

## RESEARCH PAPER

# Ontogeny of the adrenocortical response in an extremely altricial bird

Karl S. Berg<sup>1</sup>  | Soraya Delgado<sup>1</sup> | Astolfo Mata-Betancourt<sup>2</sup> | Jesse S. Krause<sup>3</sup> | John C. Wingfield<sup>4</sup> | Steven R. Beissinger<sup>5</sup>

<sup>1</sup>Department of Biology, University of Texas Rio Grande Valley, Brownsville, Texas

<sup>2</sup>Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela

<sup>3</sup>Department of Biology, University of Nevada, Reno, Nevada

<sup>4</sup>Department of Neurobiology, Physiology & Behavior, University of California, Davis, California

<sup>5</sup>Environmental Science, Policy & Management, University of California, Berkeley, California

## Correspondence

Karl S. Berg, Department of Biology, University of Texas Rio Grande Valley, One West University Blvd, Brownsville, TX 78520. Email: karl.berg@utrgv.edu

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## Abstract

Life history theory predicts that physiological and behavioral responsiveness to stress should be delayed in development until the benefits of heightened reactivity outweigh the costs of potentially chronic glucocorticoid levels. Birds often acquire stress-responsiveness at locomotor independence, however, both stress-responsiveness and locomotor ability are delayed in birds with altricial developmental strategies. Parrots (Psittacidae) are extremely altricial, but it is not known whether they also postpone physiological responsiveness to stress until locomotor independence. We quantified individual variation in baseline and stress-induced plasma corticosterone (CORT) concentrations, the main avian glucocorticoid, in wild green-rumped parrotlets (*Forpus passerinus*) of Venezuela at four stages of nestling development. Parrotlet neonates are very underdeveloped and compete for parental care among extreme sibling size hierarchies, a competitive scenario that might benefit from early hypothalamic–pituitary–adrenal (HPA) functionality. Nestlings that underwent a standardized restraint stress-treatment showed higher average CORT concentrations compared to baseline in all age groups sampled, and exhibited no evidence of age-related changes in the stress response. This is 2 weeks before locomotor independence and earlier than previously documented for altricial species. Results suggest that precocity of HPA function may be advantageous to growth and survivorship in extremely altricial birds.

## KEYWORDS

adrenocortical, altricial, birds, corticosterone, glucocorticoid, hypothalamic–pituitary–adrenal axis, ontogeny, parrots, stress, Venezuela

## 1 | INTRODUCTION

Production of glucocorticoids by the adrenal glands is important for numerous metabolic processes (e.g., glucose metabolism and homeostasis), and provides individuals with the behavioral flexibility to confront unpredictable situations by responding in ecologically and socially appropriate ways (Korte, Koolhaas, Wingfield, & McEwen, 2005; Romero & Wingfield, 2016; Wingfield et al., 1998). In birds, the adult adrenocortical response to perturbations of the environment

can affect parental care, buffering offspring from adverse ecological (e.g., predation, starvation, and dehydration) and social (e.g., intraspecific aggression, sibling competition, and social foraging) conditions until they mature and can meet such challenges independently of parents (Romero & Wingfield, 2016). Inherent in this buffering is the avoidance of chronic stress of the progeny, which can suppress the developing endocrine system's ability to down-regulate glucocorticoid levels after otherwise adaptive responses (Jenkins & Porter, 2004). Unchecked glucocorticoid production

during early development has well-documented, deleterious, and sometimes permanent effects on developing neuro-immuno-endocrine axes (Apanius, 1998; McEwen, 2012; Wada, 2008). Dysregulation of glucocorticoid levels can impact bone growth and maintenance (Rath, Huff, Huff, & Balog, 2000) and metabolic control with respect to lipid and glucocorticoid metabolism (Landys, Ramenofsky, & Wingfield, 2006). Thus, there are good reasons why selection may favor parental care strategies that lessen the likelihood of deleterious glucocorticoid production in neonates.

