



Corticosterone exposure during development has sustained but not lifelong effects on body size and total and free corticosterone responses in the zebra finch



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ABSTRACT

Animals exposed to stress during development experience sustained morphological, physiological, neurological, and behavioral consequences. For example, elevated glucocorticoids (GCs) during development can increase GC secretion in adults. Studies have examined the sustained effects of elevated developmental GCs on total GC responses, but no study to date has examined the effect of developmental stress on corticosteroid binding globulin (CBG). CBG is a protein which binds to GCs and facilitates their transportation in blood. When bound to CBG, GCs are unavailable to interact with target tissues. Exposure to stress can decrease CBG capacity and, thus, increase free GCs (the portion of unbound GCs). We examined the long-term effects of elevated corticosterone (CORT) during development (12–28 days post-hatch) on acute stress responses, negative feedback, and CBG capacity at 30, 60, and 90 days post-hatch in zebra finches. Additionally, we evaluated the effect of CORT treatment on body size and condition at 28, 60, and 90 days post-hatch. CORT exposed birds had higher acute stress responses at 30 days post-hatch compared to control birds. However, there was no treatment effect at 60 or 90 days post-hatch. CBG levels were not affected by treatment, and so free CORT estimations reflected patterns in total CORT. CORT treatment decreased growth and condition in zebra finches at 28 days post-hatch, but these differences were not present at later life history stages. However, brood size had a sustained effect on body size such that birds reared in medium sized broods were larger at 28, 60, and 90 days post-hatch. These results demonstrate the complexity of early environmental effects on adult phenotype and suggest that some conditions may have stronger programmatic effects than others.

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1. Introduction

Glucocorticoids increase in response to external perturbations and promote behavioral and physiological changes to restore homeostasis. In this way, glucocorticoids (GCs) have *activational* effects on adult phenotype and behavior. In developing animals, GCs can have similar effects on short-term behavior and physiology. In addition, there is a growing body of literature from across taxonomic groups which suggest that GCs have *organizational* effects on developing animals (a process known as developmental programming; (McMillen and Robinson, 2005). Specifically, animals exposed to elevated levels of GCs during development can experi-

ence sustained morphological, physiological, neurological, and behavioral consequences (reviewed in Matthews (2005), Nesan and Vijayan (2005), Schoech et al. (2012)). In some cases, these phenotypic effects appear to be life-long and can even be transmitted across generations (Catalani et al., 2000; Liu et al., 1997; Schöpper et al., 2012; Weaver et al., 2000).

Recent research has focused on the organizational effects of developmental stress on hypothalamic–pituitary–adrenal (HPA) axis activity in birds. Exposure to pre- and postnatal stress (via elevated GCs, reduced maternal condition, food restriction), can significantly affect HPA axis function at later life history stages. In general, developmental stress causes sustained elevation of HPA function such that animals exposed to stress during development respond more strongly to stressors as adults (e.g. Marasco et al., 2012; but see Lendvai et al., 2009; Love and Williams, 2008). For example, chicks from CORT-implanted Japanese quail (*Coturnix coturnix japonica*) grew more slowly and had significantly higher HPA responses to stressors at eight weeks of age compared to controls (Hayward and Wingfield, 2003). Postnatal GC exposure has

Abbreviations: CBG, corticosteroid binding globulin; CORT, corticosterone; EPC, extra pair copulation; GC, glucocorticoid; HPA, hypothalamic pituitary adrenal.

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been shown to have similar effects on HPA axis function. Zebra finches (*Taeniopygia guttata*) fed CORT dissolved in peanut oil during the nestling period (12–28 days post-hatch) had elevated levels of CORT following an acute stressor compared to control finches at 60 days post-hatch (Spencer et al., 2009). Finally, western scrub jays (*Aphelocoma californica*) raised on a food restricted diet (65% of *ad libitum*) had higher levels of baseline CORT as nestlings and elevated levels of stress-induced CORT at one year of age (Pravosudov and Kitaysky, 2006). These studies, along with many studies from mammals (e.g. Liu et al., 1997, reviewed in Matthews, 2005), demonstrate that exposure to developmental stress (i.e. elevated GCs and food restriction) can have sustained effects on HPA activity at later life history stages.

Although there is substantial support demonstrating the sustained effects of developmental stress (i.e. elevated GCs) on HPA function, no study to date has examined the long-term effects of developmental stress on corticosteroid binding globulin (CBG) capacity. CBG is a protein that binds to GCs with high affinity and facilitates transportation of lipophilic GC molecules through the blood. CBG regulates stress responsiveness by binding to GCs and preventing them from interacting with target tissues (the “free hormone hypothesis;” Mendel, 1989; Malisch and Breuner, 2010; Breuner et al., 2013). Elevated GCs can transiently decrease CBG levels which may increase the amount of CORT available to interact with target tissues. For example, Breuner et al. (2006) showed a reduction in CBG capacity following 60 min of restraint stress in zebra finches suggesting a regulatory role for CBG in response to acute stress. Other studies have shown a decline in CBG capacity 24 h following acute stressors (Malisch et al., 2010, but see Mueller et al., 2009). Therefore, measuring free CORT (the portion of CORT not bound to CBG) can provide additional information about how animals respond to stressors.

