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Seasonal Variation in Fecal Glucocorticoid Levels and Their Relationship to Reproductive Success in Captive Populations of an Endangered Parrot

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Abstract: Many species are threatened with extinction, and captive breeding programs are becoming more common to avoid this outcome. These programs serve to prevent extinction and produce individuals for eventual reintroduction to natural populations in historical habitat. Captive animals experience different energetic demands than those in the wild, however, and as a result may have different levels of glucocorticoid hormones. Glucocorticoids help with responses to energetically expensive and potentially stressful situations. Elevated glucocorticoid levels can also potentially alter reproduction and other key behaviors, thus complicating successful captive breeding. The Puerto Rican parrot (*Amazona vittata*) is a critically endangered parrot that currently exists in only two wild and two captive populations. Its recovery program provides a good platform to better understand how glucocorticoid levels may relate to reproductive success under captive conditions. We validated a corticosterone assay in this species and used non-invasive techniques of measuring fecal glucocorticoid metabolites of males and females from two captive populations (Rio Abajo and El Yunque) of Puerto Rican parrots over two consecutive breeding seasons, 2017 and 2018, and the pre-breeding season of 2018, which occurred just after Hurricane Maria struck Puerto Rico. Our results show that levels of fecal glucocorticoid metabolites of males measured during the breeding season of 2018 negatively correlated to the number of total eggs and fertile eggs laid by pairs. In contrast, there was a positive relationship of female fecal glucocorticoid metabolite levels during the pre-breeding season of 2018 with total eggs laid. In males from the Rio Abajo population, we found seasonal differences in fecal glucocorticoid metabolite levels, with higher levels during the pre-breeding season of 2018 compared to both 2017 and 2018 breeding seasons. There was no difference in the mean value of male fecal glucocorticoid metabolites between the 2017 breeding season and 2018 breeding season which started four months after Hurricane Maria struck Puerto Rico. We did find sex differences during the pre-breeding season of 2018 in birds from the Rio Abajo population. Adjustments in the care routine of both populations that could reduce circulating baseline glucocorticoids and avoid frequent, sudden elevations of glucocorticoids should be considered. These results provide a baseline for future comparison with reintroduced populations of this endangered species and other species with captive breeding programs.

Keywords: captive populations; endangered species; glucocorticoids; parrot; reproductive success; seasonality

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1. Introduction

Changes in the local environment of an organism can promote responses or changes in both physiological processes and behavior, specifically an increase in glucocorticoids [1]. Environmental challenges can be either predictable or unpredictable, but in either case, can have a major impact on individual fitness and evolutionary adaptation [1]. One of the primary mechanisms to promote adaptive responses to environmental challenges

in vertebrates is the elevation of glucocorticoid hormone levels by the hypothalamic-pituitary-adrenal (HPA) axis [2,3]. Glucocorticoids primary role in an organism is to aid the metabolism of protein and lipids into carbohydrates for energy consumption and multiple other functions [4,5]. During a disturbance, an animal will release glucocorticoid hormones, resulting in a process of prioritization of energy for a survival response [2,6,7] and subsequent recovery. The frequency and duration of perturbations can cause an animal to have either persistently high glucocorticoid levels, or repeated short-term elevations of glucocorticoids, sometimes termed 'allostatic overload', with potentially negative consequences [1,5]. For example, in birds, elevated levels of corticosterone (the primary avian glucocorticoid) may inhibit the production of luteinizing hormone and prolactin [8–10] and reduce affiliative behaviors, potentially affecting reproductive success [8,11,12].

Captivity is a prime example of an environment that can potentially alter normal glucocorticoid levels and reproductive success [8]. Captive breeding programs are becoming more common as human-mediated habitat changes and other anthropogenic disturbances threaten more species with population reduction and even extinction. A high production of individuals from captive breeding populations of endangered species can allow managers to develop better strategies to enhance reintroduction efforts and sustain wild populations [13,14]. But under captive conditions, animals may have altered levels of glucocorticoids compared to individuals in the wild, modifying behavior otherwise characteristic of the species [15]. In captivity, individuals may experience increased social interactions, atypical photoperiods and limited space overall [16], which can lead to an increase in glucocorticoid levels and altered behavior [17]. Captive conditions for individuals born and raised in the wild may cause a drastic increase in glucocorticoids levels, especially when these individuals spend long periods of time in captivity, with substantial effects on reproduction [17]. In black-legged kittiwakes (*Rissa tridactyla*) artificial increase of the glucocorticoid corticosterone, reduced the production of prolactin, significantly reducing reproductive success [8]. Furthermore, increased glucocorticoids could negatively impact reproductive success by altering parental behaviors, such as feeding patterns of offspring, incubation consistency in birds and the timing of nesting [8,15]. Although the endocrine system is well conserved across vertebrates, the reaction to captivity may vary depending on the species [16]. There may also be physiological differences in responses among populations and individuals [18–23]. Some species may never be able to reproduce effectively in captive conditions even after generations, while in others, artificial selection can lead to changes in reproductive physiology [16]. Therefore, studying the effects of glucocorticoid levels on parental care behavior and reproductive success in captive breeding individuals and understanding the difference among populations, sex and seasonal variation can help program managers develop improved techniques to increase reproduction.

Another factor to consider in species conservation is ongoing anthropogenic-induced climate change which has led to unpredictability in weather, potentially increasing the frequency, duration, and severity of climate events. In particular, hurricanes can be a major threat to vulnerable and threatened populations of wildlife [24]. For wild populations, direct wind effects during the hurricane can kill individuals, and those that survive must deal with limited food resources [25,26], potentially experiencing altered glucocorticoid levels. Furthermore, species that coordinate their breeding with local fruiting patterns can experience reduced reproductive success after such events [26]. Although captive populations are buffered from many weather events, hurricanes may still affect them. During and after an extreme weather event, housing facilities may suffer damage and loss of power, and caretaking staff may have limited access to facilities. Food scarcity, overcrowding and isolation from normal light cycles could alter the physiology of captive individuals in these situations. Despite these potentially important effects, there are few studies examining changes in glucocorticoid levels in captive populations after hurricanes or other natural disasters [27].