One proposed developmental strategy for avoiding the negative effects of unchecked glucocorticoid production in juveniles is to delay maturation of hypothalamic-pituitary-adrenal (HPA) functionality until the time of locomotor independence when individuals could, for example, plausibly escape a predation attempt or find food independently of adult caregivers. Precocial species, which obtain early locomotor independence, show HPA maturation at an earlier life history stage than altricial species (Wada, 2008). Precocial birds hatch with relatively well-formed central nervous systems, and attain thermoregulatory, locomotor, and foraging capabilities within hours or days (Starck & Ricklefs, 1998). They also show an adult-like adrenocortical response during the late embryonic stage (Jenkins & Porter, 2004; Wise & Frye, 1973). In contrast, altricial songbirds (passerines) hatch naked, deaf, and blind; are often confined to a nest structure; and do not attain locomotor or thermoregulatory ability for a week or more after hatching. They also undergo more brain growth compared to precocial birds (Iwaniuk & Nelson, 2003; Ricklefs & Starck 1998). As a result, the nestling period for altricial birds often involves sensitive periods in development when the greatest organizational changes in brain and behavior are likely to occur (Adkins-Regan, 2005; Hogan & Bolhuis, 2005; Nowicki, Searcy, & Peters, 2002; Wada, Hahn, & Breuner, 2007; Will et al., 2014). Altricial species are predicted to delay maturation of HPA functionality until the benefits of reactivity outweigh the risks of higher, potentially deleterious glucocorticoid levels (Blas, Baos, Bortolotti, Marchant, & Hiraldo, 2005; Rensel & Schoech, 2011; Schwabl, 1999; Sims & Holberton, 2000; Wada, 2008). In support of this idea, known as the Developmental Hypothesis, altricial nestling songbirds show increasing HPA responsiveness with age, and delay attainment of adult-like HPA sensitivity until around the time of fledging (Blas et al., 2005; Rensel & Schoech, 2011; Schwabl, 1999; Wada et al., 2007) or nutritional independence (Sims & Holberton, 2000). Given the potential for HPA feedback on developing phenotypes, determining when during development HPA becomes functional in lineages with diverse developmental strategies may provide unique insights into the evolution of altriciality.

Parrots (Psittacidae) are very altricial birds, and are closely related to songbirds (Hackett et al., 2008; Suh et al., 2011), but little is known about endocrine function in free-ranging populations. There are good reasons to think parrots might differ in how the endocrine system mediates environmental conditions with internal physiology. Parrots are highly social and develop some the

most complex of vocal learning abilities (Petkov & Jarvis, 2012), an area of behavioral endocrinology extensively studied in songbirds (Ball & Balthazart, 2010). Parrots undergo a disproportionate amount of brain growth during their protracted nestling period (Iwaniuk & Nelson, 2003; Pacheco, Beissinger, & Bosque, 2010; Ricklefs & Starck 1998), which increases the time period during which developing systems are vulnerable to neuroendocrine-environment feedback processes (Eyck, Buchanan, Crino, & Jessop, 2019). Development is further delayed owing to asynchronous hatching (Krebs, 1999; Stamps, Clark, Arrowood, & Kus, 1985; Stoleson & Beissinger, 1997), which in large clutches results in extreme age and size hierarchies within broods. Size hierarchies select for complex patterns of nutritional provisioning by adult caregivers, in which underdeveloped neonates must compete for food with considerably older, larger, and more experienced siblings (Budden & Beissinger, 2009; Krebs, 2001; Krebs & Magrath, 2000; Stamps et al., 1985). Maternal deposition of corticosterone (CORT) in egg yolks reinforces the competitive effects of the age hierarchy in cockatiels (*Nymphicus hollandicus*; Kozłowski & Ricklefs, 2010). In parrot species studied to date, female parents ameliorate effects of the age hierarchy by biasing resources to younger hatchlings (Budden & Beissinger, 2009; Krebs & Magrath, 2000; Stamps et al., 1985). However, developmental endocrinology has been little studied in extremely altricial species (Adkins-Regan, 2005), and it is not clear when during ontogeny juveniles begin to regulate their own responsiveness to environmental stress.

Here we report on the ontogeny of adrenocortical response to a standardized restraint stressor in free-ranging green-rumped parrotlets (*Forpus passerinus*) of Venezuela. Parrotlets are small (25–30 g), granivorous birds and are extremely altricial (Waltman & Beissinger, 1992). Neonates hatch naked with fused eyelids and middle ear canals, and remain so for 7–10 days, the age at which similar-sized altricial white-crowned sparrows (*Zonotrichia leucophrys*) fledge from their nest (Wada et al., 2007). Parrotlet nestlings grow slowly, become endothermic at 3 weeks of age (Pacheco et al., 2010), and fledge at approximately 30 days posthatching (dph; Stoleson & Beissinger, 1997). Given that adrenocortical maturation appears around the time of fledging in altricial songbirds (Schwabl, 1999), an adult-like adrenocortical response might be similarly postponed in parrotlets until the fledging stage (i.e., 28–35 dph). On the other hand, nestling parrotlets engage in competitive begging contests amidst extreme size hierarchies (Budden & Beissinger, 2009), in which case early adrenocortical maturation could be adaptive. While altricial songbird siblings also compete for parental care, age hierarchies, if they exist, are often on the order of hours or days and are the result of competitive advantages, whereas parrotlet broods range in age from 1 to 3 weeks, resulting in up to an order of magnitude differences in body mass that are not the direct result of sibling competition. Therefore there are good ecological reasons to predict either that (a) HPA functionality occurs at fledging, as in songbirds; or (b) HPA functionality is obtained before fledging to aid sibling competition exacerbated by the size hierarchy.