We investigated the long-term effects of elevated CORT during development on HPA function, CBG capacity, negative feedback, body size, and condition in zebra finches. We elevated endogenous CORT by orally administering CORT dissolved in peanut oil to nestling zebra finches for 16 days during the nestling period (from 12 to 28 days post-hatch). We predicted that CORT-exposed nestlings would have elevated total CORT, lower CBG capacity, and reduced negative feedback as adults, compared to controls. We also expected that CORT treatment would result in reduced body size and body condition metrics. These results would support and expand recent studies demonstrating sustained effects of elevated developmental GCs on phenotype and physiology.

2. Methods

2.1. Parental birds – housing and breeding

Ten female and ten male zebra finches were purchased from six pet stores across Montana and Washington. We banded the birds with a unique combination of color bands in order to identify individual birds. Throughout the course of the experiment, 3 males and 2 females were replaced due to mortality. Breeding finches were housed in a 20 × 25 ft. room where they were allowed to interact freely with all other birds. We housed the birds on a 14:10 light/dark cycle at 26–27 °C with 20–30% humidity. Birds had access to 12 nest boxes and shredded burlap nesting material. We fed birds commercial finch seed (Silver Song West) and spray millet *ad libitum* and supplemented their diet daily with hard boiled eggs, spinach, and crushed egg shells. Nest boxes were monitored daily for signs of nest building and egg laying. Over the course of the experiment, 48 clutches of nestlings were produced.

2.2. Nestlings– treatment and measurements

Starting on hatch day, we marked nestlings with an individual combination of leg markings using a black Sharpie marker. Between three and four days after hatching, we banded nestlings with a numbered plastic leg band. Twelve days after hatching, we weighed nestlings to the nearest 0.1 g and measured tarsus length (posterior to anterior tarsus) and wing chord (carpus to longest primary feather) to the 0.1 mm. Within clutches, nestlings were randomly assigned to treatment groups such that roughly half of each clutch was CORT-treated and half control. Nestlings exposed to the CORT treatment were fed oral boluses (25 µl) of CORT (Sigma Aldrich) dissolved in peanut oil twice daily approximately 5 h ± 1 h apart. From 12 to 15 days post-hatch, nestlings received 0.124 mg/ml of CORT in peanut oil for a total daily dose of 6.2 µg of CORT. Starting 16 days post-hatch, the dose was increased to 0.163 mg/ml for a total daily exposure of 8.15 µg of CORT. Control nestlings were fed 25 µl of peanut oil on an identical feeding schedule. Nestlings were exposed to treatments from 12 to 28 days post-hatch (methods as per Spencer et al., 2009).

Nestling zebra finches fledge as early as 17 days post-hatching. Before fledging, we identified the social parents of a nest by observing incubating and provisioning behaviors. If we were unable to identify parents based on these behaviors, we recorded parental behavior using VehoMuvimicroDV camcorders and identified parents from the resulting videos. After determining social parents, we moved the nest box and parents to wire cages (70 × 40 × 44 cm³) where they were housed until the nestlings reached nutritional independence at 28 days post-hatch (Spencer et al., 2009). Following nutritional independence, we returned the parents to the breeding aviary. Nestlings remained in the cages and were fed a diet of commercial finch food, spray millet, boiled eggs, and spinach. We measured tarsus, wing chord, and mass at 28, 60, and 90 days post-hatch. We calculated condition for birds at 28, 60, and 90 days post-hatch using the scaled mass index (Peig and Green, 2009; Peig and Green, 2010). The scaled mass index accounts for errors associated with residual body mass measurements by using a scaling relationship derived from the population of interest to calculate the expected mass of each individual at a fixed body size. In this way, the scaled mass index standardizes all animals to the same growth phase or body size and is considered to be a more accurate measure of condition (Peig and Green, 2010).

2.3. HPA function at 30, 60, and 90 days post-hatch

We measured the effects of CORT treatment during development on HPA function 30, 60, and 90 days post-hatch ($n = 29, 25,$ and 38, respectively). We measured stress responses by exposing finches to a standardized restraint stress protocol (Wingfield, 1994). We obtained one blood sample within three minutes of disturbing birds (baseline CORT). After initial blood samples were obtained, we placed finches in cloth bags and collected two more samples 10 and 30 min after initial disturbance. To collect blood, we punctured the alar vein with a 26-gauge needle and collected 25–50 µl of blood with heparinized microcapillary tubes. Immediately after collection, blood was kept in a 4 °C refrigerator on ice (<1 h) until it could be centrifuged to separate plasma from red blood cells (3000 rpm for seven minutes). After separation, the plasma was isolated and stored at –20 °C.

