Puerto Rican parrots (*Amazona vittata*) found that elevated levels of male fecal glucocorticoid metabolites during the breeding season had a negative relationship with the total number of eggs laid and the fertility of those eggs. Furthermore, 1 study[7] found that Hawai'i 'amakihi (*Chlorodrepanis virens*) with artificially elevated levels of corticosterone from a hormonal implant experienced higher avian malaria loads of *Plasmodium relictum* compared to birds that were not given a corticosterone implant. This supports the general belief that chronically elevated levels of corticosterone can negatively impact the ability of a bird to cope with an infectious agent.[7] Elevated levels of corticosterone have also been associated with stereotypic behaviors in captive birds, such as feather-destructive behavior, a common behavioral disease presentation of companion psittacine species, and other anxiety-related behaviors.[4,8,9] Thus, chronically elevated levels of corticosterone appear to have a wide range of negative effects on the health status of birds.

In veterinary medicine, understanding the role of corticosterone in avian species is imperative to improve and maintain the health of these patients. Most veterinary procedures can be considered stressful for an animal; therefore, one would expect to see elevations in corticosterone levels in response to these procedures. Even activities seemingly as innocuous as being held can induce a stress response in birds. For example, 1 previous study[10] focused on how different captive-bred and wild-caught parrot species varied in their response to restraint. They found that both groups of birds showed elevated levels of corticosterone, but captive-bred parrots showed decreasing levels of corticosterone at 45 minutes, while wild-caught parrots maintained elevated corticosterone levels.[10] Although this study was not conducted from a veterinary standpoint, its findings show how restraining birds induces a stress response and how that stress response can vary depending on how the bird was reared. Furthermore, the stress parrots experience from routine veterinary care has been correlated with changes in hematologic values.[11] While it is known that birds can become easily stressed from handling, few studies have quantified corticosterone levels in psittacines in response to the routine capture and handling that occurs in veterinary clinics.

The goal of this study was to measure baseline plasma corticosterone levels and perform a clinically relevant stress test in Louisiana State University's Hispaniolan Amazon parrot research colony. We hypothesized that the parrots would have a change in their corticosterone levels in response to being held. Because the parrots were hatched in captivity, we predicted that corticosterone levels would start low, peak at the 30-minute mark, and then subsequently decrease due to negative feedback of the hypothalamic-pituitary-adrenal axis. We also predicted that individuals exhibiting feather-destructive behavior would have elevated baseline corticosterone concentrations compared to parrots that did not show this behavior.

Materials and Methods

Study animals

Ten male (average weight, 299 g) and 12 female (average weight, 279 g) adult Hispaniolan Amazon parrots of unknown age from Louisiana State University's Hispaniolan Amazon parrot research colony were used as subject animals for this study. Each individual parrot was subjected to 60 minutes of restraint and blood collection for samples 1 time during this project. There were 2 males and 5 females with feather-destructive behavior. All experimental procedures were approved by the Louisiana State University Institutional Animal Care and Use Committee. The parrots were kept individually housed in cages that measured either 24 X 18 X 29 inches or 29 X 24 X 31 inches in length by width by height. Enrichment items, such as acrylic toys, rope toys, and wiffle balls stuffed with paper, were provided to the birds throughout the study. Perches were manzanita branches and measured 12 inches long and 1 to 1.5 inches in diameter. All parrots had ad libitum access to tap water and Kaytee Exact Rainbow Parrot and Conure diet.

Sample collection

The sampling period was between May 25 and June 3, 2022. Each day, blood samples were collected at either 0700 or 1500, except for one 1100 collection time. We attempted to process a total of 4 parrots each day, 2 in the morning and 2 in the afternoon. Prior to our scheduled collection times, the birds were left undisturbed for at least 2 hours. To collect valid baseline plasma corticosterone samples, a timer was used so that all initial samples were collected in < 3 minutes upon researchers' entrance into the room that housed the parrots.[12] Because of this time constraint to collect the initial blood sample, the parrots selected to be processed each day were selected based on cage order in the housing room. While the first bird of a sampling time was caught with a net and wrapped in a towel, and the baseline blood sample was collected by a board-certified avian specialist (TNT), the second bird was caught and wrapped in a towel by an assistant (ALS). After a blood sample was collected from the first bird, the 2 birds were swapped so a sample could be collected from the second bird. All baseline blood samples were collected in < 3 minutes. Blood samples were collected from the right jugular vein using a 26-gauge needle and 3-mL syringe **(Figure 1)**. Blood samples were then transferred into heparinized microcapillary tubes. If the right jugular vein could not be identified, venipuncture of the right basilic vein was achieved by pricking the vein with a needle and collecting the blood directly into a heparinized microcapillary tube. After baseline corticosterone samples were collected, the parrots being sampled continued to be held wrapped in towels