## 2 | METHODS

### 2.1 | Study site and species

All experiments were reviewed and approved by the Institutional Animal Care and Use Committee (2014-003), University of Texas Rio Grande Valley and the Ministerio del Poder Popular para el Ecosocialismo y Aguas in Venezuela (No. 1430). Plasma samples were imported under U.S.D.A. Research Permit (#127114) and U.S.F.W.S. Permit (#MA78044B-0). We studied a population of green-rumped parrotlets at Hato Masaguaral (8°34' N, 67°35' W), State of Guarico, Venezuela between June–December 2012 and June–December 2013. The habitat consists of seasonally flooded tropical savanna and gallery forest. Parrotlets are year-round residents, but breed monogamously during the wet season between June and December (Waltman & Beissinger, 1992). Parrotlet breeding, social system, and population ecology have been monitored at this site since 1988, facilitated by 106 artificial nest cavities fashioned from 1×0.08 m PVC pipes (Beissinger, 2008). Clutch size varies from 4 to 12 eggs (mean of 7), which hatch very asynchronously over a 10–18-day period creating pronounced age and size hierarchies within broods (Stoleson & Beissinger, 1997). Nestling sex ratios do not vary significantly from parity and nestlings can be sexed based on plumage color difference by 15 dph (Budden & Beissinger, 2004). Both parents typically feed nestlings once an hour throughout the day and individuals fledge at on average 30 dph (Beissinger & Waltman, 1991; Waltman & Beissinger, 1992).

### 2.2 | Nest monitoring

Contents of nest boxes were determined at 3-day intervals throughout the study periods to determine laying and hatching dates. Eggs and nestlings were marked with nontoxic felt-tip markers. After phlebotomy (see below), we measured culmen and tarsus length with a caliper ( $\pm 0.1$  mm); estimated crop fullness on a quarter scale; measured their wing chord ( $\pm 1$  mm); and weighed them ( $\pm 0.1$  g). We monitored the fate of 75 nest attempts during the study.

### 2.3 | Sampling design and phlebotomy

We randomly selected 33 broods, from which we sampled 126 individual nestlings. Broods were divided into one of two treatment groups, baseline and restraint stress-treatment (see below), and one of four evenly spaced age groups (13, 18, 23, and 28 dph). This age range encompassed the stage at development in which nestlings were large enough to safely provide a blood sample and ended when fledging was imminent. Each nestling was sampled once to avoid habituation and to safeguard the amount of blood collected from very small (<15 g), underdeveloped nestlings. Sampling took place between 7:00 and 10:00 a.m. At each sampling session the nest box was opened and the focal

nestling removed and bled within 2 min of removing the nestling (baseline group), or removed from the nest box and held alone in a cloth bag and bled at 30 min (restraint stress-treatment group). Nineteen adults were sampled after capture in mist nets between 7:00 and 10:00 a.m. Adults were sampled within 2 min of being caught in the net and were sampled again after they were held alone in a cloth bag for 30 min. To begin to understand how nutritional stress may affect variation in baseline levels, in a subset of eight nestlings we collected baseline samples before nestlings had been fed, and 5–7 days later in development, we collected a second sample immediately after the parents exited the box at the conclusion of a feeding session. If nestlings are able to mount a significant stress response to hunger, then CORT levels should be lower after nestlings were recently fed. Individuals' brachial arteries were punctured with a 26-G needle and a maximum of 75  $\mu$ l of blood collected into heparinized capillary tubes. Tubes were immediately placed on ice and centrifuged within 2 hr. Plasma was separated with a Zipocrit centrifuge (LW Scientific, Lawrenceville, GA) at 11,000 rpm for 10 min. Hematocrit was measured to provide an independent assessment of physiological changes during development. Plasma was extracted from the capillary tube with a Hamilton syringe (100  $\mu$ l) and placed in an Eppendorf tube (0.60 ml volume). Tubes containing plasma samples were immediately stored in a  $-20^{\circ}\text{C}$  freezer. Tubes were transported on dry ice to freezers in the laboratory of AMB at the Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela, and were later shipped in dry ice to the laboratory of JCW at the University of California, Davis, where CORT assays were conducted.

### 2.4 | Radioimmunoassay

CORT concentrations were quantified using a radioimmunoassay (Wingfield, Vleck, & Moore, 1992). Plasma steroids were extracted using HPLC-grade dichloromethane having been redistilled within 24 hr before use. Plasma samples were measured to the nearest  $\mu$ l and 20  $\mu$ l of tritiated CORT (2,000 cpm/ $\mu$ l) added to each sample to determine extraction efficiency. Each sample was mixed with 4 ml of redistilled dichloromethane for 3 hr to extract steroids from plasma. Extracts were dried under nitrogen at  $35^{\circ}\text{C}$ , and reconstituted in 550  $\mu$ l phosphate-buffered saline with gelatine. A 100  $\mu$ l aliquot was added to a scintillation vial and combined with scintillation fluid to determine percent recoveries, which averaged 81% ( $\pm$ standard deviation [SD] 5.6%, range = 65–96%). Duplicate 200  $\mu$ l aliquots were assayed by adding 100  $\mu$ l ( $\sim 10^4$  cpm) of tritiated CORT (Perkin Elmer NET399250UC) and 100  $\mu$ l of antibody (Esoterix Inc. B3-163). Unbound steroid was separated from bound steroid with 500  $\mu$ l of dextran-coated charcoal followed by centrifugation at 3,000g. The supernatant was decanted and combined with scintillation fluid (Perkin Elmer Ultima Gold: 6013329) and counted for 5 min or within 2% accuracy on a Beckman 6500 LS counter. Sensitivity of the assays

was estimated at 7.8 pg per tube and intra-assay variation for each sample ranged from 7% to 17%.