Plasma CORT typically does not increase within three minutes of stress exposure (Romero and Reed, 2005). However, samples obtained within three minutes of disturbance in our experiment ($\bar{x} = 1.80$ min, $SD = 0.77$) did show a significant increase in CORT ($N = 118, F_{1,115} = 24.99, r_s = 0.18, P < 0.01$). To account for this, we used the time to collect the initial blood sample as a scaled weight variable in our analyses.

2.4. Negative feedback at 30 days post-hatch

We examined negative feedback of HPA activity for finches 30 days post-hatch ($n = 26$). To quantify negative feedback we obtained blood samples within three minutes of initial disturbance (baseline CORT), after 30 min in a cloth bag (stress induced CORT) and then again after 15 min of release into their home cage (recovered CORT). To minimize the amount of blood we collected, we did not collect blood samples after 10 min of restraint stress for the negative feedback experiment. For this reason, we used separate birds to measure HPA activity and negative feedback at 30 days post-hatch.

2.5. Corticosterone and corticosteroid binding globulin assays

Corticosterone was quantified with Enzyme Immunoassay (EIA) kits (Cat No. 901-097, Assay Designs), previously optimized for zebra finches (Wada et al., 2008). Following the protocol used by Wada et al. (2009), we used a raw plasma dilution of 1:40 to determine CORT levels. We ran our samples against a six point standard curve ranging from 20,000 to 15.53 pg/ml. An external standard of 500 pg/ml was run on every plate and used to calculate inter-plate variation. All samples and standards were run in triplicate. Plates were read on a Multiskan Ascent microplate reader at 405 nm corrected at 595 nm. Intra- and inter-plate variation was 8.4% and 15.5%, respectively.

Corticosteroid binding globulin (CBG) is a protein that interacts with CORT in the plasma and likely modulates the amount of CORT exposed to target tissues (Breuner et al., 2013; Breuner et al., 2003; Malisch and Breuner, 2010). Therefore, measuring free CORT (the portion of CORT not bound to CBG) can provide additional information about glucocorticoid function. We quantified CBG using a ligand-binding assay with tritiated CORT (as described in Breuner et al., 2003). This assay has been optimized for zebra finch adults (Wada et al., 2008) and used for zebra finch nestlings (Wada et al., 2008). We thawed nestling plasma at 4 °C and stripped plasma with activated charcoal at a 1:3.5 ratio. Samples were incubated at room temperature for 20 min and then vortexed at 4 °C, 10,000 rpm for 10 min. The stripped plasma supernatant was removed and stored at -20 °C until assayed. We determined total CBG binding using 50 µl buffer, 50 µl tritiated CORT, and 50 µl stripped, diluted plasma (for a 1:1050 final dilution of raw plasma). Non-specific binding (NSB) was determined using 50 µl of 1 µM unlabeled CORT, 50 µl tritiated CORT, and 50 µl stripped, diluted plasma. Intra- and inter-filter variation for CBG point samples were 11.4% and 17.4%, respectively. Separate assays were run for samples from birds 90 days post hatch and all other samples. For this reason, we do not compare changes in CBG capacity, or free hormone estimates across life history stages.

We calculated free CORT levels using the mass action-based equation by Barsano and Baumann (1989):

$$H_{free} = 0.5 \left[H_{total} - B_{max} - \frac{1}{K_a} \pm \sqrt{2(B_{max} - H_{total} + \frac{1}{K_a}) + 4(\frac{H_{total}}{K_a})} \right] \quad (1)$$

In this equation, H_{free} = free hormone, H_{total} = total hormone, B_{max} = total binding capacity for CBG, and $K_a = 1/K_d$ (nM). The affinity of CORT for CBG was determined from equilibrium saturation binding analysis on pooled plasma samples from Wada et al. (2009). Individual CBG capacity estimates were approximately 85% of B_{max} so capacity values were increased to 100% for free CORT calculations. CBG capacity was assayed from baseline samples, and that value used to calculate free hormone levels from 0 to 3, 10, and 30 min samples (CBG capacity does not decline in zebra finches with 30 min of handling stress, Breuner et al., 2006).

We compared nestling CORT physiology by examining the amount of total and free CORT circulating before restraint stress (baseline CORT) and after 10 and 30 min of stress exposure. Additionally, we examined total integrated CORT for total integrated CORT which is the total amount of CORT circulating during 30 min of stress exposure. We calculated these values by integrating the amount of CORT at 0, 10, and 30 min after stress exposure. Total integrated CORT represents the total amount of CORT target tissues are exposed to during an acute stressor.