The parrots and cockatoos (Order Psittaciformes) are one group for which increased knowledge of the relation of glucocorticoid levels to captive reproduction is critical. Psittaci-

formes have a worldwide species distribution with 419 known species, 42.2% of which are classified from near threatened to critically endangered [28]. Major threats include habitat loss and capture for the pet trade, leading to captive breeding being increasingly used as a conservation tool to protect these species [29–33]. One endangered species in which captive breeding programs play a critical role is the Puerto Rican parrot (*Amazona vittata*). This species is endemic to the island of Puerto Rico and underwent a drastic decline during the 20th century due primarily to habitat loss as the island's native forest was converted to agriculture [34]. The periodic threat of hurricanes combined with low population numbers has stalled population growth in the wild [35,36] and potentially perpetuates the genetic bottleneck observed in the species [37].

The Puerto Rican Parrot Recovery Program is an excellent model in which to test the relationship of glucocorticoids to parental care and reproductive success in captivity. The program currently consists of two captive populations (Rio Abajo and El Yunque) in different locations on the island that are closely monitored with good record-keeping practices. Climate and husbandry methods differ between each population. The urgent need for the establishment of wild populations in historical habitat demands high productivity of individuals from the captive populations for release. Birds are reintroduced at both wild populations' sites at least once a year, with the number of released birds varying among releases depending on the yearly production of the captive populations. Captive breeding is an essential part of the plan to save this species, but no study to date has investigated the relationship between glucocorticoid levels and reproductive success in this, or any other, captive breeding program for parrots.

In this study, we validate a commercial corticosterone assay and use it to examine fecal glucocorticoid metabolite levels in both captive populations of Puerto Rican parrot in 2017 and 2018 and relate these measures to reproductive success of captive individuals. Assessment of fecal glucocorticoid metabolites is non-invasive and can provide a broader picture of general circulating levels of glucocorticoids in animals [38]. We predicted that high glucocorticoid metabolite levels during the pre-breeding season and the beginning of the breeding season would have a negative relationship to reproductive success. If so, we expected to see females with lower levels of fecal glucocorticoid metabolites during the pre-breeding season produce more fertile eggs and males with lower levels of fecal glucocorticoid metabolites during the breeding season produce more chicks and fledglings. Additionally, we were provided with an opportunity to test for variation of glucocorticoid metabolites of captive individuals before and after a major environmental perturbance in the form of a hurricane. We also explore the effects of Hurricane Maria, a Category 5 hurricane that caused extensive damage to local forests and impacted the captive breeding facilities and their staff, on fecal glucocorticoid metabolites levels before and during the following breeding season, while also testing for differences among season, populations, and sexes.

2. Materials and Methods

The Puerto Rican parrot has a monogamous mating system with biparental care in which only the females incubate, and the male provides food to the female and the chicks. Chicks hatch asynchronously, and once the youngest can thermoregulate (at approximately 14 days post-hatch), the female joins the male in foraging for food for the chicks. In captivity, first eggs are laid from the end of January to the beginning of February. Hatching time is on average 26 days after egg laying and a range of 55–75 days until chicks fledge from the nest [34]. Clutch size and the number of chicks that fledge from nests vary among mated pairs, but typically 3 eggs are laid per pair each year in a single clutch [34]. The production of fledglings varies with population. Fledgling production from 2015 to 2018 in the Rio Abajo captive population was 1.02 per pair, and in the Rio Abajo wild population was 1.29 per pair. In the El Yunque captive population fledgling production was 0.71 per pair. The El Yunque wild population was extirpated by Hurricane Maria in 2017 but releases have

been ongoing since summer 2019. The birds produced in captivity are used for eventual reintroductions into the wild or as new breeding individuals in the captive populations.

Each captive population houses around 220 individuals in outside cages with yearly fluctuations due to births, deaths, translocations, and reintroductions. Each year there are at least 80 to 120 individuals that form mated pairs at each captive population. Housing conditions and daily care routines differ somewhat between the two captive populations. At Rio Abajo, the captive breeding season starts between January 15–25 when the breeding pairs are each placed in their own breeding cages and ends when the last chicks fledge in July-August. During the breeding season at Rio Abajo, personnel enters the breeding areas in the morning to feed the birds and in the afternoon to collect food dishes. All nests are inspected on Monday mornings by personnel, with secondary nest checks done on some nests on Thursday or Friday mornings. After all the chicks have fledged from the nest, mated pairs are placed in retention cages in which some pairs are maintained together in the same cage and other pairs are separated with males and females placed in individual cages next to each other. All pair members at Rio Abajo remain in these cages until the beginning of the next breeding season.

At El Yunque, the captive breeding season also starts between January 15–25 and ends when the last chick fledges from the nest in July-August. El Yunque personnel feed the birds in the morning and nest checks are done throughout the week at variable times, with personnel potentially entering breeding areas multiple times during a day. At the end of the breeding season, the nest entrance is closed until the initiation of the next breeding season and pairs remain in these breeding cages year-round except for a week-long period, during which their cage is washed and prepared for the next breeding season. The activity of cleaning the breeding cages can occur at any moment between August to December.

2.1. Ethical Considerations

The Puerto Rican parrot is classified as critically endangered by the IUCN Red List [28]. This status imposes particular ethical considerations regarding the type of handling, sampling, and experimental manipulations that might be conducted in a study of this type. For example, frequent bleeding for measurement of circulating hormone levels or experimental manipulation of the HPA axis via adrenocorticotropic hormone or dexamethasone challenges, are considered overly invasive and are not permitted by the two managing authorities for this species (Puerto Rico Department of Natural and Environmental Resources and United States Fish and Wildlife Service). To minimize disruption to reproductive efforts in this species we primarily used non-invasive sampling of fecal glucocorticoids metabolites collected as part of the regular daily care routine already established at the two breeding facilities. This study was conducted under NMSU IACUC protocols 2014-030 and 2021-014, approved by the Puerto Rican Parrot Recovery Program Interagency Operational Team, supported by the management under the Puerto Rico Department of Natural and Environmental Resources and conducted under the United States Fish and Wildlife Service Permit TE125521-4.

2.2. Reproductive Information

We collected reproductive information for the pairs that were included in our study from captive population managers. This information included the production of total eggs, fertile eggs, first egg fertility, chicks, and fledglings.