## 2.5 | Statistical analysis

We used generalized linear mixed models (GLMM) in SAS (v9.4; SAS Inc.) to determine differences between baseline and stress-induced CORT groups at each stage in development. Treatment (baseline or restraint stress), Status (nestling or adult), Sex (female or male), and interactions were included as fixed factors. AICc were calculated for each model and used to rank models according to their AICc weights (Burnham, Anderson, & Huyvaert, 2011). We conducted a second AIC analysis on a separate set of models excluding information on adults. These models included Treatment (baseline or restraint stress) and Hatch sequence (early vs. late) as categorical fixed factors, and Brood size and Age (dph) as continuous fixed factors. Hatching sequence was standardized to include first and middle sequence (early) compared to penultimate- and last-hatched individuals (late). Individual nest attempt was included as a random effect to control for nonindependence between siblings within a brood. Three samples had recovery values below 55% and were excluded from the analysis. One individual from a nest where both parents died 2 days before sampling had baseline CORT levels of 35 ng/ml, more than 1 SD above the global maximum of the study. This nestling exhibited signs of undernourishment and dehydration, and died the following day, so was also excluded from statistical analysis. In three cases, we were unable to collect blood samples within 2 min and excluded these samples from statistical analysis. Baseline levels did not vary significantly with time since capture ( $r^2 = 0.00$ ,  $F_{1,59} = 0.49$ ,  $p < .48$ ). CORT levels were modeled as a Gaussian response distribution linked to an identity function. All models converged, however all had chi-square to degrees of freedom ( $df$ ) ratios of  $>20$ , suggesting over dispersion and non-normality. Values were  $\log_{10}$  transformed, which normalized the distribution and resulted in chi-square-to- $df$  ratios closer to 1. Because there were no significant differences between age groups within treatments, we also report results of a GLMM pooling all age groups.

**TABLE 1** Results from generalized linear mixed models comparing ( $\log_{10}$ ) baseline and stress-restraint plasma corticosterone concentrations in nestling ( $N = 126$ ) and adult ( $N = 19$ ) green-rumped parrotlets in Venezuela

Model	$k$	AIC	$\Delta$ AIC	$w_i$
Treatment + Status + Treatment $\times$ Status	9	127.84	0.00	0.367
Treatment	3	128.07	0.23	0.327
Treatment + Status	5	128.32	0.48	0.289
Treatment + Sex	6	134.06	6.22	0.016
Status	3	165.87	38.03	0.000
Sex	4	171.90	44.06	0.000

Note: Treatment (baseline or stress-restraint), Status (nestling vs. adult), Sex (female or male) of individuals were included as fixed effects. AICc scores were used to rank models according to their AICc weights ( $w_i$ ).

Abbreviation: AICc, Akaike Information Criteria, corrected for small samples size.

