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Developmental stress in birds: phenotypic and fitness

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DEVELOPMENTAL STRESS IN BIRDS: PHENOTYPIC AND FITNESS CONSEQUENCES

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The environment animals experience during development can have important effects on phenotype, performance, and fitness across multiple life-history stages. Environmental cues experienced during development can provide information to animals about the environment they will soon inhabit and promote phenotypic changes which affect fitness. Increasing evidence suggests that *physiological stress* may be one such cue that conveys environmental information to developing animals.

Here, I explore the short- and long-term consequences of developmental stress in captive and free-living birds. In chapter one, I explore the effects of developmental stress on body size and physiological stress responses across life-history stages in zebra finches (*Taeniopygia guttata*). I found that developmental stress increases stress response and decreases body size in juvenile zebra finches.

In chapter two, I examine the effects of developmental stress on learning in zebra finches. Developmental stress has well-known suppressive effects on song learning in passerines. I examine whether this is generalizable for other types of learning, specifically learning that relates to foraging. I found that adult zebra finches exposed to developmental stress learned a novel foraging task faster compared to control siblings.

In chapter three, I investigated the effects of developmental stress on male reproductive success in zebra finches. I found that developmentally stressed males invested more in parental care and reared nestlings in better condition compared to control males. Developmentally stressed males also sired more offspring and were less likely raise non-genetic nestlings compared to control males.

In chapters four and five, I explore the causes and consequences of stressors in a free-living model species, the white-crowned sparrow (*Zonotrichia leucophryus oriantha*). I examine the effects of an anthropogenic stressor (a high traffic road) on nestling stress responses, growth, and survival. I found that proximity to a road increased both nest failure due to predation and nestling stress responses.

Cumulatively, these studies expand our understanding of the phenotypic and fitness consequences of developmental stress. In contrast to most studies, I find several beneficial outcomes in response to developmental stress. Hence, early life stress appears to shape phenotype and performance in some ways that are beneficial.

Chapter 1: Developmental stress has sustained but not lifelong effects on body size and total and free corticosterone responses in the zebra finch

Abstract

Animals exposed to stress during development experience sustained morphological, physiological, neurological, and behavioral consequences. In particular, elevated glucocorticoids (GCs) during development can increase GC secretion in adults. Studies have examined the effects of developmental stress 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 effect of elevated corticosterone (CORT) during development on long-term GC activity, CBG capacity, body size, and condition at 30, 60, and 90 days post-hatch in the zebra finches. CORT exposed birds had higher GC activity 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 30 days post-hatch, but these differences were not present at later life history stages. However, clutch size had a sustained effect on body size such that birds reared in medium sized clutches were larger at 30, 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.

Keywords: Developmental stress, corticosterone, nestling, passerine, body size, clutch size

Abbreviations used

1. **CBG –** corticosteroid binding globulin
2. **CORT –** corticosterone
3. **EPC –** extra pair copulation
4. **GC -** glucocorticoid
5. **HPA –** hypothalamic pituitary adrenal

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; 20). Specifically, animals exposed to elevated levels of GCs during development can experience sustained morphological, physiological, neurological, and behavioral consequences (reviewed in 27, 34, 45). In some cases, these phenotypic effects appear to be life-long and can even be transmitted across generations (6, 18, 46, 52).

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. 24, but see 17, 19). 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 (14). Postnatal GC exposure has 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 (49). 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 (37). These studies, along with many studies from mammals (e.g. 18, reviewed in 27), 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 on HPA function, no studies have 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," 28, 22, 5). Stress 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 minutes 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 hours following acute stressors (23, but see 31). 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 developmental stress 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 higher 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 developmental stress 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 X 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. Nestlings were then randomly assigned to treatment groups (CORT or 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 hours ± 1 hour 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 -28 days post-hatch (methods as per 49).

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 cues, 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 (70X40X44cm) where they were housed until the nestlings reached nutritional independence at 28 days post-hatch (49). 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 60 and 90 days post-hatch. We calculated condition for birds at 28, 60, and 90 days post-hatch using the scaled mass index (35, 36). 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 (36).

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 (53). 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 minutes after initial disturbance. To collect blood, we punctured the alar vein with a 26-gauge needle and collected 25-50µl of blood with heparinized microcapillary tubes. Immediately after collection, blood was kept in a 4°C refrigerator on ice (<1 hour) 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=116, $F_{1,115}$=24.99, r_s=0.18, P<0.01). To account for this, we used linear regression with the amount of time to collect initial blood samples as an independent variable and CORT measurements as a dependent variable. We obtained unstandardized residuals from this analysis which we used in all analyses of baseline CORT.

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 minutes in a cloth bag (stress induced CORT) and then again after 15 minutes of release into their home cage (recovered CORT).

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., 2009). 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 (5, 3, 22). 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 3). This assay has been optimized for zebra finch adults (51) and used for zebra finch nestlings (51). 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 minutes and then vortexed at 4˚C, 10,000rpm for 10 minutes. The stripped plasma supernatant was removed and stored at -20˚C until assayed. We determined total CBG binding using 50µl buffer, 50µl tritiated CORT, and 50µl stripped, diluted plasma (for a 1:1050 final dilution of raw plasma). Non-specific binding (NSB) was determined using 50µl of 1 µM unlabeled CORT, 50µl tritiated CORT, and 50µl 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):

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

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 0-3, 10, and 30 minute samples (CBG capacity does not decline in zebra finches with 30 minutes of handling stress, 4).

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 minutes of stress exposure. Additionally, we examined the amount of total and free CORT released during 30 minutes of stress exposure (total integrated CORT). Total integrated CORT represents that total amount of CORT target tissues are exposed to during an acute stressor.

2.5. *Statistical analyses*

All CORT data were non-normally distributed (Shapiro-Wilk's $P<0.02$). Log transformations failed to normalize data. For this reason we used non-parametric tests to statistically evaluate differences in CORT physiology between treatment groups. CBG values were normally distributed and we used analysis of variance (ANOVA) to statistically evaluate differences between treatment groups. To assess differences in negative feedback, we calculated the rate of change of CORT secretion between CORT titers after 30 minutes of restraint stress and after 15 minutes of recovery. We compared slopes between treatment groups using ANCOVA. There was no effect of sex on CORT output at any age ($P>0.17$ for all).

Clutch size affected body size and condition at 28, 60, and 90 days post-hatch. We used general linear models to examine the effects of treatment on body size with clutch size and treatment as fixed factors. There was no effect of sex on body size or condition at any age ($P>0.17$, $F<2.01$ for all). 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.

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 10 minute CORT levels (Fig. 1, Table 1, N=54, 28, $P<0.001$, 0.003 respectively) but no difference at 30 minutes post-handling, and no difference in CBG (Fig. 1, Table 1, N=28, $P>0.24$). Integrated CORT levels were elevated in CORT-exposed individuals as compared to controls (Fig. 2, Table 1, N=28, $P=0.004$). Calculated free CORT levels reflect patterns in total CORT, except that the 10 minute sampling time shows a non-significant trend (Fig. 1, Table 1, N=28, $P=0.058$).

There were no differences in any measure of total or free CORT output or CBG levels in zebra finches 60 or 90 days post hatch (Figs. 2, 3, 4, Table 1, $P>0.13$ 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; U=29.0, 29.0, P=0.004, 0.02). There was no difference in total CORT after 30 minutes of stress exposure between treatment groups (Fig. 5, N=12, 14, U=52.0, P=0.11). After 15 minutes of recovery, there was no difference between treatment groups levels in absolute levels of total and free CORT (N=12, 14, U=66.5, 50.0, P=0.37, 0.35) nor any difference in slopes (rate of return to baseline) for total or free CORT (N=12, 14, U=66.0, 36.0, P=0.37, 0.12).

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 ($F_{1,28}$=6.09, 5.58, 5.54, P=0.02, 0.03, 0.03 respectively, Table 2). There were no differences in body size or condition between treatment groups at 60 and 90 days post-hatch (F<1.46, P>0.24 for all, Table 2).

In contrast to the transient effects of treatment on body size, clutch size affected all body size measurements (F > 4.20, p<0.05 for all) except mass at 28 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). Clutch 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, clutch size affected body size in an inverted-U-shaped function with zebra finches reared from clutches of five nestlings being larger compared to zebra finches reared in smaller and larger clutches (e.g. Fig. 6). Clutch size had similar effects on condition.

Discussion

Previous studies in birds have shown that developmental stress 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. We found that CORT exposure decreased body size and condition in birds 30 day post-hatch, but there were no effects of treatment on body size or condition at 60 and 90 days post-hatch. In contrast, there were sustained effects of clutch size on body size present 30, 60, and 90 days post-hatch suggesting that clutch size has programmatic effects on morphology.

4.1. The effects of developmental stress on stress physiology at 30, 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. In mammals, developmental stress 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 (16, 26, 47). We investigated a possible change in neural receptors through measurement of negative feedback once the stressor had ended. We found no difference in CORT recovery between treatment groups suggesting that developmentally induced changes in CORT secretion are not occurring via changes in negative feedback. Future studies could directly test this by measuring the effects of CORT exposure during development on glucocorticoid receptor density or mRNA expression in the hippocampus (a section of the brain implicated in negative feedback of the HPA axis; e.g. 18).