2.3. Pair Selection and Breeding Stages

For this study, we randomly selected 46 pairs from all the breeding pairs in the two captive populations as follows: 21 pairs for the 2017 breeding season (Rio Abajo = 12 and El Yunque = 9), 12 for 2018 breeding season (Rio Abajo) and 13 for the intervening pre-breeding season of 2018 (Rio Abajo). We resampled 10 pairs from 2017 in the 2018 breeding seasons. At the Rio Abajo captive population, the breeding areas are clusters of breeding pairs, and each area is located in a different section of the facility. To minimize the

potential effect of breeding area the pairs selected were placed in Area I. At the El Yunque captive population, all breeding cages are in close proximity compared to the structure used at the Rio Abajo captive population, for this reason, there was no potential effect of area. We define the breeding season for the captive population as the period of the year from January when pairs are placed in their breeding cage (Rio Abajo) or when the nest box is opened (El Yunque) up to the date the last chick in the population fledged. We define pre-breeding as the time before pairs are placed in their respective breeding cages (November to December in both populations); during this period, it is common to see birds showing pair-bonding behaviors (synchronized flights, duets, allopreening, copulations, allofeeding) and territorial displays.

2.4. Assay Validation

We used the DetectX Corticosterone Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor MI, USA) to measure fecal glucocorticoids. To validate this assay for the measurement of corticosterone in plasma and fecal glucocorticoids in the Puerto Rican parrot, we conducted a small study with a limited subset of birds and under supervision of a veterinarian. We employed two different manipulations that were each anticipated to be stressful to the birds and collected blood and fecal samples at regular intervals after each to determine whether we could detect a rise in plasma corticosterone and fecal glucocorticoids following these stressful events. The first manipulation was to capture birds from a group flight cage and transfer them to individual cages, with subsequent collection of fecal samples to monitor fecal glucocorticoids. The second manipulation was capture and immediate blood collection followed by 30 min of restraint and then a second blood collection. Individuals were returned to cages and additional fecal samples were collected following blood collection. This manipulation allowed us to compare baseline and stress response circulating corticosterone to fecal glucocorticoid metabolites measured before and after the stressful event. Assessment of circulating corticosterone and levels to which it elevates with handling were key to assessing later secretion of glucocorticoids into fecal material and to confirm that general handling procedures are seen as stressful to this species.

To conduct our validation, we selected twelve birds from the captive population at Rio Abajo, six males and six females. All birds were two years old at the time of the study. For the first manipulation, we captured individuals with a large butterfly net from their standard group housing in a large flight cage and placed them into the smaller cages for fecal sampling. This method of capture, while standard in the facility, is thought to be stressful for the birds as it involves chasing them with the net, during which time they often produce alarm calls. We captured birds at 7:00 and then collected fecal samples every 2–3 h on the day of capture and the following 4 days to assess changes in fecal glucocorticoid levels after this putatively stressful event. For this study, we only used the day of capture and the following day after capture. If this event caused an increase in circulating glucocorticoids, as anticipated, then we also expected to see a transitory rise in fecal glucocorticoids later on the day of capture compared to samples collected in following days.

For the second part of the validation, we collected blood samples and performed a capture restraint on the same 12 birds after two weeks of individual housing in the same cages. Approximately 0.3 cc of whole blood was collected from the jugular vein from each bird between 8:00 am and 9:30 am. Samples were collected in under 3 min from approaching each cage, and again after 30 min of restraint in a small wooden box to measure baseline and stress-response circulating corticosterone. The blood samples were centrifuged, and plasma extracted and frozen immediately. We collected fecal samples for our baseline values before the restraint protocol was conducted. We then continued to collect fecal samples from birds for the remainder of the day after blood samples were collected. For the stress response analysis, we used from each individual the fecal sample with the highest value after restraint collected between 13:30 to 18:00.

2.5. Fecal Sampling and Analysis

For our main study, we collected fecal samples from males in both the breeding and pre-breeding seasons and from females during the pre-breeding season only. Pre-breeding (November to January) samples were collected only at Rio Abajo where the members of each pair are separated, which allowed us to distinguish which individual produced each sample. Breeding season samples were collected from males in both populations due to their presence outside nesting cavities while females incubate. To collect the fecal samples, we placed a clean PVC plank under a perch where individuals roosted at night after sunset between 7:30 to 8:00 PM and collected the plank before the following sunrise between 5:30 to 6:00 AM. The droppings collected at sunrise were thus collected no more than 10 h after defecation and when no rainfall occurred overnight. A study examining the effects of environmental changes on fecal glucocorticoid metabolites found no effect of room temperature for 12h on fecal glucocorticoid metabolite concentration but it did find effects of rainfall on the concentration of fecal glucocorticoid metabolites [39]. Samples were collected in a 2ml microcentrifuge tube and then stored at −20 °C until drying and analysis.

Fecal samples were dried using a gravity convection oven (Fisher Scientific, No: 3511FS) preheated to 90 °C, the samples remained in the oven for at least 2 h until fully desiccated. A study examining the effect of this procedure on glucocorticoid metabolites levels found that samples that are frozen and then dried in conventional ovens do not change significantly in glucocorticoid metabolite concentration [40]. After drying the samples were pulverized and centrifuged, then stored at −20 °C until analysis. Rainfall and small amounts of fecal material in the samples of some individuals, lead to some individuals having low sample collection during the pre-breeding season of 2018. We randomly selected 3 samples per individual during the pre-breeding and a range of 3 to 15 samples per individual during the breeding seasons that had ≥0.2 g of dry feces to measure glucocorticoids metabolites. We extracted glucocorticoid metabolites from fecal samples following the DetectX Steroid Extraction Protocol (Arbor Assay), after drying, 2 mL of ethanol was added to the solid to initiate the extraction of the glucocorticoids. We did minor adjustments to the centrifugation step during which extracted samples were centrifuged at 4000 rpm for 20 min. We used a SpeedVac (Eppendorf) centrifuge to dry down extracted samples, after which samples were stored at −20° until they were assayed (within 24 h), sample recovery was assumed to be 100% in subsequent analyses. We adjusted the kit recommended reconstitution procedure slightly and dissolved extracted samples in 50 µL of ethanol followed by 600 µL of Assay Buffer. Fecal glucocorticoid metabolite levels were then analyzed using the DetectX® Corticosterone Enzyme Immunoassay kit (EIA, Arbor Assays, Inc., #K014-H5). This kit has high affinity for corticosterone (100%), with much lower cross-reactivity for other glucocorticoids and their metabolites (Desoxycorticosterone—12.30%, Tetrahydrocorticosterone—0.76%, Aldosterone—0.62%, Cortisol—0.38%, Progesterone—0.24%, Dexamethasone—0.12%, Corticosterone-21-Hemisuccinate—<0.1%, Cortisone—<0.08%, Estradiol—<0.08%); since in fecal samples there is low presence of corticosterone, we report on glucocorticoid metabolites as opposed to corticosterone.