Figure 1—Image depicting the restraint and blood sampling techniques used on the Hispaniolan Amazon parrots in this study. We attempted to collect 0.5 mL of blood from the right jugular vein using a 26-gauge needle and 3-mL syringe each time. Parrots were held wrapped in a towel for the duration of the 60-minute sampling period.

and were moved into a different room located down the hall from their housing room for the duration of the sampling period. This was done to minimize the disturbance of other parrots in the research colony. Birds were restrained in hand in a towel for the duration of the 60-minute period to reflect a clinical scenario. We collected additional blood samples from both birds at 15, 30, 45, and 60 minutes as previously described. If blood could not be drawn from the right jugular vein during the last 4 collection time points, either the left jugular vein, left basilic vein, or right basilic vein was utilized. We attempted to collect 0.5 mL of blood each time; however, this was not always possible, as blood samples collected from the basilic veins were collected directly into a microcapillary tube. In these instances, approximately 2 full microcapillary tubes containing 120 µL of blood were collected. At the end of the 60-minute period, parrots were weighed and assessed for signs of feather-destructive behavior. A bird was considered to have feather-destructive behavior if it had evidence of plucked feathers and bare areas of exposed skin from areas of the body the bird could reach, such as the chest, abdomen, and back. After this information was collected, birds were returned to their home cages and the room was prepared for the next sampling period.

Blood samples were stored on wet ice and centrifuged at the end of the 60-minute period. Microcapillary tubes were centrifuged at 21,100 X *g* for 10 minutes or 17,000 X *g* for 12 minutes in a hematocrit rotor (7500 3473; Thermo Fisher) in a Heraeus Pico 21 centrifuge (Thermo Fisher), both of which resulted in the appropriate separation of plasma from packed red blood cells. A Hamilton syringe was used to collect the plasma from microcapillary tubes. Plasma samples were stored in microcentrifuge tubes at −81 °C until the corticosterone assays were performed.

Corticosterone assays

To measure the concentration of corticosterone in the collected samples, enzyme-linked immunoassay plates (Arbor Assays; K014-H1/H5) that had previously been successfully used for several avian species were utilized.[13,14] A total of 110 plasma samples were collected for the study, and each assay plate could hold 35 samples plus standards. Seven birds and their 5 respective samples were randomly selected using the random number generator function in Microsoft Excel to run on each plate, for a total of 4 plates. Each plate contained a mix of males and females and individuals exhibiting and not exhibiting feather-destructive behavior, across different sampling dates and times. Extracting corticosterone from plasma as well as measuring its concentration was a 2-day process. Prior to data collection, the enzyme-linked immunoassay for Hispaniolan Amazon parrots was validated by assessing the parallelism of diluted pooled parrot plasma samples with a corticosterone standard curve (see Results). The pooled parrot plasma came from spare plasma obtained from the 45 minutes samples of 2 birds. Moreover, a pooled plasma sample was included on each plate to determine our interplate variability (coefficient of variation, 21%). Intraplate variability was determined using the coefficient of variation of duplicate samples and averaged 15%. Extraction efficiency for this assay was determined using 2 plasma samples per plate that were stripped of endogenous corticosterone using Dextran-coated charcoal and spiked with a known amount of corticosterone (average recovery, 90%). Because recovery was high and consistent, we did not correct the final values. The sensitivity of this assay was 20.9 pg/mL.