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 minutes 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 (11). 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. 7, 15, 10). The stress response has a strong genetic component (43) 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 minutes 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 (4, 23). However, exposure to developmental stress 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.

4.2. The effects of developmental stress on body size and condition at 30, 60, and 90 days post-hatch

Developmental stress (elevated CORT) 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*; 31, 42, 44). 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 (14, 32, 44). Such compensation in growth may not be possible in scenarios where developmental stress organizes HPA axis such that animals are exposed to higher levels of endogenous CORT throughout their lifetime. However, because we did not observe differences in HPA axis function at 60 and 90 days post-hatch, birds in our experiment would have had the opportunity to compensate for reduced growth once CORT treatment was terminated.

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

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 (32, 9, 41, 48,). In zebra finches, the effects of clutch 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 (33). 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 clutch sizes). These differences were present at 30 days post-hatch with differences in body size persisting 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. 41). Examining the effects of clutch size on body size and condition across a natural range of clutch sizes in this study revealed that nestlings from small and large clutches do worse compared to nestlings from medium sized clutches. Nestlings from large clutches may do poorly because they compete for limited resources. Although parents can increase provisioning rate for large broods (12, 29, but see 39) the increase might not be enough to compensate for the increased demand of additional nestlings in very large clutches (13, 25, 40, 50). Nestlings from small clutches 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 clutch does not outweigh the costs in forgone extra pair mating opportunities (21). In other words, with small clutches, males could maximize their fitness by provisioning nestlings less and pursing more EPCs. Alternatively, small clutches 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 (8). Future studies in free living birds could evaluate these hypotheses.

5. Conclusions

Previous studies have shown that developmental stress 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 stress does not always have programmatic responses on HPA activity and that stress responses may reflect 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, clutch size had strong effects on body size and condition. These findings suggest that some developmental conditions (e.g. clutch size) may have stronger programmatic effects than others (e.g. CORT exposure).

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Variable	Day 30							Day 60							Day 90						
	Control (n=15)		CORT (n=13)		Difference			Control (n=11)		CORT (n=13)		Difference			Control (n=18)		CORT (n=20)		Difference		
	Mean	SD	Mean	SD	P	U		Mean	SD	Mean	SD	P	U		Mean	SD	Mean	SD	P	U	
Total baseline CORT (ng/ml)	1.06	1.01	2.41	1.63	**0.001**	166.0		0.54	0.81	0.38	0.40	0.53	60.0		0.51	0.66	0.41	0.50	0.87	174.5	
Total CORT 10 min (ng/ml)	5.43	2.54	9.65	3.89	**0.003**	38.0		3.01	2.67	3.87	1.69	0.13	45.0		4.01	2.32	4.00	2.40	0.92	176.0	
Total CORT 30 min (ng/ml)	4.58	2.63	8.26	6.60	0.41	79.0		5.68	3.93	5.15	2.59	0.93	63.0		6.51	3.32	5.77	3.89	0.35	147.0	
Total integrated CORT (ng/ml)	117.85	33.27	213.24	96.51	**0.004**	37.0		90.20	71.54	94.21	27.50	0.38	50.0		107.52	48.90	100.90	54.45	0.57	160.0	
CBG (ng/ml)	155.54	62.74	171.09	54.12	0.24	252.0		75.57	37.81	74.36	27.66	0.85	55.0		1065.76	591.35	1360.22	745.64	0.180	133.0	
Free baseline CORT (ng/ml)	0.06	0.07	0.10	0.08	**0.02**	184.0		0.05	0.10	0.03	0.04	0.56	49.0		0.002	0.003	0.002	0.002	0.59	161.0	
Free CORT 10 min (ng/ml)	0.30	0.30	0.40	0.19	0.058	57.5		0.30	0.46	0.34	0.36	0.36	44.0		0.02	0.02	0.01	0.002	0.36	148.0	
Free CORT 30 min (ng/ml)	0.18	0.10	0.45	0.66	0.58	285.5		0.40	0.46	0.33	0.36	0.99	58.0		0.03	0.02	0.02	0.03	0.20	135.0	
Free integrated CORT (ng/ml)	5.94	4.77	9.56	6.83	**0.03**	51.0		7.55	10.25	6.42	6.57	0.43	46.0		0.49	0.38	0.38	0.36	0.17	132.0	

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

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 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

Table 2. Means values of tarsus, wing, mass, and condition for CORT exposed and control zebra finches at 30, 60, and 90 days post-hatch.

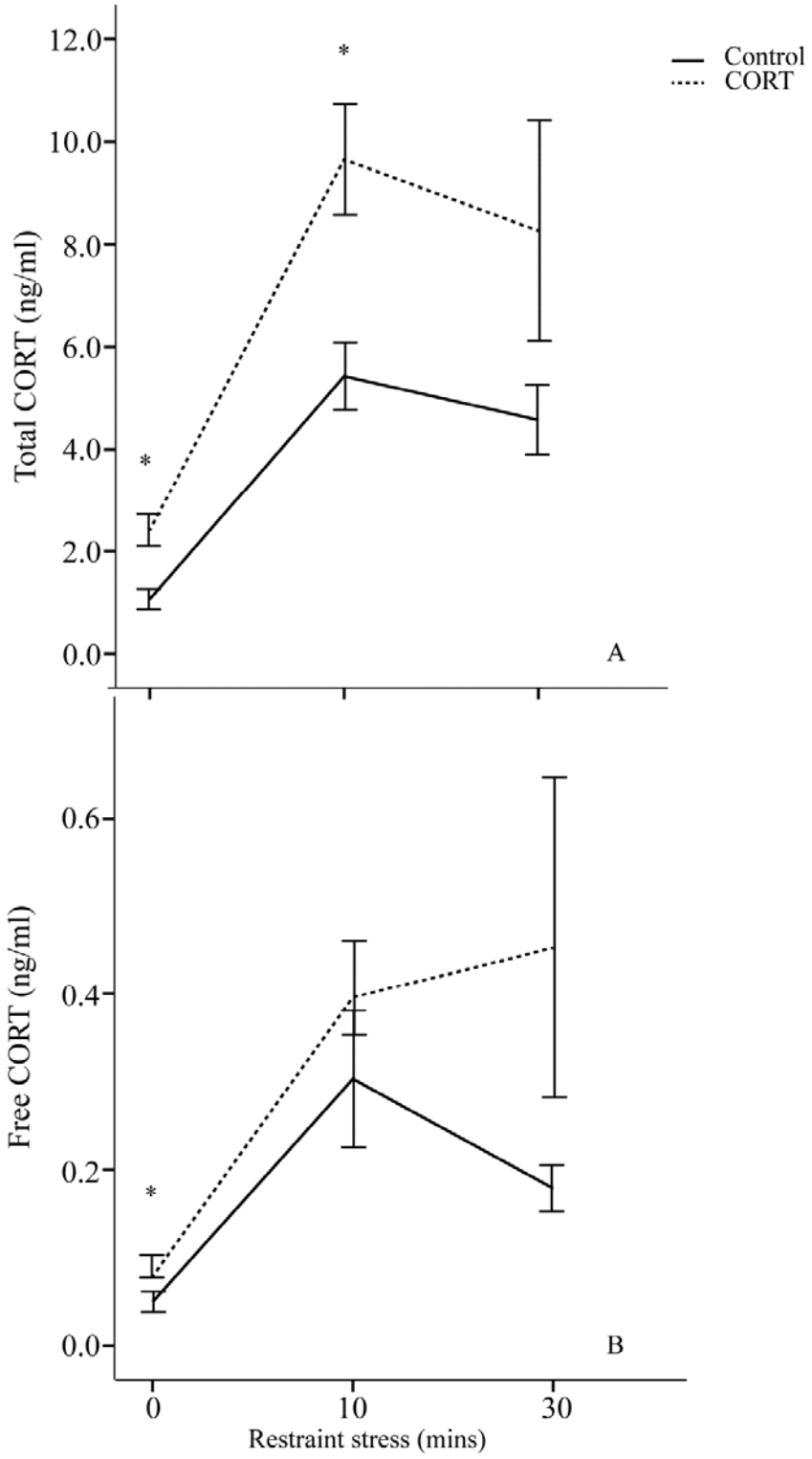

Figure 1. CORT-fed zebra finches had A) elevated total baseline CORT and CORT after ten minutes of restraint stress and B) elevated free baseline CORT and free CORT after ten minutes of restraint stress.