2.6. Statistical Analysis

Since we analyzed samples in multiple plates and years we corrected our glucocorticoid estimates using a correction factor applied to all samples to account for inter-assay variation that could be linked to variation in kits or lab conditions as glucocorticoid analyses spanned across years [as in 41]. We used the estimated levels of glucocorticoid metabolites from the fourth point of the standard curve as a basis for our correction factor. We used the grand mean for all point 4 data points from all standard curves and divided this by the mean point 4 standard curve point for each individual plate to develop a correction factor [41–47]. We then multiplied each hormone value on each plate by the corresponding correction factor. Inter-assay variation was measured as the coefficient of variation (N = 21, Mean = 1241.44, SD = 131.78, CV = 0.106 equaling an inter-assay variation of 10.6%, intra-assay variation range = 3.22–9.09%). The adjusted glucocorticoid values were then

log-transformed for subsequent analyses. For all analyses, we used the mean glucocorticoid values obtained for each individual across all measures within a season.

From the full set of data available, we used a subset to address different questions depending on the data available. We used twelve males for the 2017 and 2018 breeding seasons, and ten for the 2018 pre-breeding from the Rio Abajo captive population. From the Rio Abajo captive population, nine males had data across all seasons, two males had data only for the 2017 breeding season, only one male had data for pre-breeding and breeding season of 2018. We had data from nine males for 2017 breeding season from the El Yunque captive population. For females, we had data from only eleven females from the Rio Abajo captive population from the 2018-pre-breeding season. After a Shapiro Wilk Test we Log transform our glucocorticoid values for all the analyses. For the determination of the relation of reproductive success measures and glucocorticoid metabolite levels at each season, we used only males (see Table 1) from the Rio Abajo captive population and performed a Spearman correlation analysis. To compare glucocorticoid metabolite levels between the pre-breeding and two breeding seasons we used an ANOVA followed by Tukey post hoc tests using only the males (14) from the Rio Abajo captive population because this was the only population sampled over all three seasons. Since the breeding season of 2017 occurred before Hurricane Maria and the breeding season of 2018 occurred after, we used the results from the previous analysis to compare levels before and after Hurricane Maria. We performed a paired *t*-test to investigate differences among males (10) and females (11) from the Rio Abajo captive population during the pre-breeding season. For population differences in glucocorticoid metabolite levels, we compared the data from the breeding season of 2017 of only males (21) from each population using a paired *t*-test. We used a logistic regression to determine the relationship of glucocorticoids metabolite levels during the pre-breeding to first egg fertility. Statistical analysis was performed using the JMP statistical software package, version 14.0.0 (SAS Institute, Inc. 2018, Cary, NC, USA).

Table 1. Relationship between male fecal glucocorticoid metabolite levels and reproductive success variables †.

Reproductive Success	Season	*n*	r	*p*
Total eggs	Breeding 2017	12	0.2587	0.4169
	Pre-breeding 2018	10	0.4411	0.2019
	Breeding 2018	12	−0.7255	**0.0076**
Fertile eggs	Breeding 2017	12	−0.0431	0.8942
	Pre-breeding 2018	10	−0.0187	0.9591
	Breeding 2018	12	−0.6538	**0.0211**
Chicks	Breeding 2017	12	−0.4139	0.1810
	Pre-breeding 2018	10	0.0781	0.8301
	Breeding 2018	12	−0.3897	0.2105
Fledglings	Breeding 2017	12	−0.4193	0.1810
	Pre-breeding 2018	10	0.0855	0.8143
	Breeding 2018	12	−0.3528	0.2607

† All tests Spearman correlations.

3. Results

3.1. Validation of Corticosterone Assay

In the first potentially stressful event of our validation study (capture and placement in cages), we saw a sharp but transitory rise in estimated fecal glucocorticoid metabolite levels in the 12 birds following initial capture from group housing and movement to individual housing. Although we were not able to obtain fecal samples from all individuals immediately upon capture, all samples collected in the 6 h following capture had estimated fecal glucocorticoid metabolite levels under 20,000 pg/mg (Figure 1a). After 6 h there was a general rise in CORT levels that persisted for 30 h after capture, at which time

levels decreased (Figure 1a). In our second stressful event (capture and restraint) we saw a significant rise in circulating plasma CORT from baseline to stress-response levels following 30 min of a standard capture restraint protocol for the whole group (Figure 1b, paired *t*-test, df = 20.12, t = 5.92, $p = 0.001$). When we compared levels based on sex, we found that at time zero there was no difference in plasma glucocorticoid levels between the sexes (paired *t*-test, df = 7.41, t = 0.02, $p = 0.987$) but after restraint males showed higher plasma glucocorticoid levels than females (paired *t*-test, df = 9.77, t = 2.76, $p = 0.021$). Both sexes demonstrated a restraint response with an increase of plasma glucocorticoids (paired *t*-test males, df = 11.04, t = 5.59, $p = 0.0002$ and for females df = 4.93, t = 2.86, $p = 0.036$). There was a concomitant rise in estimated fecal glucocorticoid levels in fecal samples collected on the same day before and after the capture restraint (Figure 1c, paired *t*-test, df = 13.09, t = 4.76, $p = 0.0004$). Comparing the sexes, both sexes demonstrated a significant increment of fecal glucocorticoids after the restraint protocol (paired *t*-test for males, df = 5.89, t = 3.48, $p = 0.0136$ and for females df = 5.47, t = 6.59, $p = 0.0009$). Before the restraint protocol females had higher fecal glucocorticoid levels than males (paired *t*-test, df = 10.00, t = −3.82, $p = 0.003$) but there was no significant difference between the sexes after the restraint protocol (paired *t*-test, df = 6.83, t = −1.46, $p = 0.1998$). As a final assessment concerning the ability of this assay to detect fecal glucocorticoids in this species, we used extra, mixed fecal samples from individuals that we then measured as split into two aliquots ($n = 10$). These split aliquots were analyzed on the same plate with final calculated levels of fecal glucocorticoid metabolites compared for similarity. Levels from split samples were highly correlated, supporting the reproducibility of this assay and its ability to detect fecal glucocorticoid metabolites for this species (Pearson Correlation, r = 0.963, $n = 10$, $p < 0.0001$). These results validate that the Arbor Assay Detect-X ELISA Assay is effective in detecting both CORT in plasma and feces of this species. No birds were harmed during this procedure and veterinary monitoring detected no adverse impacts on their health.