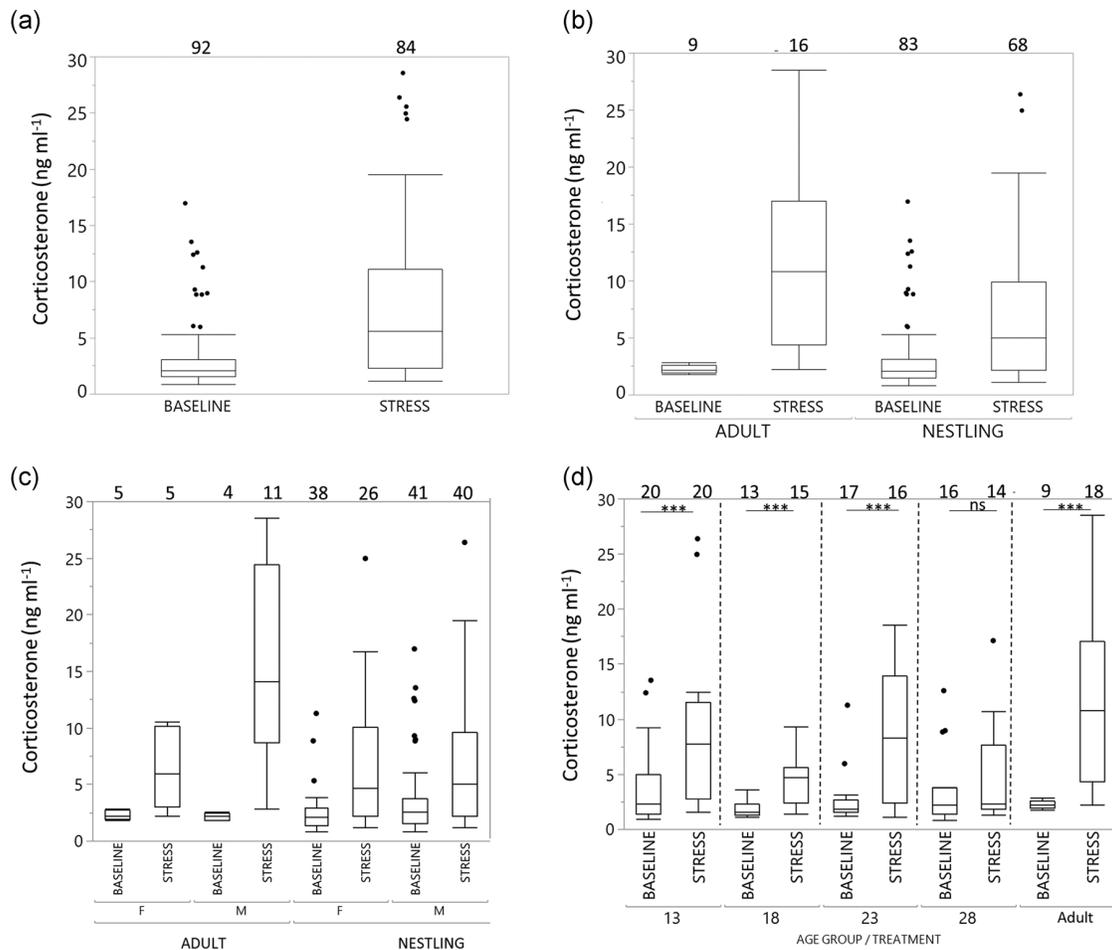
## 3 | RESULTS

### 3.1 | Stress response in adults and nestlings

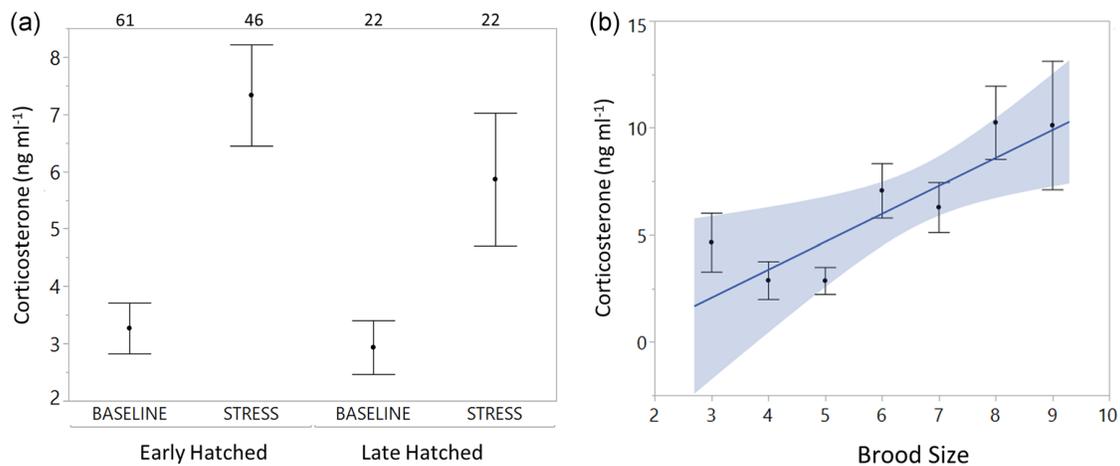
The models including Treatment, Status and their interaction, Treatment and Status, as well as Treatment by itself ranked highest among models describing individual differences in plasma CORT concentrations, with AICc weights ranging between 0.29 and 0.37 (Table 1). CORT concentrations were significantly higher in the restraint stress-treatment group ( $\bar{X} = 7.89$  ng/ml  $\pm 0.73$  SE,  $N = 84$ ) compared to the baseline group ( $\bar{X} = 3.09 \pm 0.32$  SE,  $N = 92$ ; Figure 1a). Nestlings as a whole had lower average restraint stress-treatment CORT concentrations compared to adults (Adults:  $\bar{X} = 12.29 \pm 2.08$  SE,  $N = 16$ ; Nestlings:  $\bar{X} = 6.86 \pm 0.71$  SE,  $N = 68$ ; Figure 1b). However, this was partially caused by the higher concentrations exhibited by adult males compared to adult females, though we had smaller sample sizes in adult females (Figure 1c), and lower CORT concentrations recorded in the 18 and 28 dph age groups (the 13 and 23 dph groups were similar to adults; Figure 1d). Pooling adults and nestlings, there were no significant sex differences in restraint stress-treatment (Females  $\bar{X} = 6.5 \pm 0.98$  SE,  $N = 31$ , Males:  $\bar{X} = 8.7 \pm 1.03$  SE,  $N = 51$ ), nor were there significant sex differences between age groups within treatments (baseline:  $F_{3,39} = 0.90$ ,  $p < .448$ ; stress-treatment:  $F_{3,25} = 1.09$ ,  $p < .373$ ; Figure 1c) and Sex was not among the top models identified by AIC. Interestingly, adults appeared to exhibit more variability in the restraint stress-treatment and invariably low baseline concentrations compared to nestlings, despite the former group being less exhaustively sampled (Figure 1b-d).