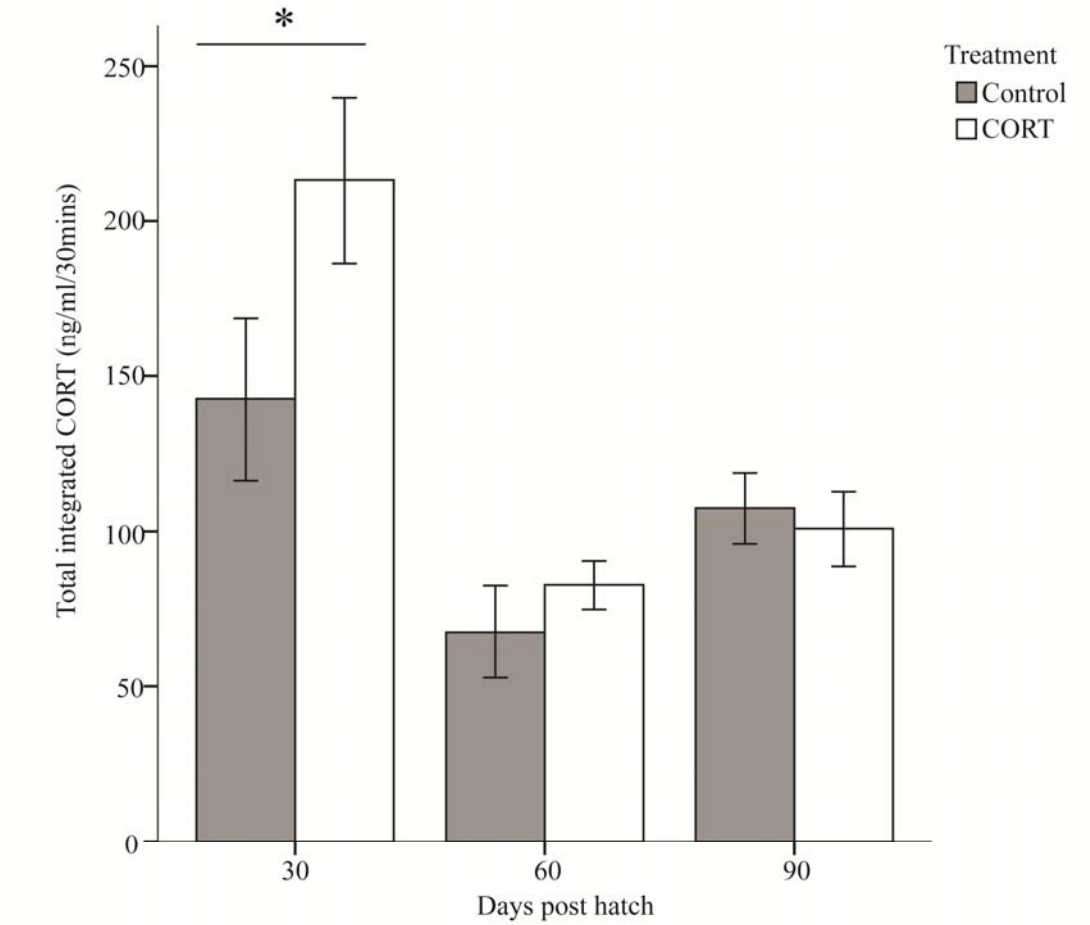

Figure 2. At 30 days post-hatch, zebra finches exposed to CORT had higher total integrated CORT responses. There were no differences in total integrated CORT responses at 60 and 90 days post-hatch.

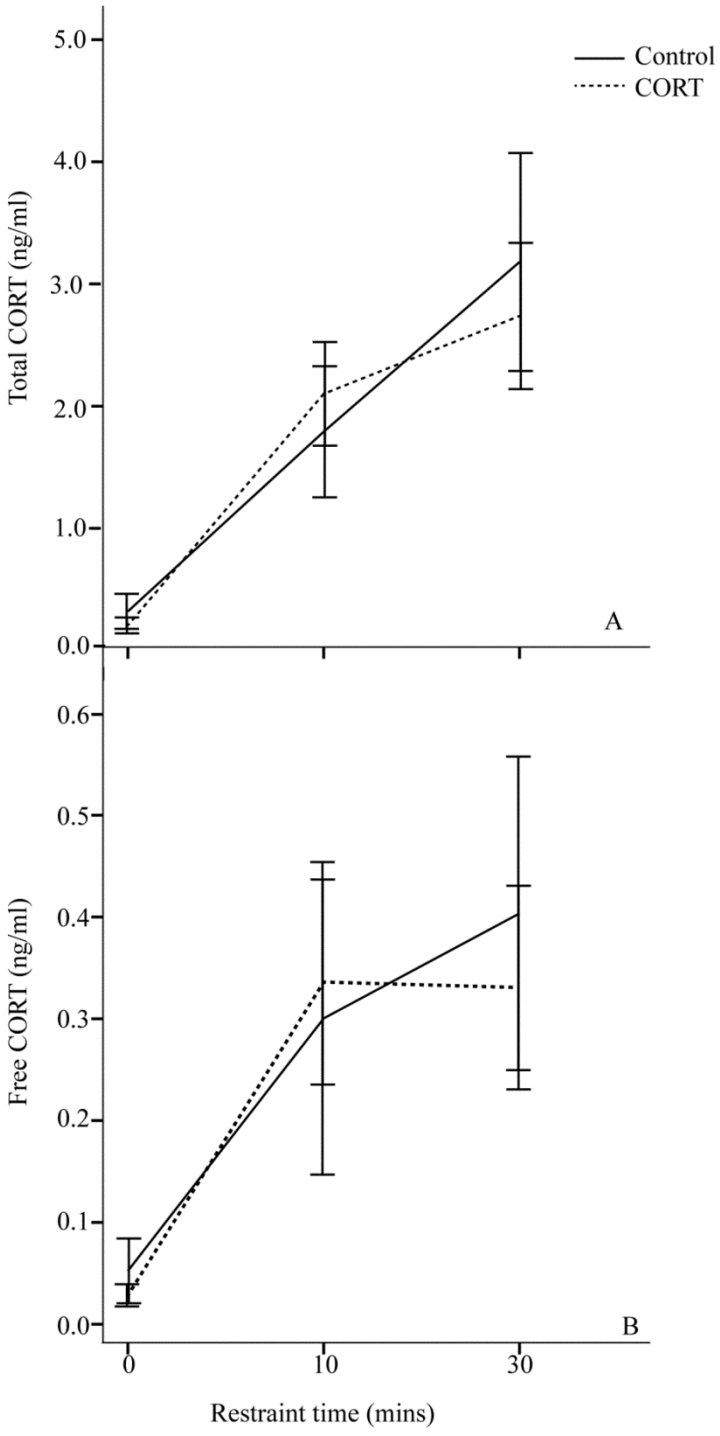

Figure 3. At 60 days post-hatch, there was no differences between treatment groups in total and free baseline CORT or CORT secretion following restaint stress.

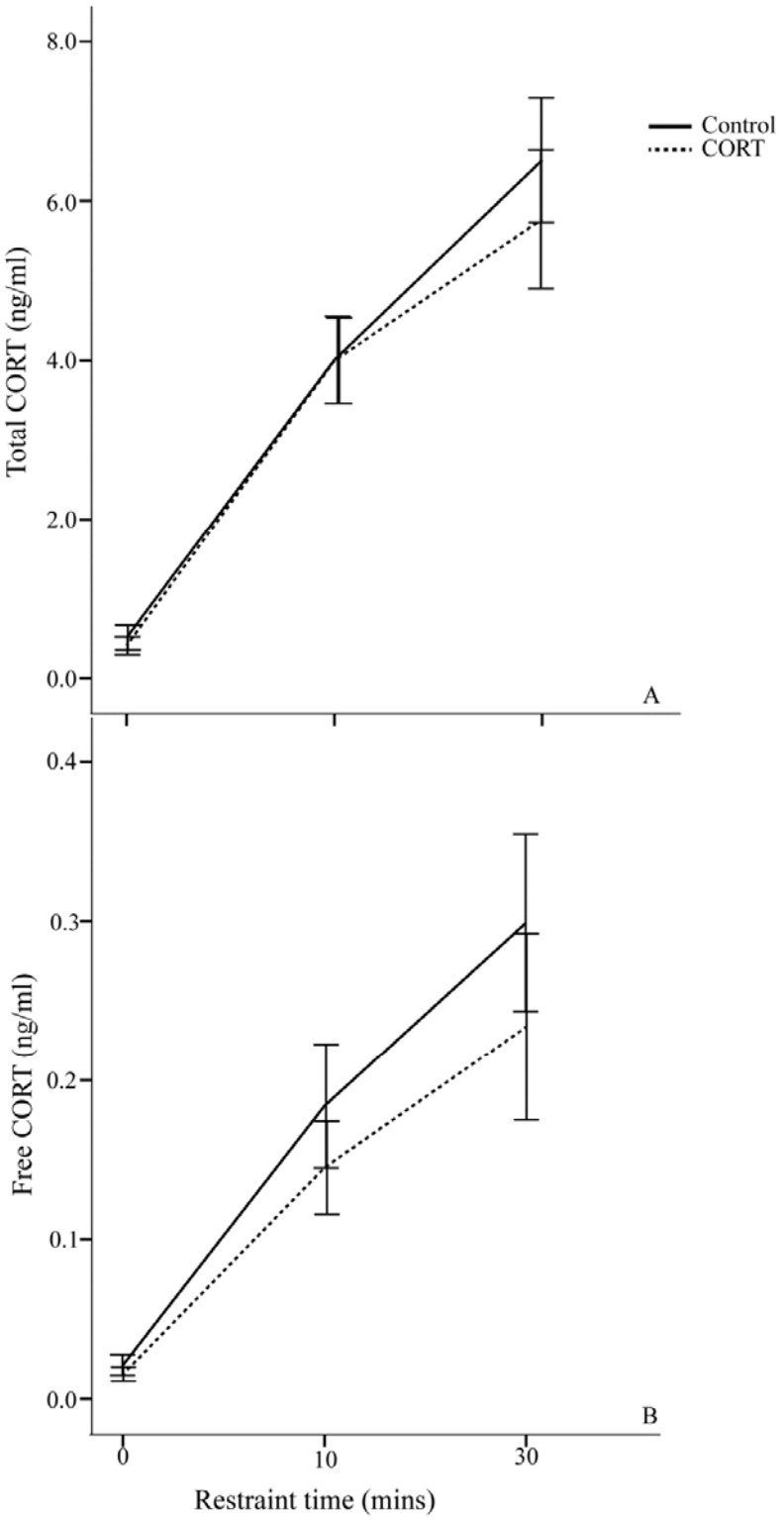

Figure 4. At 90 days post-hatch, there was no differences between treatment groups in total and free baseline CORT or CORT secretion following restaint stress.