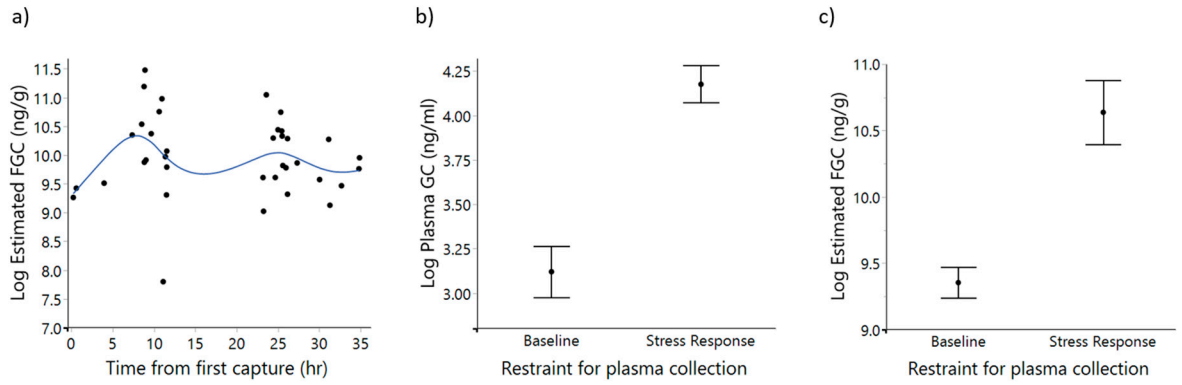

Figure 1. Glucocorticoids levels in fecal and plasma samples. A total of 12 individuals were used for this test with 6 males and 6 females. (**a**) Fecal glucocorticoids fluctuation same day of capture up to 40 h after first capture. Multiple samples per individual, with a total of 37 samples. (**b**) Plasma glucocorticoids levels before and after bleeding, $n = 12$, 6 males and 6 females. Figure shows means ± SE. (**c**) Fecal glucocorticoids levels before and after restraining for blood capture, $n = 12$, 6 males and 6 females. Figure shows means ± SE.

3.2. Glucocorticoid Metabolite Levels and Measures of Reproductive Success

We assessed how male fecal glucocorticoid metabolite levels in different seasons related to reproductive success. For the 2018 breeding season we found a significant relationship between fecal glucocorticoid metabolite levels in males and measures of reproductive success in the egg stage (total eggs and fertile eggs), but not at the post-egg stage (chicks and fledglings) (Table 1). Fecal glucocorticoid metabolite levels had a negative relationship with both the number of eggs laid and the number of fertile eggs (Figure 2). In contrast, we did not find any relationship between male fecal glucocorticoid metabolite

levels during either the 2017 breeding season or the pre-breeding season of 2018 with measurements of reproductive success at the egg stage (total eggs laid, number of fertile eggs) or chick stage (number of chicks hatched, number of fledglings).

In females, fecal glucocorticoid metabolite levels during the pre-breeding stage of 2018 were positively related to the total number of eggs laid but were not related to the number of fertile eggs, chicks produced, or fledglings (Table 2, Figure 2). There was no significant relationship between fecal glucocorticoids metabolite levels of either males or females during the pre-breeding stage in 2018 to the fertility of the first egg (Female df = 1, $r^2 = 0.1748$, $X^2 = 2.1361$, $p = 0.1439$; Male, df = 1, $r^2 = 0.0727$, $X^2 = 0.6927$, $p = 0.4052$).

Table 2. Relationship between fecal glucocorticoid metabolite levels in females during the pre-breeding season and reproductive success variables †.

Reproductive Success	*n*	r	*p*
Total eggs	11	0.6404	**0.0338**
Fertile eggs	11	0.3202	0.3371
Chicks	11	0.2741	0.4147
Fledglings	11	0.2513	0.4561

† All tests Spearman correlations.

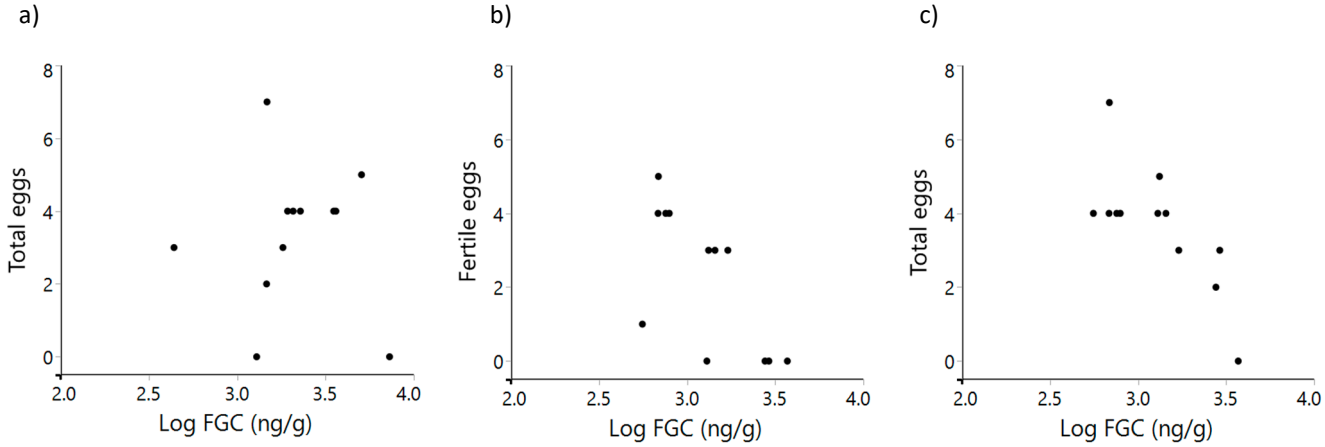

Figure 2. Significant relationships of fecal glucocorticoid metabolites to reproductive success measures. (**a**) Females showed a positive relationship of fecal glucocorticoid metabolites during the pre-breeding to the total eggs laid by female in the following breeding season (2018). Males have a negative relationship of fecal glucocorticoid metabolites to (**b**) total eggs and (**c**) total fertile eggs laid in the following breeding season (2018).