2.6. Statistical analyses

We used generalized linear models (GzLM) to analyze the effect of developmental treatment on CORT levels following 0, 10, and 30 min of restraint stress. CORT values were not normally distributed (Shapiro–Wilk's $P < 0.01$) and we could not normalize data using transformations. For this reason, we used GzLM's with gamma distributions to account for right skewed data and logistic link function resulting in GzLM that approaches a logistic regression (McCullough and Nelder, 1989). We included treatment, sex, and brood size as fixed factors in initial models. We tested each explanatory variable for significance using a standard backward procedure where non-significant terms were removed from the model. CBG values were normally distributed and we used general linear models (GLM) with treatment, sex, and brood size as fixed factors to evaluate differences between treatment groups. To assess differences in negative feedback, we calculated CORT recovery by subtracting CORT titers after 15 min of recovery from CORT titers after 30 min of restraint stress. We used general linear models (GLM) to examine the effects of treatment on body size with treatment, brood size, and sex as fixed factors. To examine the data graphically, we used a principle components analysis to reduce tarsus, wing chord, and body mass to one component score which explained 58.88% of variation.

3. Results

3.1. Stress responses at 30, 60, and 90 days post-hatch

At 30 days post-hatch, CORT-exposed zebra finches had elevated baseline and stress-induced CORT after 10 min of restraint (Table 1, $N = 54$, 28, $P = 0.001$, 0.004, $\chi^2 = 10.30$, 8.17, respectively). There was a non-significant trend for nestlings exposed to CORT during development to have higher CORT after 30 min of restraint (Table 1, $N = 28$, $P = 0.07$, $\chi^2 = 3.26$). Neither sex nor brood size affected baseline and stress-induced CORT (10 and 30 min; $P > 0.14$ for all). Nestlings exposed to CORT during development had significantly higher levels of total integrated CORT compared to control nestlings (Fig. 1, Table 1, $N = 28$, $P = 0.02$, $\chi^2 = 9.18$). There were no effects of sex or brood size on total integrated CORT ($P > 0.25$). There was no effect of treatment, sex, or brood size on CBG capacity ($P > 0.36$). Calculated free baseline CORT and free CORT after 30 min of restraint were higher in nestlings exposed to CORT during development compared to controls (Table 1, $N = 49$, 28, $P = 0.014$, 0.006, $\chi^2 = 6.09$, 7.67). CORT treatment had no effect on free CORT levels after 10 min of restraint ($N = 28$, $P = 0.34$, $\chi^2 = 0.91$). Sex had no effects on stress-induced free CORT ($P > 0.14$), but did affect baseline free CORT with females having higher levels compared to males ($N = 42$, $P = 0.05$, $\chi^2 = 4.00$). Brood size had no effect on free baseline CORT ($P = 0.14$), but did affect free CORT after 10 min of restraint ($N = 28$, $P = 0.034$, $\chi^2 = 8.69$) with nestlings from broods of four having higher levels of free B10 compared to nestlings from broods of other sizes ($P = 0.012$, $\chi^2 = 6.34$). There was a non-significant effect of brood size on free CORT after 30 min of restraint ($P = 0.07$, $\chi^2 = 6.97$) with nestlings

Table 1

Mean values of total and free CORT and CBG capacity for CORT exposed and control zebra finches at 30, 60, and 90 days post-hatch.

Variable	Day 30			Day 60			Day 90		
	Control (n = 15) Mean ± SD	CORT (n = 13) Mean ± SD	Diff. P	Control (n = 11) Mean ± SD	CORT (n = 13) Mean ± SD	Diff. P	Control (n = 18) Mean ± SD	Control (n = 20) Mean ± SD	Diff. P
Total baseline CORT (ng/ml)	1.06 ± 1.01	2.41 ± 1.63	0.001	0.54 ± 0.81	0.38 ± 0.40	0.20	0.51 ± 0.66	0.41 ± 0.50	0.96
Total CORT 10 min (ng/ml)	5.43 ± 2.54	9.65 ± 3.89	0.004	3.01 ± 2.67	3.87 ± 1.69	0.96	4.01 ± 2.32	4.00 ± 2.40	0.92
Total CORT 30 min (ng/ml)	4.58 ± 2.63	8.26 ± 6.60	0.07	5.68 ± 3.93	5.15 ± 2.59	0.66	6.51 ± 3.32	5.77 ± 3.89	0.35
Total integrated CORT (ng/ml)	117.85 ± 33.27	213.24 ± 96.51	0.020	90.2 ± 71.54	94.21 ± 27.50	0.73	107.52 ± 48.90	100.90 ± 54.45	0.57
CBG (ng/ml)	155.54 ± 62.74	171.09 ± 54.12	0.36	75.57 ± 37.81	74.36 ± 27.66	0.71	1065.76 ± 591.35	1360.22 ± 745.64	0.18
Free baseline CORT (ng/ml)	0.06 ± 0.07	0.10 ± 0.08	0.01	0.05 ± 0.10	0.03 ± 0.04	0.31	0.002 ± 0.003	0.002 ± 0.002	0.14
Free CORT 10 min (ng/ml)	0.3 ± 0.30	0.4 ± 0.19	0.340	0.3 ± 0.46	0.34 ± 0.36	0.40	0.02 ± 0.02	0.01 ± 0.002	0.59
Free CORT 30 min (ng/ml)	0.18 ± 0.10	0.45 ± 0.66	0.006	0.40 ± 0.46	0.33 ± 0.36	0.76	0.03 ± 0.02	0.02 ± 0.03	0.95