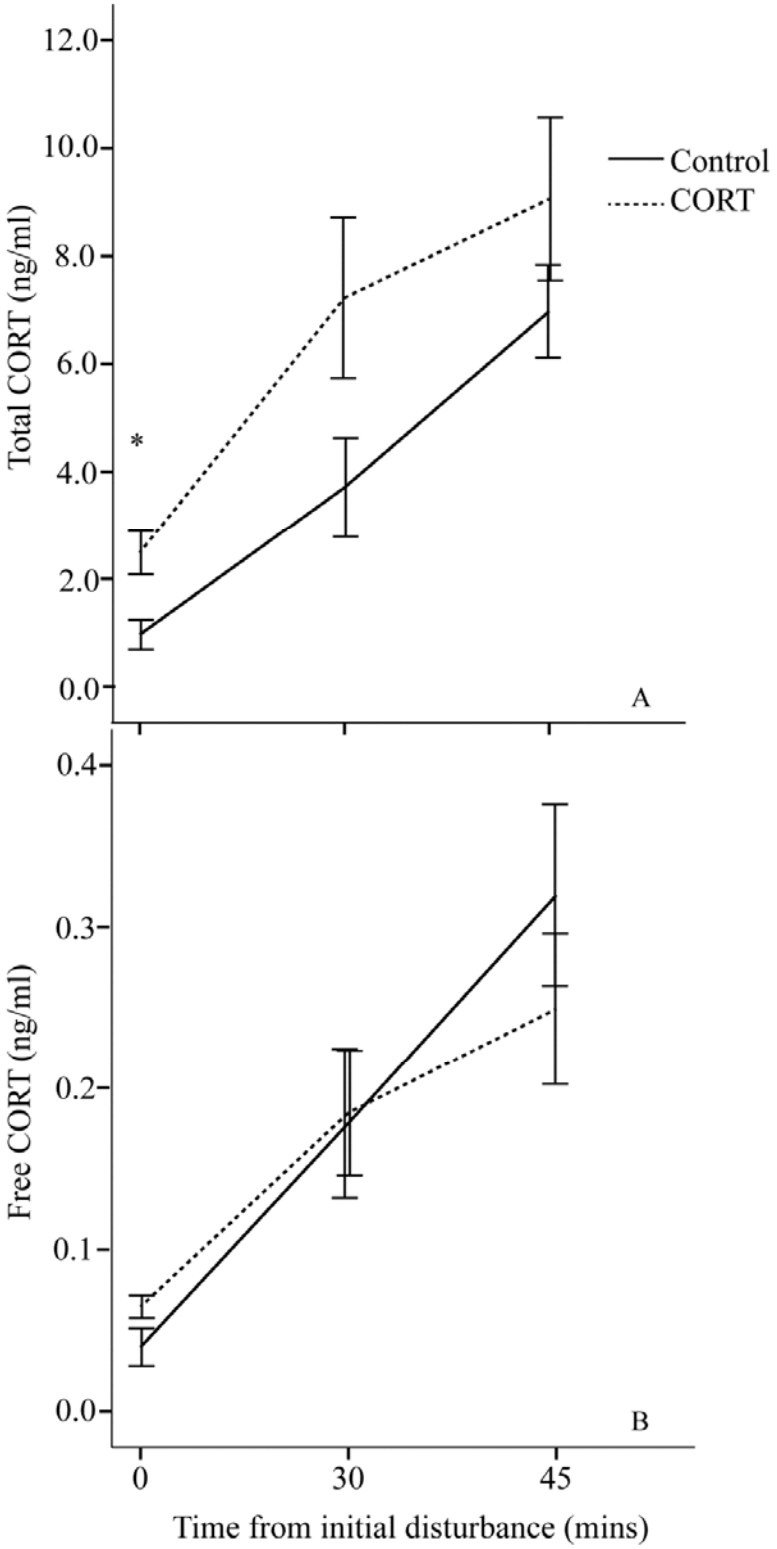

Figure 5. At 30 days-post-hatch, there was no difference between treatment groups in the rate of return of stress-induced CORT to baseline CORT,

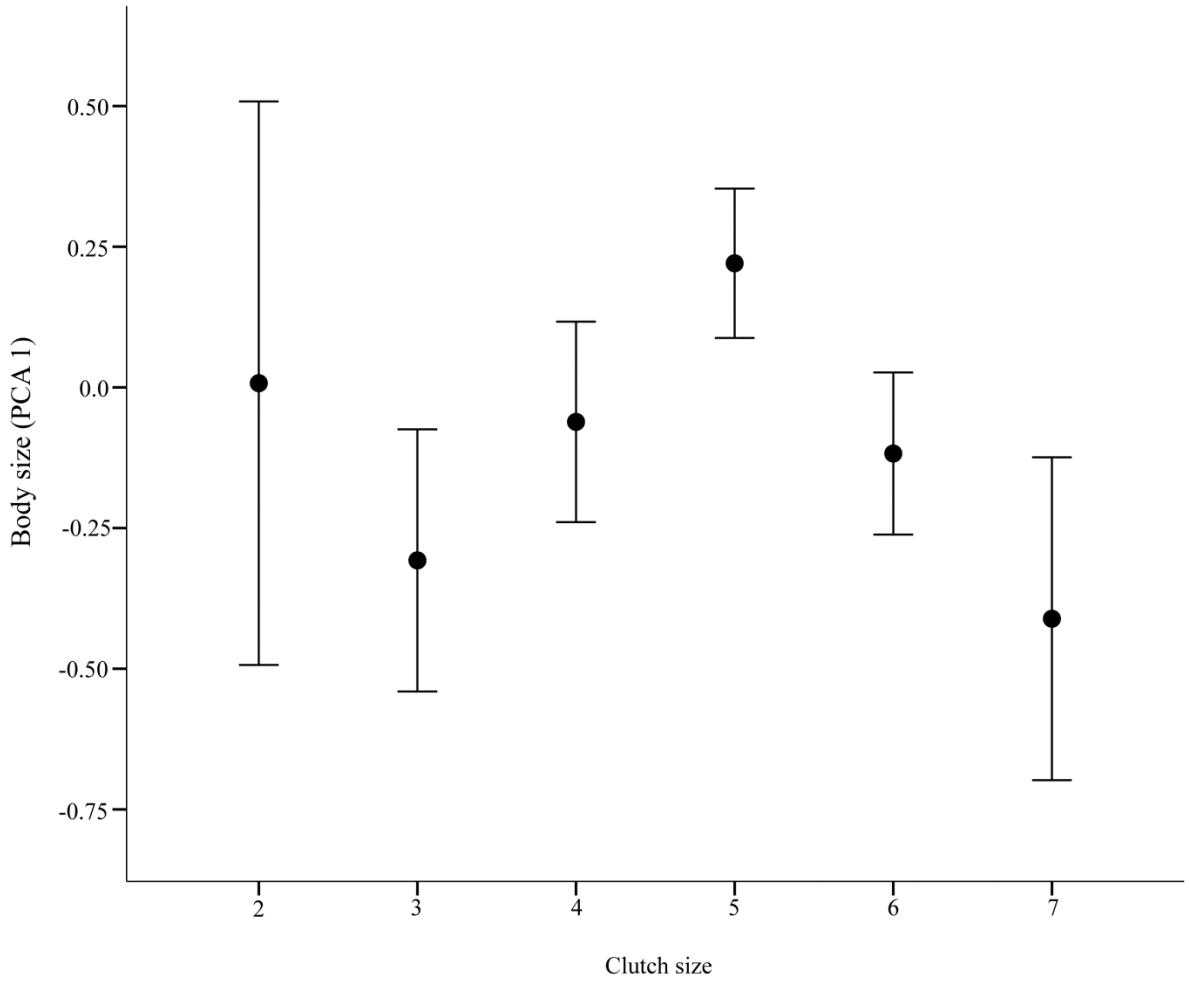

Figure 6. Clutch size affects body size at 30 days post-hatch with zebra finches reared in clutches of five nestlings being larger compared to zebra finches from smaller

Chapter 2: Developmental stress improves performance on a novel foraging task in zebra finches

Abstract

Exposure to stress during development is known to affect a range of phenotypic traits in later life history stages. For example, developmental stress has well known negative effects on the ability of song birds to learn their species specific song. Bird song is an important sexually selected trait and it has been proposed that the link between early development and adult song indicates an individual's ability to cope with early environmental adversity and, thus, provides important information to prospective mates (the developmental stress hypothesis). Developmental stress appears to affect learning broadly, but the direction of effect is not always consistent between studies. The effects of developmental stress on phenotype are broad and it is possible that developmental stress elicits physiological changes that indirectly influence learning ability. Here, we examine the effect of developmental stress on the ability of zebra finches to learn a novel foraging task. Additionally, we evaluate whether developmental treatment affects metabolic rate which could explain variation in learning food-based tasks. We found that birds exposed to developmental stress solved a novel foraging task in fewer trials compared to control siblings. We found no difference between treatment groups in metabolic rate. Variation in the ability to solve the foraging paradigm was largely explained by the time it took birds to pass an initial learning stage which involved associating with the learning apparatus for the first time. Therefore, it is possible that the differences we observed in the ability to solve the foraging task were driven by differences in neophobia between the treatment groups rather than difference in learning *per se.* Our data suggest that alternative modes of learning can be differentially affected

by developmental stress, and support the potential for early environmental stress to increase performance in adult tasks.