3.3. Hurricane Maria and Seasonal Variation

For the Rio Abajo captive population, we did not identify differences between the 2017 and 2018 breeding seasons (d.f = 16.53, t = −1.018, $p = 0.323$), which represented pre- and post-Hurricane Maria, respectively (Figure 3). We found a difference in male fecal glucocorticoid metabolite levels between seasons (df = 2, F = 7.015, $p = 0.003$; Figure 3). Here, male birds had higher fecal glucocorticoid metabolite levels during the pre-breeding season of 2018 (N = 10, mean ± SE = 3.83 ± 0.15 ng/g) compared to the breeding season of 2017 (N = 12, mean ± SE = 3.28 ± 0.13 ng/mL) and the breeding season of 2018 (N = 12, mean ± SE = 3.108 ± 0.13 ng/mL). After a Tukey HSD post-hoc comparison, pre-breeding levels in 2018 were significantly different from the 2017 breeding season (df = 12.821, t = −3.795, $p = 0.0023$), as well as the 2018 breeding season (df = 19.144, t = 2.354, $p = 0.029$).

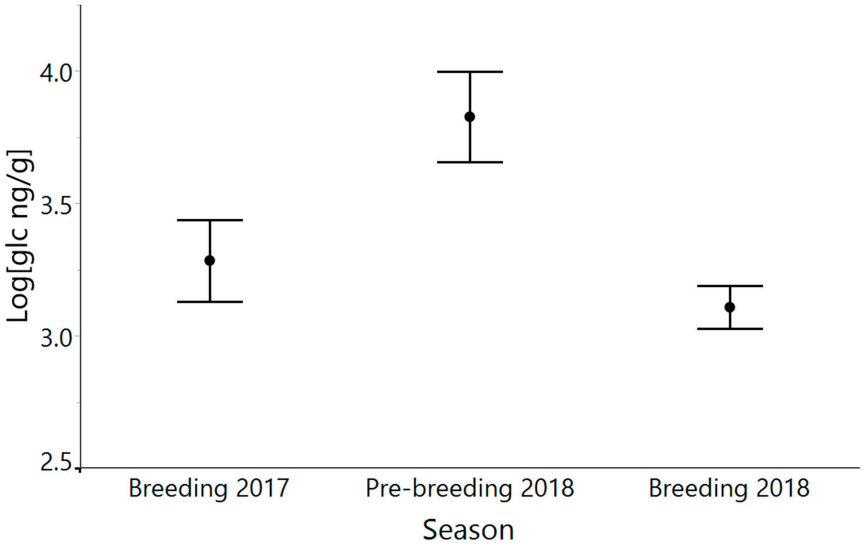

Figure 3. Seasonal variation in fecal glucocorticoid metabolite of males at Rio Abajo. During pre-breeding 2018 fecal glucocorticoid metabolites were higher relative to both the 2017 and 2018 breeding seasons, which did not differ from each other. The 2017 breeding season occurred prior to Hurricane Maria while the pre-breeding and breeding season of 2018 occurred after Hurricane Maria. Graphs show means ± SE.

3.4. Glucocorticoid Metabolite Levels Differed between Sexes and Populations

We found that the fecal glucocorticoid metabolites ranged from 10.988 to 133.750 ng/g for males and 14.057 to 40.606 ng/g for females. We compared pre-breeding season fecal glucocorticoid metabolite levels in males and females and found that males had higher levels (mean ± SE = 3.827 ± 0.17 log ng/g) than females (mean ± SE = 3.284 ± 0.86 log ng/g; df = 1, t = 2.836, p = 0.00138; Figure 4). In addition, we found a trend towards males' glucocorticoid levels differing between populations during the 2017 breeding season (Figure 5). Males at the Rio Abajo captive population had lower glucocorticoid metabolite levels (mean ± SE = 3.285 ± 0.15 log ng/g) than males at the El Yunque captive population (mean ± SE = 3.647 ± 0.11 log ng/g), but this trend was not statistically significant (df = 1, t = −1.895, p = 0.0737).

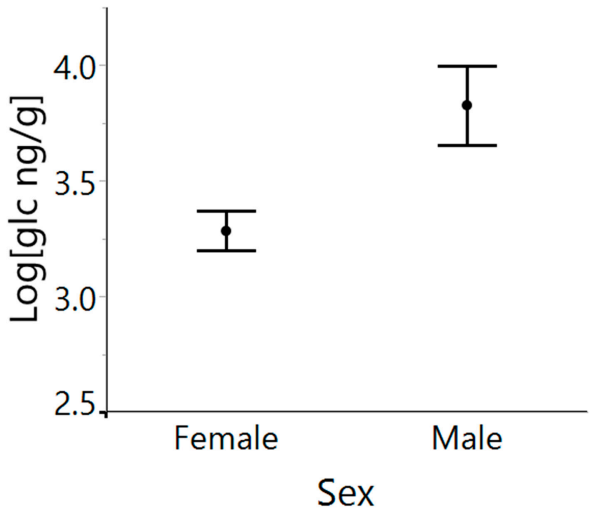

Figure 4. Sex difference in fecal glucocorticoid metabolites during the pre-breeding 2017–2018. Males had higher fecal glucocorticoid metabolite levels than females during the pre-breeding season. These samples include individuals from Rio Abajo captive populations (Rio Abajo males = 10, Rio Abajo females = 11). Graphs shows means ± SE.