Total B0 n = 27,27. Free B0 n = 26,24.

from broods of five having higher free CORT compared to nestlings from broods of other sizes ($P = 0.012$, $\chi^2 = 6.28$).

There were no differences between treatment groups in any measure of total or free CORT output or CBG levels in zebra finches 60 days post-hatch (Fig 1., Table 1, $P > 0.20$ for all). Sex affected total baseline, total CORT after 10 min of restraint, total integrated CORT, free baseline, and free CORT after ten minutes of restraint with females having higher levels of CORT at all measures than males (Table 1, $N = 19$, $P = 0.003$, 0.001 , 0.003 , 0.023 , 0.001 , $\chi^2 = 8.77$, 13.93 , 8.99 , 5.21 , 10.51). There were no sex by treatment interactions ($P > 0.11$). Brood size did not affect any measure of total or free CORT ($P > 0.19$).

There were no differences between treatment groups in any measure of total or free CORT output or CBG levels in zebra finches 90 days post-hatch (Fig. 1, Table 1, $P > 0.15$). Likewise, neither sex nor brood size affect any measure of total or free CORT output or CBG levels in zebra finches 90 days post-hatch ($P > 0.11$ for all).

3.2. Negative feedback at 30 days post-hatch

At 30 days post-hatch, CORT-exposed zebra finches had higher total and free baseline CORT compared to control nestlings ($N = 12$, 14 , $P = 0.009$, 0.03 , $\chi^2 = 6.74$, 4.78). After 30 min of restraint, CORT-exposed zebra finches had higher total CORT ($N = 12$, 14 , $P = 0.026$, $\chi^2 = 4.97$) and a non-significant trend for

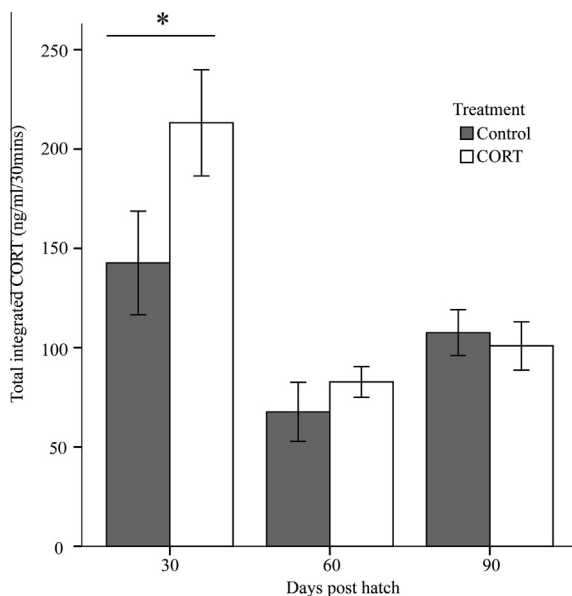


Fig. 1. At 30 days post-hatch, zebra finches exposed to CORT had higher total integrated CORT responses. There were no differences in total integrated CORT responses at 60 and 90 days post-hatch.

higher free CORT ($N = 12$, 14 , $P = 0.053$, $\chi^2 = 3.75$). Brood size had a significant effect on total baseline CORT ($N = 12$, 14 , $P = 0.03$, $\chi^2 = 9.17$) and total and free CORT after 30 min of restraint stress ($N = 12$, 14 , $P = 0.001$, 0.001 , $\chi^2 = 16.77$, 15.71) with nestlings from broods of five having higher CORT compared to nestlings from broods of other size ($P = 0.003$, 0.001 , $\chi^2 = 8.69$, 11.23). After 15 min of recovery, there was no difference between treatment groups levels in absolute levels of total and free CORT ($P > 0.32$). Brood size had no effect on CORT recovery ($P > 0.21$). Sex had no effect on any measure of total or free CORT ($P > 0.61$).