Introduction

Stress during development can affect a range of physiological and behavioral systems resulting in outcomes such as reduced growth, impaired immunocompetence, and altered neurological function (e.g. Loiseau et al. 2008, Müller et al. 2009, Liu et al. 1997, Weaver et al. 2004). Phenotypic effects shaped by developmental stress can be sustained across an animal's lifetime and, in this way, may have important effects on fitness across life-history stages (reviewed in Matthews 2005, Nesan et al. 2005, Schoech et al. 2011, Spencer and Mac-Dougall-Shackleton 2011). For example, songbirds learn their species-specific song early in life (Marler 1970, Beecher and Brenowitz 2005, Brenowitz and Beecher 2005). Developmental stress decreases development of the brain regions which control song learning and production (Buchanan et al. 2004, Nowicki et al. 2002). As adults, birds exposed to stress during development (e.g. food limitation or elevated glucocorticoid stress hormones) sing less complex songs as adults and are, consequently, less preferred by females (Buchanan et al. 2003, Nowicki et al. 2002, Spencer et al. 2003, 2005). In this way, adult song signals an individual's ability to cope with an adverse environment during development and is a reliable signal for mate choice (i.e. the developmental stress hypothesis, Nowicki et al. 1998, 2002, Spencer et al. 2003).

Over the years, substantial evidence from studies in both free-living and captive birds has supported the developmental stress hypothesis (reviewed in Spencer and MacDougall-Shackleton 2011). Recently, the evaluation of this hypothesis has been

expanded to examine how developmental conditions affect learning tasks other than song learning. For example, Bonaparte et al. (2011) restricted the protein content of food for developing zebra finches (*Taeniopygia guttata)* and found that food restricted birds had reduced ability to solve an associative learning task as adults (175 days post-hatch). Elevated levels of glucocorticoids (GCs) during development similarly affect learning. Black-legged kittiwakes chicks exposed to elevated levels of corticosterone (CORT; the dominant avain GC) had a reduced ability to complete an associative learning task as a juvenile and continued to perform poorly 8 months later as an adult (Kitaysky et al. 2006). Other studies have found a positive effect of developmental stress on learning. Domesticated chickens (*Gallus gallus*) subjected to social stress during development performed better at an associative learning task compared to control birds (Goerlich et al. 2012). Likewise, Japanese quail exposed to stress during development displayed enhanced behavioral flexibility in a spatial memory task (Calandreau et al. 2011).

Developmental stress is known to affect a range of phenotypic traits that could indirectly influence learning and potentially explain variable results between studies. For example, in zebra finches, treatment with CORT during the nestling period increased variability in overnight standard metabolic rate (Spencer and Verhulst 2008). However, this effect was only observed during the treatment period and not in adulthood (Spencer and Verhulst 2008). In contrast, Schimidt et al. (2012) showed that developmental stress permanently increases standard metabolic rate in female song sparrows (*Melospiza melodia*). Developmental stress has also been show to affect activity level and behaviors such as neophobia which could confound the results of experiments which measure learning using novel objects. Studies which comprehensively evaluate the effects of

development stress on phenotypic traits that could affect learning could help elucidate the relationship between development stress and learning.

We examined the effects of elevated CORT during the nestling period on adult learning in zebra finches. We fed zebra finches CORT during the nestling period and measured learning in adult birds (60 days post-hatch) using a foraging paradigm that quantifies the ability of birds to learn to access a hidden seed reward. This learning paradigm has been used previously to measuring learning in zebra finches (Boogert et al. 2008, Grindstaff et al. 2012). Based on the known effects of developmental stress on learning, we predicted that zebra finches fed CORT during development would solve the learning task more slowly than control birds. In addition, we tested a possible mechanism that could explain differences between treatment groups. We investigated the effect of developmental stress on basal metabolic rate. Developmental stress can affect metabolic rate in birds (e.g. Schmidt et al. 2012, Spencer and Verhulst 2008). Differences in metabolic rate between CORT-fed and control birds would indicate greater motivation to feed in one group. This may help explain variation in the performance on the food-based learning test.

Methods

Study population

All research was approved by the University of Montana Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1985). We obtained adult domesticated zebra finches 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. Breeding finches were housed in a 20 X 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.

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. Nestlings were then randomly assigned to treatment groups (CORT or 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 hours ± 1 hour 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 -28 days post-hatch (methods as per Spencer et al. 2009).

Learning paradigm

We measured learning ability in zebra finches 60 days post-hatch (± 2 days) using a foraging paradigm with four levels of escalating difficulty (methods as per Boogert et

al. 2008). We presented birds with a plastic grid (26 x 22 x 2 cm; see figure 1) containing ten wells (0.8 cm deep and 1.3 cm wide) covered with lids fitted with rubber bumpers on the bottom (3.5 cm in diameter). For each level, we placed two seeds of millet in every well of the testing apparatus. In order to proceed to the next level of difficulty in the paradigm, birds had to access and eat the seeds from at least two wells. For the first level of difficulty, we placed the lids next to the holes. For the second level, we placed the lids so they covered half of each well. For the third level we covered the wells with the lids entirely and for the fourth level we pushed the rubber bumpers into the wells. In order to pass the fourth level of difficulty, birds had to pry the lids off with their beaks to access the seeds. Birds that solve the task in the fewest trials are considered superior learners (Boogert et al. 2008).

We isolated test birds in wire cages (33 x 38 x 43 cm) 24 hours preceding the learning test. To prevent the birds from seeing each other during the test we placed opaque barriers between the cages. Twelve hours before the learning test we removed all food from the cages to ensure that test birds were equally motivated to solve the foraging paradigm. Throughout the course of the experiment birds had access to water *ad libitum*. We started the learning trials at 0730 hours. For each learning trial, birds had 15 minutes to solve the task and pass to the next stage. If a bird did not pass a stage, it was given access to all the seeds for 45 minutes before starting the next trial. After failing to pass a stage, bird were exposed to the previous stage in the next trial. For example, if a bird failed to pass stage three, it was presented with the stage two paradigm in the next trail and had to pass this stage once again before attempting stage three a second time. The learning test spanned two days with a total of 17 trials to solve the task: nine trials on the

first day and eight trials on the second day. We used the cumulative number of trials birds needed to solve the final stage of the learning test as the measure of learning performance. If birds failed to solve the final stage of the learning test they were assigned a learning score of 18.

Basal metabolic rate

We measured basal metabolic rate (BMR) of birds 60 days post-hatch (± 2 days) as oxygen consumption ($\dot{V}O_2$) in an open flow system using an oxygen analyzer (Foxbox; Sable System). On the day of the measurement, food and water were removed from the cage three hours before the beginning of the metabolic recording to have the sample in post absorptive state. The test was performed starting at 2000 h to overlap the night cycle of the birds and measure metabolic rate in a resting state. We recorded $\dot{V}O_2$ of birds between 58 and 62 days of age (with day zero being hatch date). We measured body mass of our samples using an ACCULAB portable electronic scale (ACCULAB, Elk Grove, IL, USA) with an accuracy of $\pm$ 0.001 g. Each bird was put in a stainless steel airtight metabolism chamber 3.2 liters in size, where it could perch on an iron mesh. The chamber sat in a large insulated box with a Peltier device (Pelt-4; Sable Systems) to maintain T at 30 ± 0.1°C. This temperature is within the thermo neutral zone of zebra finches. The chamber was connected to an open flow system and flushed with 200 milliliters per minute flow of atmospheric air scrubbed from CO_2 and water vapor. This flow rate guarantees a stable proportion of oxygen always available for a bird of 13-16 grams in size. Exiting air was filter through scrubbers with soda lime, magnesium perchlorate and Drierite to remove water and CO2. We allowed birds to acclimate to the

chamber for one hour and then measured $\dot{V}O_2$ continuously every 0.5 seconds until a plateau (maximum oxygen consumption) was reached and maintained. The total amount of time needed to complete a measurement ranged from 210 to 320 minutes. $\dot{V}O_2$ (mL h-1) was calculated as the O_2 concentration value for the most stable 10 minutes of oxygen consumption within the plateau using ExpeData (ver. 1.3.2) software of Sable Systems.

Statistical analyses

The number of trials needed to pass all four learning stages were non-normally distributed (Shapiro-Wilk, $P<0.01$ for all). We log transformed the number of trials and used the resulting values in unpaired analyses. We used univariate analysis to analyze the learning data with sex and treatment as fixed factors. We found no effect of sex on learning ($P>0.57$, $F<0.11$, d.f.=57 for all), and so sex was not used as a factor in our final analysis. We compared siblings from different treatment groups using paired t-tests with raw values for number of trials as dependent variables. We used univariate analysis of variance (GLM) to analyze differences in the rate of learning between treatment groups (i.e. how rapidly birds progressed through learning stages) with the number of trials to pass each stage as a dependent variable and treatment and stage as fixed effects. We used a treatment by stage interaction to test for differences in rate of learning (slopes) between treatment groups.