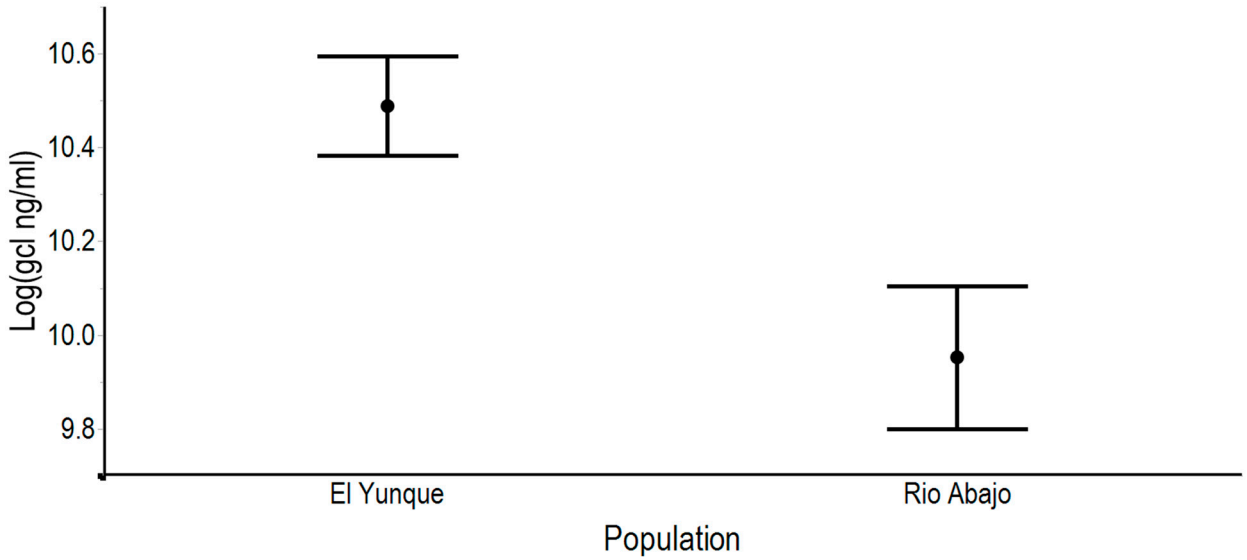

Figure 5. Populations comparison in fecal glucocorticoid metabolites between males in the Rio Abajo (12) and El Yunque (9) captive populations during the 2017 breeding season. Graphs show means ± SE.

4. Discussion

Captive breeding can be an important tool for many conservation efforts. Understanding how a threatened species is affected by captive conditions is critical for conservation success [16]. We evaluated fecal glucocorticoid metabolite levels of captive populations of Puerto Rican parrots using a newly validated commercial corticosterone assay and related this to reproductive success over two breeding seasons and the intervening non-breeding season. We expected to see a negative relationship between the reproductive success of captive breeding pairs and fecal glucocorticoid metabolite levels of males and females during pre-breeding, and glucocorticoid metabolite levels of males in the breeding seasons. Glucocorticoids have been shown to negatively impact reproductive behavior and success in other avian species [8]. Our results showed a negative relationship of male fecal glucocorticoid metabolite levels to total eggs and fertile eggs during the breeding season of 2018 (Figure 2), while female fecal glucocorticoid metabolite levels during the pre-breeding season of 2018 showed a positive relationship to total eggs laid. We also found differences in fecal glucocorticoid metabolite levels between the sexes and seasonal variation in males. Below we discuss the implications of these results and put them in the context of similar work on the physiology of captive populations.

4.1. Fecal Glucocorticoid Metabolite Levels and Reproductive Success

Previous research has found that high levels of glucocorticoids can downregulate reproductive efforts [8]. In a previous study of captive breeding Puerto Rican parrots [48] visual inspection of adrenal gland and testes showed that in some males the adrenal gland was hypertrophied and the testes were small. It was suggested that these findings could be due to high levels of stress [48], however, circulating or excreted glucocorticoids were not assessed. Here we showed that a standard capture restraint protocol produced a rise in circulating corticosterone that was mirrored by a rise in fecal glucocorticoid metabolites. Using this validated assay, we found that male fecal glucocorticoid metabolites during the breeding season of 2018 had a negative relationship to total eggs and fertility of eggs providing support to the previous study's findings related to the testes [48]. For females during the pre-breeding season of 2018, we found a positive relationship of fecal glucocorticoid metabolites levels with total eggs laid. However, our results, for either sex, do not show any relation of fecal glucocorticoid metabolites to the number of chicks and fledglings produced.

In males, increases in glucocorticoids have been documented to suppress production of testosterone [49–51] and luteinizing hormone [49]. Suppression of testosterone and luteinizing hormone can negatively affect reproductive behaviors. On the other hand, in a study of wild populations of house wrens (*Troglodytes aedon*), females whose glucocorticoids levels were artificially elevated before breeding produced heavier eggs and had higher nestling feeding rates, significantly increasing chick body mass [52]. Elevated glucocorticoids levels in females may mobilize energy to prepare for the breeding season. Together, these results suggest that, in some species, males and females are differently impacted by changes in glucocorticoid levels.

The relationship between reproductive success and glucocorticoids may also be affected by a species' natural history [1,53]. Short live species may have mechanisms that suppress the effects of high glucocorticoids while long-lived species may experience selection for survival over reproduction [53]. Like many parrots, the Puerto Rican parrot is a long-lived species and may live for up to 37 years in captivity [54]. Under natural conditions, in long-lived species, individual survival may be prioritized before reproduction and males, in particular, may not have evolved the capacity to modulate the effects of frequent energetic challenges experienced in captivity. On the other hand, females may evolve mechanisms to modulate any negative effects of elevated glucocorticoid just prior to egg-laying [55] and this may be held over into captivity. Additionally, egg production is an energetically demanding process, in which the female must place energetically rich resources into the production of eggs and may use glucocorticoids for the mobilization of that energy. This may explain the positive relationship observed between fecal glucocorticoid metabolite levels in the pre-breeding season and egg production in females seen in this study.

4.2. Seasonal Variation

Many animals have seasonal variation in hormone levels in the wild [53,56–59] but it remains unclear whether that variation is retained under captive conditions. Because of the outdoor housing and seasonal breeding cycle of the Puerto Rican parrot in the captive populations assessed here, we expected to see variation among the breeding and pre-breeding seasons in fecal glucocorticoid metabolite levels. We found that male fecal glucocorticoid metabolite levels in the Puerto Rican parrot were higher during the pre-breeding than in the breeding season.