3.3. Body size and condition at 28, 60, and 90 days post-hatch

At 28 days post-hatch, CORT-exposed zebra finches had smaller tarsi, weighed less, and were in lower condition compared to control nestlings (Table 2, $F_{1,28} = 6.09$, 5.58 , 5.54 , $P = 0.02$, 0.03 , 0.03 , respectively). There were no differences in body size or condition between treatment groups at 60 and 90 days post-hatch (Table 2, $F < 1.46$, $P > 0.24$ for all).

In contrast to the transient effects of treatment on body size, brood size affected all body size measurements ($F > 4.20$, $P < 0.05$ for all) except mass at 28 days post-hatch ($F_{3,148} = 2.71$, $P = 0.07$) and mass at 90 days post-hatch ($F_{3,108} = 0.21$, $P = 0.89$). Brood size also affected condition 60 days post-hatch ($F_{3,36} = 4.73$, $P = 0.01$, but not condition at 28 days post-hatch ($F_{3,148} = 2.18$, $P = 0.12$) or condition at 90 days post-hatch ($F_{3,109} = 0.23$, $P = 0.87$). With the exception of two-nestling clutches, brood size affected body size in an inverted-U-shaped function with zebra finches reared from broods of five nestlings being larger compared to zebra finches reared in smaller and larger broods (e.g. Fig. 2). Brood size had similar effects on condition. The average brood size across the 48 clutches produced was 4.5 (stand. dev. = 1.18).

4. Discussion

Previous studies in birds have shown that elevated GCs during development can have sustained effects on HPA axis activity such that birds exposed to stress during development respond more strongly to stressors at later life history stages. We investigated the effects of CORT exposure during development on total and free CORT responses at 30, 60, and 90 days post-hatch. We found that CORT exposure during development elevated total and free CORT levels at 30 day post-hatch, but not at 60 and 90 days post-hatch. Brood size affected stress induced free CORT in birds 30 and post-hatch with birds from medium sized broods of five having higher levels of free CORT compared to birds from smaller and larger broods. At 30 and 60 days post-hatch females had higher CORT levels compared to males at some measurement. However, these effects were not present in birds at 90 days post-hatch. Sex differences in CORT responses in zebra finches have been described elsewhere (although the opposite pattern; Wada et al., 2009). We

found that CORT exposure decreased body size and condition in birds 28 days post-hatch, but there were no effects of treatment on body size or condition at 60 and 90 days post-hatch. However, there were sustained effects of brood size on body size present 28, 60, and 90 days post-hatch suggesting that brood size has programmatic effects on morphology.

4.1. The effects of elevated developmental CORT on stress physiology at 28, 60, and 90 days post-hatch

Zebra finches exposed to stress during development had elevated HPA axis activity compared to control birds at 30 days post-hatch. However, differences in HPA axis activity present at 30 days post-hatch were not sustained across life history stages. At 60 and 90 days post-hatch there were no differences in HPA activity between treatment groups. In contrast, using a similar protocol, [Spencer et al. \(2009\)](#) showed that CORT-exposed zebra finches 60 days post-hatch had elevated CORT secretion after 10 and 30 min of restraint stress, but no differences in baseline CORT compared to control nestlings. Although we expected to find similar results due to strong treatment effects, the discrepancies between our results and those of [Spencer et al. \(2009\)](#) could be attributed to differences in the genetic makeup of the study populations. Zebra finches are a widely used study organism with most research conducted on domesticated populations. Domestic populations are less genetically diverse than wild populations and have been genetically differentiated based on geographic location ([Forstmeier et al., 2007](#)). For example, North American zebra finches (such as those used in this study) are genetically differentiated from zebra finches domesticated in Europe (such as those used by [Spencer et al. \(2009\)](#)). There are many examples in the literature where one laboratory was unable to replicate the results of another laboratory using zebra finches as a study species (e.g. [Collins and ten Cate, 1996](#); [Jennions, 1998](#); [Forstmeier, 2005](#)). The stress response has a strong genetic component ([Satterlee et al. 1988](#)) and it is possible that genetic differences between our study population and that of [Spencer et al. \(2009\)](#) contributed to the differences in our results.

Similar to total CORT responses, CORT-fed birds had elevated levels of free CORT (baseline and 10 min following restraint stress) compared to control birds at 30 days post-hatch. There was no difference in CBG capacity between the treatment groups suggesting that developmental stress modulates stress physiology by acting on the total amount of CORT produced and not by modulating the amount of CORT available to target tissues via changes in CBG capacity. In adults, CBG capacity decreases in response to acute and chronic stress ([Breuner et al., 2006](#); [Malisch et al., 2010](#)). However, exposure to elevated CORT during development appears to have no long-term programmatic effects on CBG capacity suggesting that regulation of free CORT via changes in CBG capacity is not affected by early developmental conditions in the short- or long-term. The studies to date that have examined changes in CBG capacity in relation to disrupted homeostasis have focused on relatively short-term effects of hours to days ([Breuner et al., 2006](#); [Malisch et al., 2010](#)). It is possible that CBG production and its persistence in biological systems are inherently more plastic than the mechanisms which control CORT production and clearance. In this way, CORT-induced changes in CBG capacity may act to fine tune stress responses in the short-term in comparison to the more sustained effects that CORT may have on HPA axis activity.