Prior to beginning the extraction process, the plasma samples for each of the 7 birds used in an assay and a tube containing pooled plasma were removed from the freezer and allowed to thaw. A stripped and spiked plasma sample was also prepared for each assay by adding equal parts of a 10,000 pg/mL corticosterone standard and plasma that had been stripped of corticosterone using Dextran-coated charcoal. Triple ethyl acetate extraction of 5 µL of plasma samples was used as previously described.[14] After 3 rounds of extraction, sample tubes were left open in a fume hood overnight for the ethyl acetate to evaporate. The next day, 125 µL of assay buffer was added to each tube (final dilution, 1:25). At that time, the ELISAs were done according to the manufacturer's protocol. The ELISA plates were read using a SpectraMax ABS plate reader (Molecular Devices) at 450 nm.

Statistical analyses

As the parrots were sampled over successive days, mixed-effects models were performed

to determine if the sampling date affected plasma corticosterone. This was to assess possible acclimation or stress effects of our sampling procedure on nontarget individuals. These models included corticosterone concentration as a dependent variable, individual as a random effect, and sample time point (baseline or 15, 30, 45, or 60 minutes), bird sex, and Julian date as fixed effects. Similarly, samples were collected at different times to allow for the sampling of multiple individuals each day. To test whether plasma corticosterone was affected by the time of day samples were collected, another mixed-effects model was run with corticosterone as a dependent variable, individual as a random effect, and sample time point, bird sex, and sampling time as fixed effects. As neither sampling date nor time of day significantly affected corticosterone (see Results), these effects were not included in other models.

For the final 2 models, the first tested for the effect of sex on corticosterone concentrations at baseline and in response to capture and restraint. This model used corticosterone as the dependent variable, individual as a random effect, and sample time point, bird sex, and a sample time point by sex interaction. The second model tested for an effect of feather destructive behavior on corticosterone concentrations. This model used corticosterone as the dependent variable, individual as a random effect, and sample time point, presence of feather destructive behavior, and a sample time point by feather destructive behavior interaction. When individual model effects were significant, Tukey honestly significant difference tests were used for post hoc testing. In cases where interactions were significant, further testing looked for differences among groups using planned contrasts that only examined differences within each sample time point (eg, comparing male and female corticosterone at baseline, at 15 minutes, at 30 minutes, etc). For all models, the normality of the residuals using normal quantile plots was assessed and checked for homoscedasticity by inspecting plots of studentized residuals against predicted values of dependent variables. For all analyses, we used a $P < .05$ threshold for statistical significance.

Results

After triple ethyl acetate extraction, a serial dilution of pooled plasma revealed parallelism with corticosterone standards **(Figure 2)**, demonstrating that there were no substances in extracted parrot plasma interfering with the assay. The sampling date did not significantly affect corticosterone concentrations ($F_{1,27} = 0.17$, $P = .68$) nor did the time of day when samples were collected ($F_{1,19} = 2.53$, $P = .13$), so these effects were not included in other models. In a model examining the effects of sample time point, sex, and sex by sample time point interaction on corticosterone, there was a significant effect of sample time point on corticosterone concentrations (**Figure 3**; $F_{4,80} = 33.14$, $P = < .0001$), as well as significant effects of sex ($F_{1,20} = 4.9$, $P = .038$) and an interaction between the 2 (**Figure 4**; $F_{4,80} = 2.98$, $P = .024$). Tukey's post hoc tests indicated that baseline corticosterone concentrations were lower than any of the 4 stress-induced samples, but the stress-induced samples were not different from each other. Planned contrasts did not find a difference between male and female corticosterone concentrations at baseline ($F_{1,45} = .003$, $P = .96$) or after 15 minutes of restraint ($F_{1,45} = 1.20$, $P = .28$), but corticosterone was lower in males than in females after 30 minutes ($F_{1,45} = 6.27$, $P = .016$), 45 minutes ($F_{1,45} = 7.24$, $P = .0099$), and 60 minutes ($F_{1,45} = 6.44$, $P = .015$) of restraint. A model examining the effects