We used linear regression to evaluate average VO_2 consumption against mass. There was a non-significant trend for basal metabolic rate to increase with increasing body mass ($P=0.083$, $F=3.32$, d.f.=1,20, $r^2=0.38$). Body mass has well known effects on metabolic rate and the non-significant trend of our data support this relationship. For that

reason, we used residual VO_2 consumption after accounting for body mass in all analyses of basal metabolic rate. We used univariate analysis of variance (GLM) with sex and treatment as fixed factors to examine the effects of sex on basal metabolic rate. Sex had no effect on basal metabolic rate (P=0.8, F=0.18, d.f.=1,21), and so was excluded from further analyses. We used analysis of variance (ANOVA) to examine differences in basal metabolic rate between treatment groups. We calculated effect size for metabolic data as the difference between the means of the two treatment groups divided by the pooled standard deviation (Cohen's *d).* We considered values above 0.5 to indicate an adequate sample size (Cohen 1992).

Results

Learning paradigm

Zebra finches exposed to CORT during development solved each stage of the novel task in fewer trials than control siblings (Fig. 2; P<0.02, t<-2.61, d.f.=18 for all). However, once we accounted for differences in the number of trials needed for each pair to pass stage one, there were no differences in the number of trials needed to pass each subsequent stage (P>0.43, t>-0.19, d.f.=18). In other words, the differences between the treatment groups in the number of trials needed to pass stages two, three, and four were driven entirely by the difference in the number of trials needed to pass stage one (Fig. 3). Furthermore, there was no difference in the rate of learning between the two treatment groups (P>0.49, F=0.81, d.f.=3,151).

Basal metabolic rate

The average basal metabolic rate for CORT treated birds was 0.88 (mL h-1; st.dev.=0.10, n=9) and for control birds the average was 0.82 (mL h-1; st.dev.=0.11, n=13). CORT treatment during development had no effect on basal metabolic rate (Fig. 3, P=0.22, F=1.64, d.f.=1, 20, d=0.57).

Discussion

We found that zebra finches exposed to CORT during development solve a foraging paradigm in fewer trials compared to control siblings. However, this trend was heavily influenced by the number of trials needed to pass the first stage of the foraging task. Once we accounted for variation in the number of trials needed to pass the first stage, we found no difference between treatment groups in the number of trials needed to pass the remaining three stages of the learning task. These data could indicate that once zebra finches located the millet seeds in the testing apparatus they had sufficiently learned to associate the food reward with the apparatus. Increasing the difficulty of obtaining this reward did not constitute an additional measure of learning ability. In this scenario, zebra finches exposed to developmental stress would be considered better learners compared to control siblings.

An overwhelming majority of studies with passerines find that developmental stress (elevated GCs and nutritional stress) decreases learning (e.g. Buchanan et al. 2003, Nowicki et al. 2002, but see counterexamples in introduction). In particular, developmental stress is known to decrease song learning, a trait important for reproductive success. There is increasing support for an adaptive role of developmental stress in shaping animal phenotype to match environmental conditions (phenotypic

programming; reviewed in Catalani et al. 2001, Schoech et al. 2011). Developmental stress may create resource trade-offs in developing animals which cause them to invest in some neural structures at the expense of others. In this scenario, developmental stress may decrease some types of learning (i.e. song learning) while increasing other types of learning (such as learning foraging tasks).

Developmental stress could also cause programmatic effects on learning by permanently changing hypothalamic-pituitary-adrenal (HPA) axis which modulates the release of GCs. In some systems, developmental stress increases HPA axis function so that animals exposed to developmental stress have greater GC output as adults (e.g. Spencer et al. 2009, Hayward and Winfield 2003). In this way, developmental stress could affect adult learning by altering the amount of GCs animals are exposed to which affects learning. However, in our system, we found that zebra finches fed CORT during development had elevated baseline and stress-induced CORT at 30 days post-hatch, but not at 60 nor 90 days post-hatch (Crino et al. *in review*). This suggests that the effects of developmental stress on learning are not being determined via programmatic effects on the HPA axis, but rather on programmatic effects on other systems (see above).

Development stress has been shown to decrease neophobia. For example, male zebra finches exposed to developmental stress had reduced latencies to approach a novel object compared to control birds (Spencer and Verhulst 2007). We found that zebra finches treated with CORT during development solved each stage of a novel foraging paradigm in fewer trials compared to control siblings. However, variation in the number of trials to solve each stage was driven entirely by variation to complete the first stage. Passing the first stage obligated the birds to interact with the foraging grid (a novel object) for the

first time. Therefore, it is possible that our treatment had no effect on learning *per se*, but rather modulated other behaviors (i.e. neophobia) which indirectly affected the ability of birds to solve a foraging task (but see Grindstaff et al. (2012). Many experiments which examine the effects of developmental stress on learning use apparatuses that could be perceived as novel objects. Therefore, to adequately investigate the effects of developmental stress on learning (when neophobia is a confounding factor), studies should employ methods which do not activate neophobic responses.

Using a food reward in learning tasks could potentially confound results because of the sustained effects of developmental stress on metabolic rate. Essentially, birds with higher metabolic rates may be more motivated to search for food and, therefore, solve the foraging task in fewer trials regardless of learning ability. However, we found that exposure to CORT during development did not affect basal metabolic rate in our sample suggesting that differential motivation does not explain the trend for CORT exposed birds to solve the learning task faster. Long term metabolic effects of developmental stress vary among species studied to date (Schmidt et al. 2012, Spencer and Verhulst 2008). Our lack of effect matches results previously reported in zebra finches (Spencer and Verhulst 2008).

Substantial evidence supports the role of developmental conditions in shaping the ability of birds to learn species specific songs. However, how generalizable the negative effects of development stress are on learning remains to be determined. Developmental stress has broad effects on behavior and physiology and these effects could potentially confound the effects of many learning experiments which rely on the use of novel objects or food rewards to assess learning ability. Future studies could account for these indirect

effects by utilizing multiple learning assays, employing learning tasks that do not rely on food or novel objects, or by directly measuring possible confounding behavioral and physiological factors.

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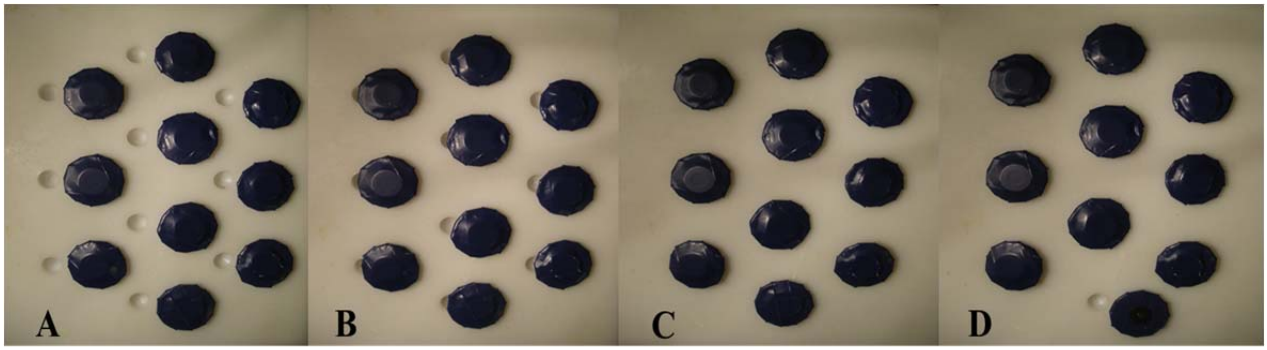

Figure 1. The foraging task had four stages: A) In stage one we placed lids next to the wells, B) in stage two we covered half of each well with the lids, C) in stage three we covered the entire well with the lid, and D) in stage four we secured the lids in the wells using rubber bumpers on the bottom of each lid (bumper shown on the inverted lid at the bottom of D).