In mammals, exposure to GCs during development may cause elevated and prolonged release of CORT in response to an acute stressor by down-regulating the intracellular receptors resulting in reduced capacity of tonic inhibition and negative feedback ([Kapoor et al., 2006](#); [Matthews et al., 2004](#); [Seckl, 2004](#)). We found that exposure to GCs during development elevated stress

responses in zebra finches 30 days post-hatch. However, after 15 min of recovery from a standardized stressor we found no evidence that GC exposure during development changed negative feedback. In fact, after 15 min of recovery (i.e. 45 min after initial disturbance), birds had higher average levels of CORT when compared to levels after 30 min of stress, suggesting that negative feedback had not terminated the stress response at this point. Future studies could more directly test for effects of GC exposure during development on negative feedback by measuring CORT after longer periods of recovery or by measuring glucocorticoid receptor density or mRNA expression in the hippocampus (a section of the brain implicated in negative feedback of the HPA axis; e.g. [Liu et al., 1997](#)).

Female zebra finches 30 days post-hatch had elevated free baseline CORT compared to males. Similarly, females 60 days post-hatch had elevated total and free CORT responses compared to males. These effects were not influenced by treatment and appear to be specific to sex. Interestingly, sex effects were only present in zebra finches 30 and 60 days post-hatch. Sexes could be differentially sensitive to environmental conditions resulting in elevated CORT output or elevate HPA activity to facilitate growth and development at certain life history stages.

4.2. The effects of elevated CORT during development on body size and condition at 28, 60, and 90 days post-hatch

Exposure to CORT during development has been associated with decreased nestling growth in a number of bird species including Eurasian kestrels (*Falco tinnunculus*), barn swallows (*Hirundo rustica*), and song sparrows (females only, *Melospiza melodia*; ([Mueller et al., 2009](#); [Saino et al., 2005](#); [Schmidt et al., 2012](#)). In our study, CORT fed birds were smaller and in lower condition compared to control birds at 28 days post-hatch. However, differences in body size between treatment groups did not persist across life history stages: at 60 and 90 days post-hatch CORT-fed birds were the same size and in the same condition as control birds. The ability to 'catch up' in growth following stress exposure during development has previously been described suggesting that birds may compensate for poor developmental environments by accelerating growth once stressors have subsided ([Hayward and Wingfield, 2003](#); [Naguib et al., 2004](#); [Schmidt et al., 2012](#)). Such compensation in growth may not be possible in scenarios where developmental GCs organizes HPA axis such that animals are exposed to higher levels of endogenous CORT throughout their lifetime. However, we did not observe differences in HPA axis function at 60 and 90 days post-hatch. Additionally, we provided birds with *ad libitum* food. For these reasons, birds in our experiment would have had the opportunity to compensate for reduced growth once CORT treatment was terminated.

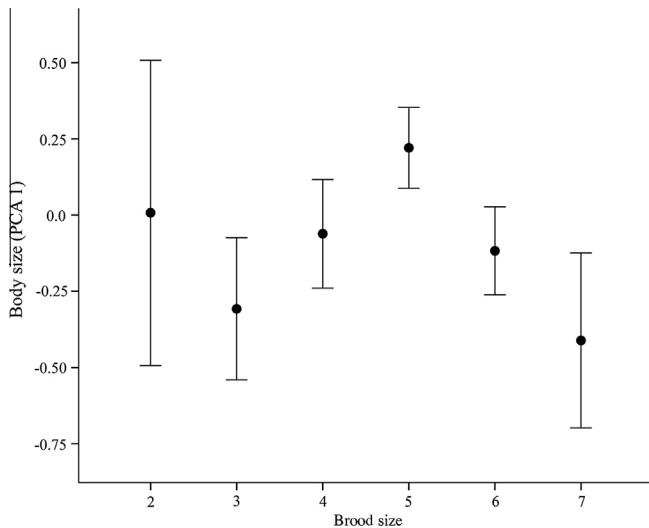
4.3. The effects of brood size on HPA activity, body size, and condition

Brood size has been shown to affect a range of phenotypic traits which ultimately shape adult fitness. Birds from experimentally enlarged broods have reduced growth, condition, immunocompetence, survival, and recruitment rates following migration ([Naguib et al., 2004](#); [de Kogel, 1997](#); [Saino et al., 2003](#); [Soma et al., 2006](#)). In zebra finches, the effects of brood size on body size has sex-specific transgenerational effects with females raised in experimentally enlarged broods raising female offspring which are smaller than male nest mates ([Naguib and Gil, 2005](#)). In our experiment, zebra finches reared in medium sized clutches were larger and in better condition compared to birds from small or large clutches (except 2-egg clutches, which overlapped in variation with all brood sizes). These differences were present at 30 days post-hatch with differences in body size persisting at 60 and 90 days post-hatch.