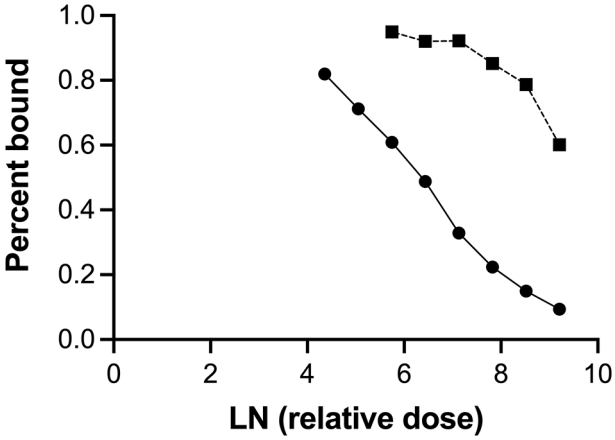

Figure 2—Natural logarithm (LN) transformed serial dilutions of Hispaniolan Amazon parrot plasma assayed via enzyme-linked immunoassay were approximately parallel to LN transformed dilutions of the kit-provided corticosterone standard. The solid line and circle points depict data from diluted standards and the dashed line and square points represent data from various dilutions of pooled parrot plasma.

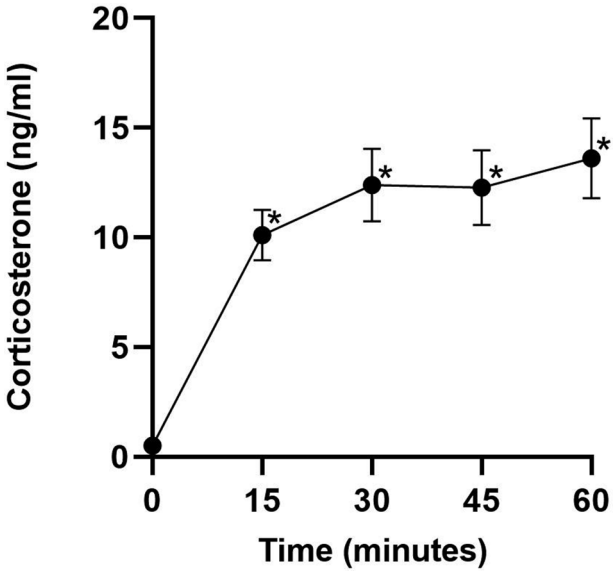

Figure 3—Average corticosterone levels of Hispaniolan parrots (n = 22) were higher after 15 minutes of restraint compared to baseline (t = 0 min; collected in < 3 min) and stayed high for the entire 60-minute period. Vertical lines represent the standard error. *Values that significantly differed ($P < .05$) from baseline.

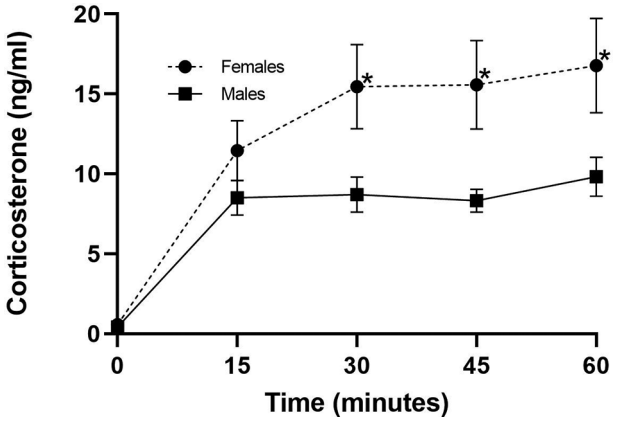

Figure 4—Corticosterone levels of female Hispaniolan parrots (n = 12) were higher than males (10) after 30 minutes ($F_{1,45}$ = 6.29, P = .016), 45 minutes ($F_{1,45}$ = 7.27, P = .0098), and 60 minutes ($F_{1,45}$ = 6.67, P = .013) of restraint. There was no significant difference at baseline or 15 minutes. Vertical lines represent the standard error. *Values that were significantly different between males and females (P < .05). The dashed line represents the average corticosterone level of females, and the solid line represents the average of males.

of sample time point, feather-destructive behavior, and feather-destructive behavior by sample time point interaction again found effects of sample time point ($F_{4,80}$ = 30.46, P = <.0001) but not feather-destructive behavior ($F_{1,20}$ = .79, P = .38) or any interaction between the 2 (**Figure 5**; $F_{4,80}$ = 1.66, P = .17).