### 3.2 | Effects of brood size, hatch sequence, and feeding

When we restricted the analysis to CORT concentrations in nestlings, Treatment ranked highest among models (weight = 0.76). Treatment and Hatching sequence or Brood size also provided information (Figure 2a, Table 2). Earlier hatched nestlings within a brood had higher mean restraint stress CORT concentrations, but did not differ in baseline concentrations (Figure 2a). Brood size was similarly unrelated to baseline CORT concentrations, but positively related to restraint stress CORT concentrations (weighted least squares regression, adjusted  $r^2 = 0.60$ ,  $F_{1,5} = 10.1$ ,  $p < .02$ ,  $N = 68$ ; Figure 2b).



**FIGURE 1** Box plots of plasma corticosterone (CORT) levels in baseline and stressed-induced green-rumped parrotlets in Venezuela (2012–2013). (a) Overall differences between baseline and restraint-induced plasma CORT concentrations, pooling nestlings and adults; (b) differences between baseline and restraint-induced CORT in nestlings and adults; (c) differences in baseline and restraint-induced CORT, controlling for sex of nestlings and adults; (d) four nestling age groups were compared to adults. Samples sizes of individuals are given above each age category. All age groups showed significantly higher CORT levels in stress groups, with the exception of the 28-day posthatch age group. Untransformed CORT levels are displayed for diagnostic purposes, while statistical models were based on results of generalized linear mixed models, using log-transformed CORT measurements



**FIGURE 2** (a) Mean ( $\pm$ SE) plasma corticosterone (CORT) concentrations comparing baseline and restraint stress-treatment as a function of hatching sequence (early vs. late) in 126 nestling green-rumped parrotlets. Samples sizes of individuals within each group are given above each category. (b) Mean ( $\pm$ SE) restraint stress-treatment CORT concentrations as a function of brood size in 68 nestling parrotlets [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

**TABLE 2** Results from generalized linear mixed models comparing ( $\log_{10}$ ) plasma corticosterone concentrations in nestling ( $N = 126$ ) green-rumped parrotlets in Venezuela

Model	$k$	AIC	$\Delta$ AIC	$w_i$
Treatment	3	99.19	0.00	0.758
Treatment + Hatch sequence	4	102.59	3.40	0.139
Treatment + Brood size	4	104.10	4.91	0.065
Treatment + Brood size $\times$ Hatch sequence	4	106.91	7.72	0.016
Treatment + Brood size + Hatch sequence	5	107.61	8.42	0.011
Treatment + Age	4	107.69	8.50	0.011
Treatment + Age + Treatment $\times$ Age	6	114.76	15.57	0.000

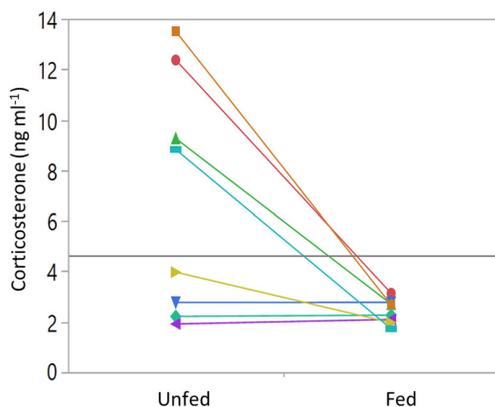
Note: Age (dph), Brood size (3–9 siblings), Hatching sequence (early or late), Sex (male or female) and their interaction were included as fixed effects. Brood identity was included as a random effect. AICc scores were used to rank models according to their AIC weights ( $w_i$ ). Abbreviation: AICc, Akaike Information Criteria, corrected for small samples size.

Age of nestlings was unrelated to baseline or restraint stress CORT concentrations and was not among the top models identified by AIC. Thus, there were no, clear age-related changes in restraint stress-treatment CORT concentrations for the age groups sampled.

In a subset of nestlings we measured crop fullness and baseline CORT concentrations before feeding sessions and, 5–7 days later, immediately after feeding sessions. Crops were on average fuller (Matched Pairs,  $F_{1,7} = 15.0$ ,  $p < .01$ ), and baseline CORT concentrations significantly lower after parental visits compared to before (Matched Pairs,  $F_{1,7} = 9.1$ ,  $p < .02$ ; Figure 3).

### 3.3 | Morphology and hematocrit

Wing chord and culmen length measurements increased linearly with age, while body mass increased in a logistic fashion, with the oldest

**FIGURE 3** Baseline corticosterone concentrations in eight unfed nestlings were higher compared to baseline concentrations in samples collected immediately after parental feedings. Unique colors and symbols connect measurements from the same individual [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

28 dph nestling group similar to the adult group (ESM Figure 1a–c). Tarsus did not change across age groups, indicating that nestlings' tarsus had attained its adult size by at least 13 dph (ESM Figure 1d). Hematocrit also increased predictably with age group (GLMM,  $F_{4,82} = 38.09$ ,  $p < .0001$ ; ESM Figure 1e). Thus, the tarsus develops early, while the other traits underwent an important period of growth and development during the age range studied.