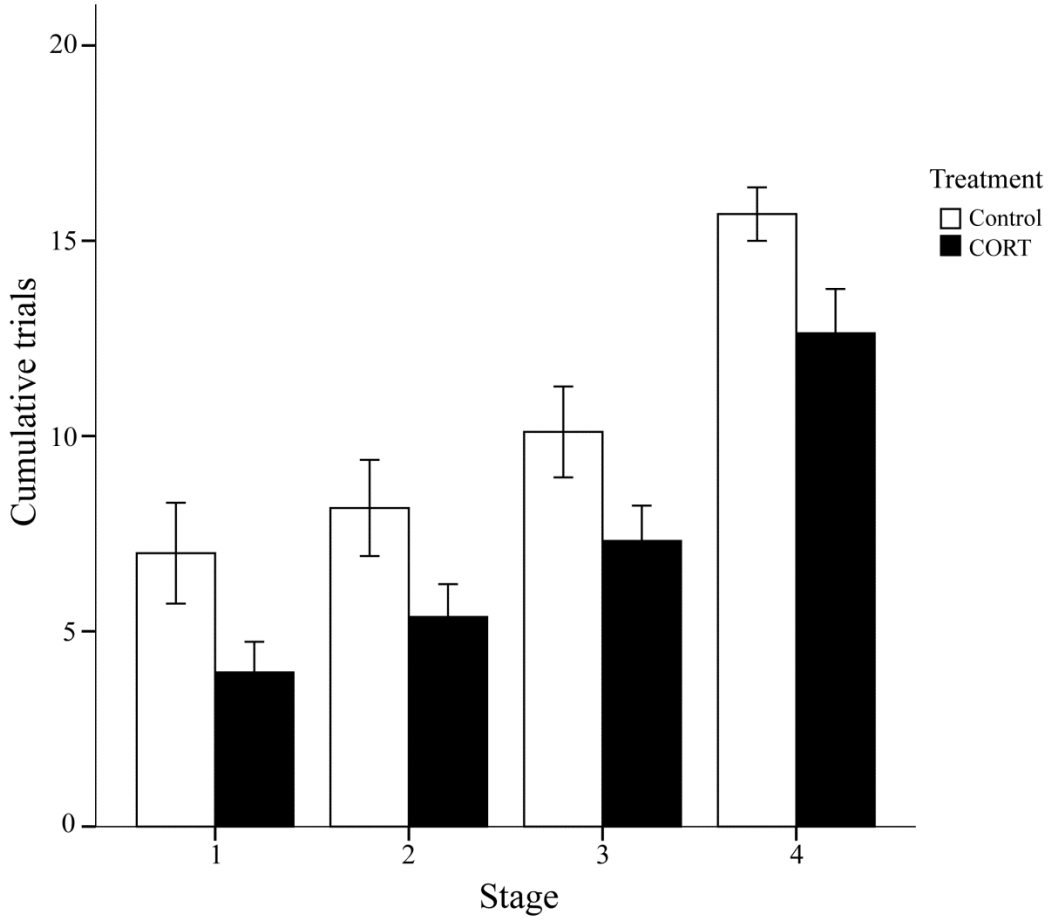

Figure 2. Zebra finches exposed to CORT during development completed all stages of the learning test in fewer cumulative trials compared to control siblings.

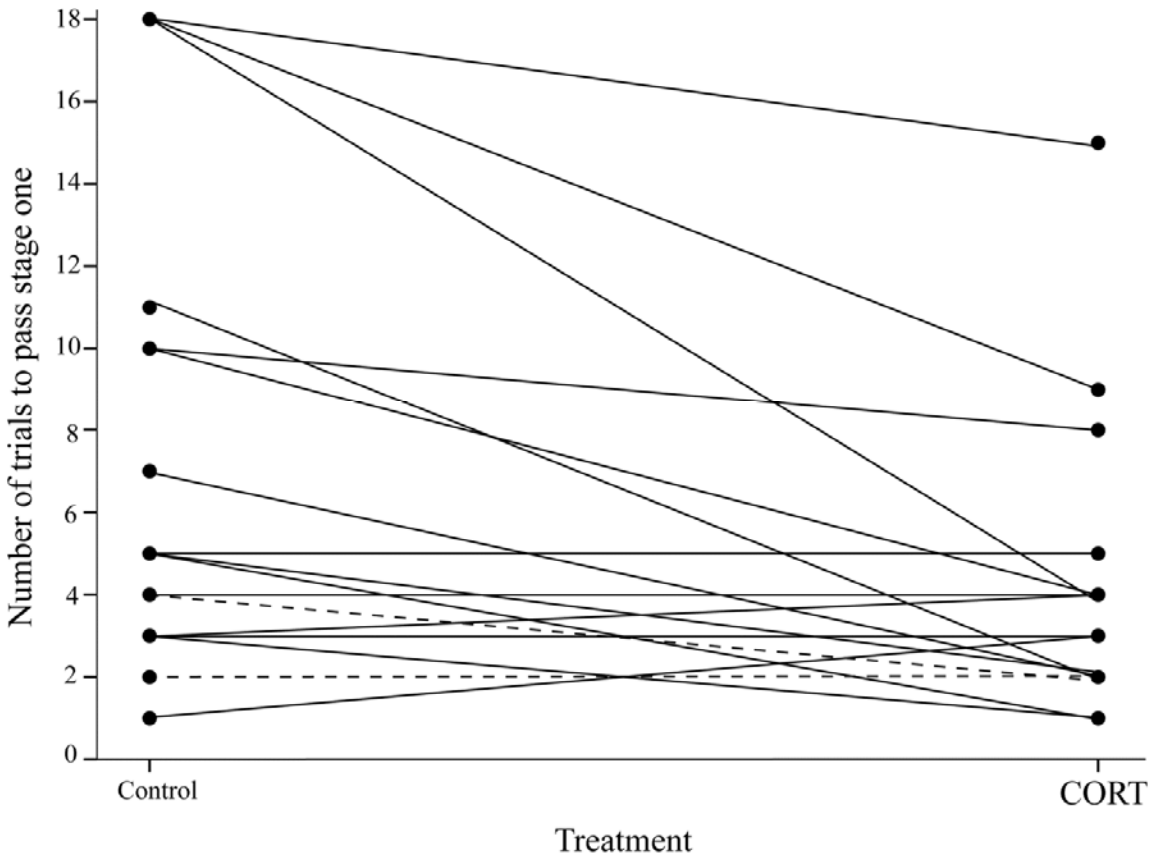

Figure3. Zebra finches exposed to CORT solved stage one in fewer trials compared to control siblings. Solid lines indicate n=1 for a pair and dashed lines indicate n>1.

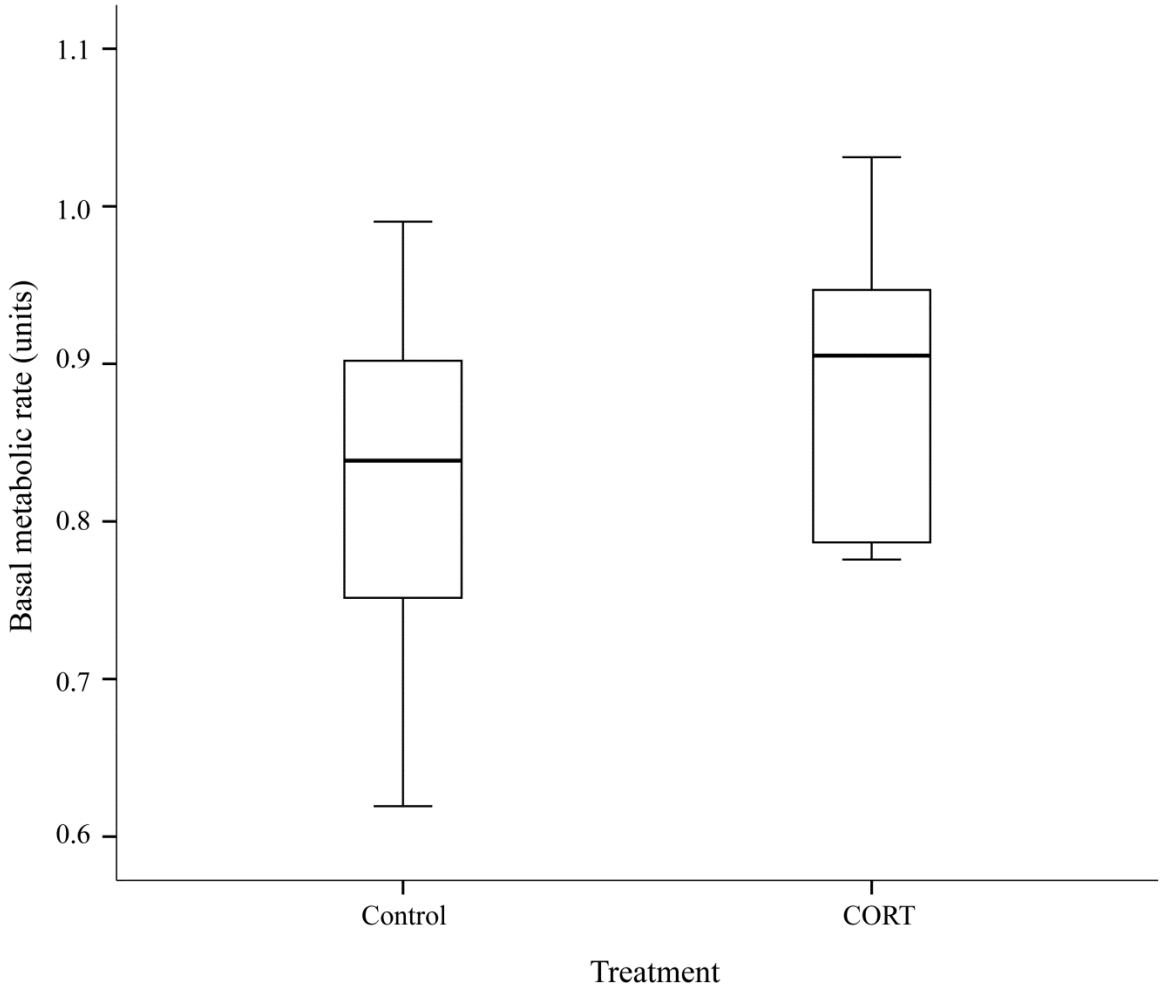

Figure 4. CORT treatment during development had no effect on basal metabolic rate.