Seasonal variation has also been documented in captive red-tailed parrots (*Amazona brasiliensis*), with mean fecal glucocorticoid metabolite concentrations highest just before breeding [60]. Research in wild populations of northern cardinals (*Cardinalis cardinalis*) has shown that females and males have higher plasma glucocorticoid corticosterone levels in the pre-breeding compared to the breeding season [47]. In northern cardinals, it was suggested that high glucocorticoid corticosterone levels during the non-breeding season could be an adaptation for energy needed to survive cold conditions. In the Puerto Rican parrot, each pair displays territorial behaviors year-round defending their nesting cavity with a peak in the pre-breeding season which could explain higher fecal glucocorticoid metabolite levels in males at that time [34], elevated plasma glucocorticoids could provide the energy needed for expensive territorial activities in this species.

Puerto Rican parrots at Rio Abajo are housed outdoors and exposed to normal daylight changes, temperature changes and rainfall, and social activity of wild parrots, all of which may affect glucocorticoids levels between seasons [1]. In European starlings (*Sturnus vulgaris*) individuals of both sexes placed in indoor housing experienced delayed reproductive behaviors while outdoor-housed individuals initiated breeding earlier [61]. Pairs of European starlings housed indoors had significantly lower sex steroids and higher expression of gonadotropin inhibitory hormone (GnIH). It is possible that the year-round outdoor housing experienced by these birds results in individuals having hormonal seasonal variation similar to what would be seen in wild individuals.

Alternatively, birds at Rio Abajo are housed in different cages during the pre-breeding versus the breeding season; these conditions may prevent the expression of the full range of natural behaviors during this period and may in turn alter glucocorticoid levels [16,61]. The cages used in the pre-breeding period are smaller than breeding cages and they are placed in closer proximity to other individuals. The proximity of the cages provokes frequent territorial displays (B. Ramos-Guivas, pers obs), potentially at a higher rate than when birds are in their breeding cages. This could result in males having higher glucocorticoid levels during the pre-breeding than during the breeding season. In addition, closer proximity to human activity and capturing for medical antiparasitic treatments (twice over two weeks) during the pre-breeding season may contribute to the seasonal glucocorticoid variations observed.

4.3. Glucocorticoid Levels Do Not Differ between Breeding Seasons, despite Hurricane Maria

The breeding seasons of 2017 and 2018 occurred pre and post-Hurricane Maria. At the Rio Abajo captive population, we found no significant differences in fecal glucocorticoid metabolite levels between the two seasons. Although initially surprising, it is important to note that captive conditions were relatively stable compared to those experienced by wild populations. Many wild populations of animals suffer increased mortality both during a hurricane (due to climatic conditions), and afterward due to increased exposure to predators and reduced food availability [26,62]. Indeed, the wild population of the Puerto Rican parrot at El Yunque, which was only 31 birds before Hurricane Maria, was totally extirpated by the hurricane. New releases started in 2019 to re-establish this wild population. The wild population at Rio Abajo fared somewhat better but is thought to have declined from 134 to 110 birds as a result of direct effect of the hurricane. Secondary effects as scarcity of food and exposure to predators reduced the population even more in the following months after the hurricane. In contrast, the Rio Abajo captive population was sheltered from the direct impacts of the storm in a hurricane shelter and had no changes in food availability.

4.4. Males Show Higher Fecal Glucocorticoid Metabolite Levels than Females

Differences between the physiology of males and females are common, therefore we expected to see a difference in fecal glucocorticoid metabolite levels between males and females during the pre-breeding season even when faced with similar captivity stimuli (variation linked to preparation for breeding). We found that males had significantly higher fecal glucocorticoid metabolite levels than females during the pre-breeding. It could be that lower levels of glucocorticoids in females compared to males represent suppression of glucocorticoid levels during the pre-breeding in preparation for egg-laying [60]. Alternatively, higher energetic demands for males than females during the pre-breeding may be due to increases in courtship or territorial behavior shown by males, which may increase glucocorticoid levels [63,64].

4.5. Differences between Captive Populations

For our study, we collected fecal samples from both extant captive populations during the 2017 breeding season. Because of the difference in location and management techniques we expected to see differences in the glucocorticoid levels between the populations. We found that fecal glucocorticoid metabolite levels among males were somewhat higher at the El Yunque population than at the Rio Abajo population, although the difference was not quite statistically significant. Housing conditions, climate and daily routines differ between the two captive populations. At the El Yunque captive population, the breeding cages are more exposed to personnel activity, so recurrent perturbations by people walking by may stimulate continuous energetic challenges [65]. In addition, the nest checks and daily feeding are not performed in a strict schedule at El Yunque captive population adding an element of unpredictability for individuals. Recurrent intrusion to breeding areas can maintain glucocorticoids levels above baseline, eventually impacting reproductive

success [65]. At the Rio Abajo captive population, the breeding cages are located behind vegetation that may serve as a screen from personnel, potentially reducing glucocorticoid levels. Another difference between the populations is the daily feeding routine. At El Yunque, feeding time lasts longer than at Rio Abajo. The longer presence of personnel inside the breeding areas may increase baseline glucocorticoids levels. High levels of glucocorticoids during the breeding season could negatively affect reproduction.

5. Conclusions

Fecal glucocorticoid metabolite analysis provides an opportunity to study species in a minimally invasive fashion. Our results provide insight into the relationship of fecal glucocorticoids metabolites to reproductive success under normal daily routines in captivity. Although it has been reported in previous studies that captive individuals have a more attenuated response to energetic challenges than wild individuals [17,66,67], captive individuals can still be affected by high glucocorticoids levels [68–70]. The negative relationship of fecal glucocorticoid metabolite levels in males to total eggs laid and total fertile eggs may indicate a susceptibility to anthropogenic activities. For endangered species programs, strategies should be developed to reduce perturbations and regularize their occurrence during key periods of the year to minimize the potential negative effects on reproduction. The differences between populations in glucocorticoid levels suggest that managers of captive breeding species should carefully evaluate factors that may be driving these differences (e.g., housing placement and the frequency and predictability of anthropogenic activities during critical stages of the breeding season). Future research should examine the role of glucocorticoids in reproduction of captive populations of other endangered species, and where possible, compared to wild populations of these species.

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