## 4 | DISCUSSION

Parrotlets showed evidence of HPA responsiveness at an earlier life history stage than altricial songbirds studied to date. Parrots and songbirds are sister branches of the largest group of altricial birds, however, in songbirds hatching is followed by rapid growth, whereas parrotlets, and most parrots, have slow growth rates more typical of precocial species (Pacheco et al., 2010; Ricklefs, 1979). However, unlike most precocial species, parrots hatch very underdeveloped and undergo a disproportionate amount of brain growth during their slow postnatal development (Iwaniuk & Nelson, 2003;). While songbirds also undergo large neonatal brain growth increments, our results suggest that in parrots, developing phenotypes are vulnerable to neuroendocrine-environmental feedback for a longer time period before maturation and acquisition of adult phenotypes.

### 4.1 | Nestling and adult CORT concentrations

Parrotlet nestlings exhibited a significant stress response in the youngest age group sampled and no clear age-related changes 2 weeks later in development. Nestlings that underwent a standardized restraint stress-treatment showed significantly higher circulating CORT concentrations compared to the baseline group as early as 13 dph, before they had plumage; up to a week before they become endothermic (Pacheco et al., 2010); a week before the begin complex vocal behavior (Berg, Delgado, Cortopassi, Beissinger, & Bradbury, 2012); and more than 2 weeks before fledging from the nest (Stoleson & Beissinger, 1997). Adults did show higher stress responses on average compared to nestlings in general (Table 1). However, this was at least partially owing to lower levels in the 18 and 28 dph age groups; the 13 and 23 dph age groups were not statistically different from adults, and age group was not among the top models identified by AIC analysis (Table 2). Because we sampled each individual once, we cannot comment on longitudinal changes in the same individual. However, our results avoid potential habituation bias owing to repeated sampling of the same individual. Our estimate of adult restraint stress CORT concentrations was also potentially elevated owing to higher concentrations in males compared to females, though our sample size of females was too low to test statistically. All of our sampling took place during the breeding season when breeding males nutritionally provision female mates before and during early stages of brood development (Curlee & Beissinger, 1995), and as a result males may be more vulnerable to nutritional stress during this time. Our sample of adults, including

both intrasexual and intersexual variation, may also differ from studies conducted during the nonbreeding period (Romero, 2002; Romero & Remage-Healey, 2000). Thus, there were no clear differences between nestling age groups and adult responsiveness to restraint stress.

We found no evidence of sex differences in the stress response in the better-sampled nestling group. Lack of sex differences in nestlings is not surprising, given the basic metabolic and survival functions of CORT and its critical role in nestling growth, processes which likely affect both sexes equally during the nestling period. In common terns (*Sterna hirundo*), nestling males have higher food requirements than females and show higher CORT levels, which may be related to higher susceptibility to nutritional stress in males (Braasch, Becker, & Grootuis, 2014). Parrotlet sexes do not, however, differ in their developmental rates of morphological, physiological, or behavioral traits (Berg, Beissinger, & Bradbury, 2013; Pacheco et al., 2010; Stoleson & Beissinger, 1997), though the emergence of sexually dichromatic feathers by 15 dph suggests a potential role for gonadal steroids (Adkins-Regan, 2005). Thus, both sexes of parrotlets appear to be physiologically vulnerable to environmental stress during an important period in postnatal development.

#### 4.2 | Comparison of parrotlet stress response to altricial songbirds

Parrotlets demonstrated an ability to mount a significant stress response at an earlier stage in physical and physiological development than altricial songbirds studied to date, though it remains to be determined how much earlier adrenocortical responsiveness ensues. Similar-sized, domesticated canaries (21 g; *Serinus canaria*) do not exhibit an adult-like stress response until the fledging period, after they have attained adult body mass and flight capability (Schwabl, 1999). Similarly, Northern Mockingbirds (25 g; *Mimus polyglottus*) do not attain an adult-like response until nutritional independence at 28 dph, several weeks after fledging (Sims & Holberton, 2000). White-crowned sparrows (25 g; *Z. leucophrys nuttalli*) attain an adult-like stress response at 7–9 dph, encompassing the fledging stage (Wada et al., 2007). Florida scrub jays (*Aphelocoma coerulescens*), which are twice the size of parrotlets (50 g), do not show an adult-like response to stress until after fledging (54 dph; Rensel & Schoech, 2011). The smaller zebra finch (15 g; *Taeniopygia guttata*), shows an adult-like stress response by 16 dph, also around fledging age (Wada, Salvante, Wagner, Williams, & Breuner, 2009). The postponement of HPA maturation in the above altricial songbirds is thought to be a developmental strategy for avoiding adverse effects of heightened glucocorticoid production (Romero & Wingfield, 2016; Wada, 2008). Thus, our results indicating an adult-like HPA responsiveness at 13-day posthatching of a 30-day nestling period, suggest that the benefits of precocity in HPA functionality in parrotlets outweigh the costs of potentially unchecked, circulating glucocorticoid concentrations during an important period in growth and maturation.