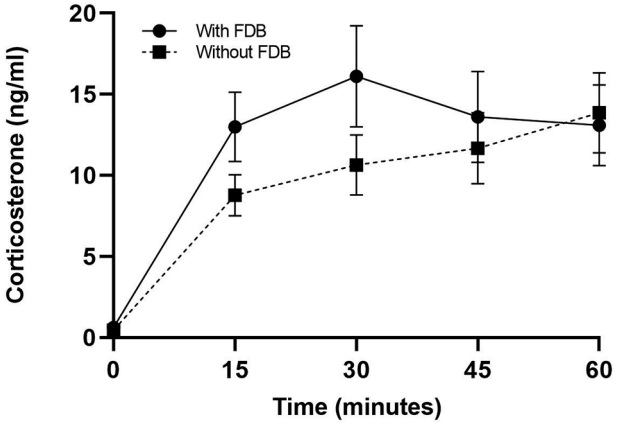

Figure 5—Corticosterone levels of birds with feather-destructive behavior (FDB) (n = 7) were not significantly different from birds without this behavior (15). Vertical lines represent the standard error. The solid line represents the average corticosterone level of birds with FDB, and the dashed line represents the average for birds without FDB.

Discussion

The goal of this study was to measure baseline plasma corticosterone levels and perform a clinically relevant stress series test in a captive colony of Hispaniolan Amazon parrots. Corticosterone is an important hormone for maintaining homeostasis and responding to stress. Although this is widely known, there is a paucity of research on psittacine species that measures baseline plasma corticosterone concentrations and how they fluctuate in response to stress. Plasma sampling provides an advantage for assessing corticosterone over fecal or feather assays because it provides a dynamic "snapshot" of circulating hormone levels over time, and 1 of the results of this project was validation of a commercially available enzyme-linked immunoassay for use with parrot plasma.[15] Based on previous work using captive-bred parrots, we predicted that corticosterone in our study parrots would initially be low, increase to a peak at 30 minutes in response to capture and handling, and then subsequently decrease. However, we found that parrots increased plasma corticosterone faster than expected, reaching peak levels at 15 minutes postrestraint (average corticosterone at 15 minutes ± SD, 10.11 ± 5.42 ng/mL) and maintained high levels throughout the 60-minute evaluation period. While restraint for an entire 60-minute period may not typically reflect what a bird would experience in a clinic, the first 15 minutes were reflective of a routine clinical scenario. Longer time points (30, 45, and 60 minutes) may be representative of handling and restraint necessary for prolonged veterinary procedures or situations in which a bird may be partially restrained for an extended period, such as bandage wraps or devices to prevent self-mutilation. Birds were restrained in hand, as opposed to being placed into a carrier between each blood sample time point, to control for any additional stress caused by repeated capture. We chose to collect blood every 15 minutes to evaluate changes in corticosterone over time. Although it is possible that frequent blood samples caused the birds additional stress, because there was no significant increase in plasma corticosterone between 15 minutes and 60 minutes, we do not believe that this was the case. However, this possibility is discussed more in depth below.

The baseline values of corticosterone measured in this study are lower than what has been found in previous studies[11,16] conducted in this same colony. However, this may be attributable to the different methods used to quantify corticosterone in each study,[11,16] and 1 of the studies did not collect baseline samples within 3 minutes upon entering the room.[16] Regardless, a common finding amongst the studies was the significant increase in corticosterone in response to standard veterinary procedures. We found that corticosterone remained high during the 60 minutes sampling period and did not decrease as predicted. This suggests that corticosterone negative feedback takes longer than 60 minutes to begin reducing corticosterone concentrations in Hispaniolan Amazon parrots, which is consistent with studies[17] in other avian species, such as European starlings (*Sturnus vulgaris*). Our study and previous work on this species suggest that parrots undergoing veterinary procedures may be especially vulnerable to potential negative effects of corticosterone on physiology and behavior and that handling and restraint time be minimized to the extent possible.