Table 2

Means values of tarsus, wing, mass, and condition for CORT exposed and control zebra finches at 28, 60, and 90 days post-hatch.

Variable	Day 28					Day 60					Day 90							
	Control (n = 81)		CORT (n = 80)		Difference		Control (n = 17)		CORT (n = 20)		Difference		Control (n = 55)		CORT (n = 54)		Difference	
	Mean	St. Dev.	Mean	St. Dev.	P	F	Mean	St. Dev.	Mean	St. Dev.	P	F	Mean	St. Dev.	Mean	St. Dev.	P	F
Tarsus (mm)	14.16	0.45	14.01	0.47	0.02	6.09	14.16	0.32	14.05	0.48	0.24	1.46	14.21	0.54	14.26	0.53	0.41	0.70
Wing (mm)	34.72	3.43	34.43	4.73	0.24	1.44	55.40	1.26	55.50	2.11	0.75	0.11	54.70	1.93	54.49	2.09	0.89	0.02
Mass (g)	11.07	1.13	10.91	1.13	0.03	5.58	14.40	0.93	14.30	0.89	0.87	0.03	14.26	1.23	14.40	1.18	0.51	0.44
Condition (g)	13.40	0.77	13.07	0.66	0.03	5.54	14.25	0.50	14.20	0.48	0.53	0.41	14.24	0.67	14.31	0.64	0.53	0.41

**Fig. 2.** Brood size affects body size at 28 days post-hatch with zebra finches reared in clutches of five nestlings being larger compared to zebra finches from smaller and larger broods.

Brood size also had a significant effect on free stress induced CORT in birds 30 days post-hatch with bird from medium sized broods having higher levels of free CORT. In altricial birds, HPA responses increase over the nestling and fledging periods (Wada et al., 2009). Birds that are the same nominal age, but larger may have greater CORT responses compared to smaller birds (Crino et al., 2011). In our experiment, birds from medium sized broods had greater free CORT responses and were larger than birds from small and large clutches. For this reason, it is likely that the elevated free CORT levels we observed in birds 30 days post-hatch result from accelerated development. The fact that brood size had no sustained effects on HPA activity at 60 and 90 days post-hatch suggests that HPA axis development can catch up in development, similar to the compensatory growth we observed in birds at 60 and 90 days post-hatch.

The majority of studies examining the effects of brood size on growth and condition focus on experimentally altered broods which are either small or large (often reduced or increased by two nestlings, e.g. Saino et al., 2003). Examining the effects of brood size on body size and condition across a natural range of brood sizes in this study revealed that nestlings from small and large clutches do worse compared to nestlings from medium sized clutches. Presumably, natural selection acts to shift the mean brood size to a size which produces the optimal number of high quality nestlings. Nestlings from large clutches may do poorly because they compete for limited resources. Although parents can increase provisioning rate for large broods (García-Navas and Sanz, 2010; Mock et al., 2009, but see Saino et al., 1997) the increase might not be enough to compensate for the increased demand of

additional nestlings in very large broods (Hainstock et al., 2010; Martin, 1987; Saino et al., 2000; Tieleman et al., 2008). Nestlings from small broods may fare poorly because of reduced paternal investment. In species with extra-pair copulations (EPCs), males may provision nestlings less because the benefit of rearing a small brood does not outweigh the costs in forgone extra pair mating opportunities (Magrath and Elgar, 1997). In other words, with small broods, males could maximize their fitness by provisioning nestlings less and pursuing more EPCs. Alternatively, small broods which were originally larger but resulted from incomplete hatching or early death of nestlings could be of overall lower quality due to poor quality parents (Costantini et al., 2009). Future studies in free living birds could evaluate these hypotheses.

5. Conclusions

Previous studies have shown that GC exposure during development has long-term effects on HPA axis activity. Conversely, we found treatment with CORT resulted in short-term elevation of HPA axis activity, but that this effect was not sustained across life history stages. This suggests that developmental GC exposure does not always have programmatic responses on the acute stress response or negative feedback and that stress responses may be more indicative of the current state of an animal rather than its developmental history. CORT exposure decreased body size and condition in our experiment. Similar to the short term effects on HPA axis activity, these effects were not permanent. In contrast to the transient effects of CORT treatment on body size, brood size had strong effects on body size and condition. These findings suggest that some developmental conditions (e.g. brood size) may have stronger programmatic effects than others (e.g. CORT exposure).

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