#### 4.3 | Brood size, hatching sequence, and feeding

HPA responsiveness to stress in nestling parrotlets might be related to competitive interactions with nestmates, because nestlings would from early in development be physiologically prepared to face challenges posed by both the quantity and quality of nest mates (Budden & Beissinger, 2009). In our study models including both hatching sequence and brood size performed reasonably well in AIC when included with treatment (Table 2). Nestlings that hatched earlier in the brood or that came from larger broods had higher mean restraint stress CORT concentrations (Figure 2a,b, respectively), while baseline concentrations did not differ. Lack of differences in baseline concentrations suggests that nestlings are generally not nutritionally stressed, corroborating evidence from experimental feedings (Budden & Beissinger, 2009). Higher stress responses in larger broods raises the question of whether early social interactions might prime HPA responsiveness depending on prevailing competitive scenarios in different brood sizes. Alternatively, adult females might adjust embryonic deposition of hormones (Kozłowski & Ricklefs, 2010), with downstream effects manifested in higher responsiveness during the nestling stage. In Nazca boobies (*Sula granti*), sibling size asymmetries select for higher CORT levels in responsiveness to stressful confrontations in subordinates, which invariably end in siblicide (Ferree, Wikelski, & Anderson, 2004; Tarlow, Wikelski, & Anderson, 2001). Parrotlets are highly social and from a young age sibling interactions are often affiliative (Arellano, 2019). Nevertheless, parrotlet parents preferentially feed nestlings that beg more (Budden & Beissinger, 2009). In support of this, in a subset of nestlings, we found significantly higher baseline CORT concentrations before nestlings had been recently fed compared to baseline estimates immediately after the same individuals had been fed (Figure 3). Adult parrotlets do not necessarily feed all nestlings during every visit, especially in larger broods (Budden & Beissinger, 2009). This suggests that nestlings produced an acute response to nutritional stress, which was exacerbated in larger broods and presumably downregulated after parental feeding sessions. However, confirmation of causes requires experimental manipulations. Nevertheless, in our study this suggests that the HPA functionality is poised to aid nestlings in competitive begging contests that ultimately influence nutritional provisioning outcomes.

#### 4.4 | Morphology, hematocrit, and adrenocortical responses

Hematocrit increased with age almost as predictably as did several morphological attributes (ESM Figure 1e), eventually obtaining an average adult level of 56% ( $\pm 0.6$  SE), within the range reported for adults in the same population (Sheridan, Beissinger, & Hughes, 2004). Increasing hematocrit during nestling development is consistent with several studies on developing birds, and is presumably caused by increased production of red blood cells as nestlings grow (Fair, Whitaker, & Pearson, 2007). In our study increasing levels of hematocrit in nestlings provides additional evidence that individuals

underwent normal physiological development during the age range studied. However, hematocrit's relationship to the developing immune system and stress physiology is complex and understudied in altricial birds (Apanius, 1998; Martin, 2009).

Hematocrit and a suit of morphological traits varied predictably across the age range studied (ESM Figure 1a–e). The main exception was tarsus length, which attains its adult size by 10 dph (Pacheco et al., 2010). Attainment of adult tarsus might be required for incipient locomotor activity used in asymmetrical begging contests (Budden & Beissinger, 2009) and may benefit from early HPA functionality. The late emergence of locomotor ability is a hallmark of altricial development. Many songbirds are predicted to acquire an adult-like stress response around the time of locomotor independence (Schwabl, 1999). Parrotlets, and most parrots, are obligate cavity nesters, and their prolonged development can result in elaboration of locomotor movements involved in competitive begging from caregivers (Budden & Beissinger, 2009); developmental precursors to flight (Berg et al., 2013); and exploratory tactile interactions with nest mates (Arellano, 2019). These behaviors are concealed from the visual systems of potential predators by the cavity. Thus, while fledging is postponed in parrotlets compared to similar-sized cavity nesting songbirds (K. S. Berg, unpublished data), cavity nesting and extreme altriciality in parrotlets may result in a relaxation on movement behaviors that appears to coincide with HPA functionality.

#### 4.5 | Conclusions

The emergence of locomotor ability is the principal driver of diversity in avian developmental mode (i.e., precocial-altricial spectrum), because it requires maturation of a number of underlying physiological, cognitive, and motor systems (Stark & Ricklefs, 1998). Parrots and songbirds are both altricial, suggesting this was the ancestral developmental mode in the sister group (Psittacopasserae; Suh et al., 2011), however parrots postponed the emergence of locomotor until well after HPA emerges. While studies of more parrot species are needed, early HPA maturation combined with slower growth rates represents a previously underappreciated departure in the evolution of altricial development.

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#### ORCID

Karl S. Berg  <http://orcid.org/0000-0002-3648-2991>

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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