We found that male and female Hispaniolan parrots, on average, did not differ in their baseline concentrations of corticosterone. This finding is consistent with previous work[6,18] in other parrot species, which have also found a lack of sex differences in baseline corticosterone from both plasma and fecal samples. Our study did however find that females had a stronger acute corticosterone response after 30, 45, and 60 minutes of restraint. Sex differences in corticosterone concentrations in response to stress are somewhat consistent with previous parrot work, although the sex with the stronger response appears to vary by species. For example, in Puerto Rican parrots, male parrots, not females, were found to have a higher concentration of plasma corticosterone after 30 minutes of restraint.[6] Studies[19] that have extracted corticosterone from feces have found sex differences in the concentration of fecal glucocorticoid metabolites, but this has been partly attributed to differences in how each sex metabolizes corticosterone. It is unclear why females in our study showed a greater release of plasma corticosterone in response to stress than males. Differences in which sex experiences higher concentrations of corticosterone after a stressful event could be due to differences in life history, social organization, or reproductive strategies between parrot species. However, further research on this topic should be conducted.

Contrary to our prediction, parrots with and without feather-destructive behavior were found to have no significant differences in their corticosterone concentrations at any time point. However, our sample size of birds displaying feather-destructive behavior was small (7 out of 22 parrots), and it is possible that a larger sample size would yield different results. There are few studies that have investigated the concentration of corticosterone in the plasma of birds with and without feather-destructive behavior. One previous study[20] conducted in African grey parrots (*Psittacus erithacus*) found no differences in plasma corticosterone concentrations between birds with and without feather-destructive behavior before or after the administration of bovine thyroid stimulating hormone. However, studies[8,21] that have investigated fecal glucocorticoid metabolites have found that the concentration of corticosterone was higher in African grey parrots with feather-destructive behavior compared to those without the condition. These studies did not investigate the concentration of corticosterone in the plasma. It is possible that parrots with and without feather-destructive behavior could metabolize corticosterone differently, similar to the sex differences seen in previous fecal glucocorticoid metabolite studies.

There were limitations to this study. Because we limited our collection time to 60 minutes, we were unable to see when plasma corticosterone concentrations begin to fall in response to negative feedback, where corticosterone binds to receptors in the brain and pituitary to shut down its own release. Subsequent studies could investigate when this decrease begins by collecting samples beyond 60 minutes. For this study, we attempted to collect blood from the right jugular vein each time but that was not always possible. While we do not anticipate that the different anatomic sites yielded different concentrations of corticosterone, it is possible that different collection sites could induce a stronger or weaker stress response in the parrots, especially because certain anatomic sites, such as the basilic vein, often develop hematomas following needle removal. To our knowledge, there are no studies showing that hematoma formation or blood collection from multiple veins can affect circulating corticosterone, although these would be valuable subjects for future investigation. Future studies could control for this by consistently drawing blood from 1 location.

Because parrots did not continue to increase corticosterone at each successive time point, we know that repeated blood draws did not induce a stronger stress response with each collection. Although previous work has shown that captive-bred parrots' reduction of corticosterone concentrations can begin as early as 45 minutes, it is possible that the repeated sampling performed in our study contributed to the elevated corticosterone and prevented negative feedback. Furthermore, although the parrots used in this study are often handled for research projects and veterinary care, their environment is not reflective of a companion pet psittacine. Thus, it is possible that parrots housed as pets may exhibit differences in their corticosterone response due to frequent handling and interactions with owners, as well as increased familiarity with routine veterinary procedures.

Understanding how corticosterone levels change over time in response to a stressful stimulus can be used by veterinarians to educate clients about the physiologic responses of parrots that are exposed to stressful conditions. This could help pet owners, conservationists, zookeepers, and other individuals who care for birds make more informed decisions to reduce stress within their bird's environment. Improved care and reduced stress could result in a decrease in chronic corticosterone levels and an overall improvement in the bird's welfare. Furthermore, this project helps lay the groundwork for future studies of the physiological stress response in psittacine species, such as studies examining the effects of adrenocorticotropic hormone stimulation or negative feedback inhibition using dexamethasone. Future studies could also evaluate to what extent specific medical procedures induce stress and explore different techniques to help minimize that stress. Moreover, future work could investigate how males and females, as well as birds with and without feather-destructive behavior, respond differently to various stressful stimuli. By understanding how birds respond physiologically to stressors, new protocols can be developed to provide better care for avian species in their home environment and in the clinic.

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The authors declare that there were no conflicts of interest.

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