Chapter 3: Developmental stress increases parental investment and reproductive success in male zebra finches

ABSTRACT

Developmental stress causes life-long physiological and behavioral changes. The effects of developmental stress on phenotype and performance have been well-studied. In comparison, the effects of developmental stress on fitness remain largely unexplored. Developmental stress is known to decrease the quality of sexually selected traits (e.g. bird song), and therefore is thought to decrease reproductive success. However, animals exposed to developmental stress may compensate for poor quality sexually selected traits by pursuing alternative reproductive tactics. Here, we examine the effects of developmental stress on adult male reproductive investment and success in zebra finches. We tested the hypothesis that males exposed to developmental stress sire fewer offspring through extra-pair copulations (EPCs), but invest more in parental care. We fed nestlings corticosterone (CORT) during the nestling period and measured their adult reproductive success using common garden breeding experiments. We found that males fed CORT during development invested more in parental care and reared nestlings in better condition compared to control males. Surprisingly, CORT-fed males also sired more offspring and were less likely to rear non-genetic offspring compared to control males. Contrary to the prediction that developmental stress decreases male reproductive success, we found that CORT-fed males had greater reproductive success. Investing in parental care and increasing offspring condition could maximize offspring survival and, hence, reproductive success.

1. INTRODUCTION

The environment animals experience during development can have important effects on phenotype, performance, and fitness across multiple life-history stages (Mousseau and Fox 1998, Sheldon 2002, Stamps 2003). Environmental cues experienced during development can provide information to animals about the environment to which they are about to enter and promote phenotypic changes which maximize fitness (Monaghan 2008). There is increasing evidence from across taxonomic groups which suggest that stress may be one such cue that conveys environmental information to developing animals and helps match prenatal animals to their postnatal environment (reviewed in Breuner 2008, Schoech et al. 2011).

In vertebrates, the endocrine response to stress involves activation of the hypothalamic-pituitary-adrenal (HPA) axis and results in the release of glucocorticoid (GC) hormones. GCs modulate physiological responses and behaviors that allow animals to cope with stressors (Wingfield at al. 1998). Developing animals can be exposed to GCs prenatally via maternal interactions (i.e. through the placenta or during egg formation in oviparous animals) or postnatally via behavioral interactions with parents and siblings, food limitation, and environmental perturbations (Champagne, et al. 2006, Hayward and Winfield 2004, Honarmand et al. 2010, Kapoor et al. 2006, Love and Williams 2008, Saino et al. 2003). GC exposure during development has been associated with a range of phenotypic effects such as decreased growth, development, and condition; impaired immunocompetence; and altered neurological function (reviewed in Schoech et al. 2011). Although the phenotypic consequences of developmental stress appear overwhelmingly negative, there is increasing evidence for an adaptive function of

developmental stress (reviewed in Breuner 2008, Gluckman and Hanson 2004, Groothius et al. 2005). Specifically, developmental stress may induce phenotypic changes which prepare developing animals to live harsh environments or match offspring needs to maternal capabilities (Gluckman and Hanson 2004, Hayward and Wingfield 2004, Love and Williams 2008). In these scenarios, stress-induced developmental changes may be adaptive.

Although the effects of developmental stress on phenotype and performance have been well-studied, there are comparatively few studies which have examined the fitness consequences of developmental stress. Numerous studies have linked CORT levels in developing animals to performance measures that could affect fitness (e.g. Saino et al. 2005, Blas et al. 2007, Wada and Breuner 2008). Fewer studies have shown that developmental conditions (e.g. enlarged broods, reduced food availability, elevated GCs) affect phenotypic traits which could affect fitness such as metabolism, immunocompentence, and flight performance (e.g. Chin et al. 2009, Grindstaff et al. 2012, Schmidt et al. 2012). Fewer studies still have examined the effects of developmental stress on female reproductive success (e.g. Naguib et al. 2005, 2006). To date, no study has examined the effect of developmental stress on male reproductive success.

Developmental stress may play an important role in male reproductive success by modulating the expression of sexually selected traits. Developmental stress can affect sexually selected traits by creating resource trade-offs during development (Nowicki et al. 2002) or by permanently up-regulating HPA activity which could affect the expression of condition-dependent sexually selected traits. Many studies have established a strong

link between GCs and the expression of sexually selected traits (Buchanan et al. 2003, Nowicki et al. 1998, 2002, Roulin et al. 2008, Spencer et al. 2003). For example, in barn owls (*Tyto alba*), black plumage is an important trait for mate choice (Roulin et al. 2008). Nestling owls exposed to experimentally elevated levels of GCs reduced the amount of melanin (black) pigment deposited in feathers as adults (Roulin et al. 2008). In, zebra finches, males selected for low HPA activity had more brilliantly colored legs and beaks (traits important for mate choice) compared to males selected for high HPA activity (Roberts et al. 2007). Additionally, zebra finches exposed to either dietary stress or experimentally elevated levels of GCs during development sing lower quality songs as adults (Nowicki et al. 1998, Spencer et al. 2003). This relationship between GCs and song persist through adulthood and males with lower HPA activity produce songs which are more attractive to females (Wada et al. 2008).

The negative effects of developmental stress on the quality of sexually selected traits have led to the assumption that developmental stress has negative effects on reproductive success. However, animals exposed to stress during development may compensate for poor quality sexually selected traits by pursing alternative reproductive tactics. For example, in birds with bi-parental care, males with poor quality sexually selected traits could maximize their fitness by investing more in parental care (e.g. nestling provisioning). In contrast, males with elaborate sexually selected traits could maximize fitness by pursuing copulations outside of their social mate (i.e. extrapair copulations; EPCs) and avoiding costly parental care (e.g. Badyaev and Hill 2002, Mitchell et al. 2007). Such tactics have been demonstrated in zebra finches (*Taeniopygia guttata*), where unattractive males invest more in parental effort and sire fewer offspring

through EPCs compared to attractive males (Burley et al. 1996). Likewise, in house finches (*Carpodacus mexicanus*), males with drab plumage provision nestlings more compared to males with elaborate plumage (Duckworth et al. 2003). Using alternative reproductive tactics, animals exposed to developmental stress may maximize their fitness by investing in offspring *quality* rather than offspring *quantity*.

We examined the consequences of developmental stress on parental behavior, bill coloration and reproductive success in male 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). After these birds reached sexually maturity, we used common garden breeding experiments to determine if developmental stress modulated male reproductive tactics. Specifically, we measured male bill coloration, parental provisioning rate, nestling condition, and the number of nestlings sired through EPCs. We predicted that males exposed to developmental stress would invest in offspring quality by increasing nestling provisioning and, consequently, rear nestlings in better condition compared to control males. In contrast, we predicted that control males would invest in offspring quantity by siring more nestlings through extra-pair copulations compared to CORT-exposed males. Therefore, males exposed to developmental stress would maximize reproductive success by investing in offspring *quality* while control males would invest in offspring *quantity*.

2. MATERIALS AND METHODS

(a) Parental birds – housing and breeding

Ten female and ten male zebra finches were purchased from six pet stores across Montana and Washington. Throughout the course of the experiment, 3 males and 2 females were replaced due to mortality. Individuals were given unique color band combinations and housed communally in a 20 X 25 ft. room (14:10 light/dark, 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.

(b) ***First generation nestlings – experimental treatment***

We marked newly hatched nestlings with an individual combination of leg markings using a black Sharpie marker. We banded nestlings with a numbered plastic leg band between three and four days post-hatch. Nestlings were then randomly assigned to treatment groups (CORT or 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 hours ± 1 hour 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 -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 incubation and provisioning behaviors (observations made in person, or using VehoMuvimicroDV camcorders if necessary). After determining social parents, we moved the nest box and parents to wire cages (70X40X44cm) where they were housed until the nestlings reached nutritional independence at 28 days post-hatch (49). 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 until reaching sexual maturity at 90 days post-hatch.

(d) ***Breeding experiment - setup***

To measure F1 reproductive success, we established common garden breeding experiments in aviaries (20 x 25 ft.) that consisted of ten novel females, five adult males from the CORT-fed treatment and five adult males from the control treatment. We obtained females from pet stores from Montana and Washington. All housing conditions (light/dark, temperature, humidity, nest boxes and nesting material and food) were identical to those described in the parental generation above. We allowed the birds to breed for one reproductive bout. The number of nests built in the experiment ranged from six to nine. We replicated the breeding experiment twice to examine the effects of developmental stress on male paternal behavior. When we examined the effects of developmental stress on paternity, our initial trends were non-significant. To further explore this effect we expanded our sample size to four replicates for the paternity analysis.

(e) ***Breeding experiment – social paternity and feeding behavior***

To measure parental behavior, we collected videos of nests using VehoMuvimicroDV camcorders for three consecutive days starting six days post-hatch. From these videos we identified social parents and measured the following parental behaviors: occurrences of nest attendance, nest association, and time spent in nest attendance or nest association. We considered birds to be engaging in nest attendance after they entered the nest box. We defined nest association as behaviors that occurred when the birds were perched outside or on top of nest boxes.

(f) ***Second generation nestlings – measurements and social paternity***

We monitored nest boxes daily for signs of egg laying and hatching. 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 and 28 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. We used these measurements to calculate condition using the scaled mass index (Peig and Green 2009, 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.

At 28 days post-hatch we also collected 25 µl of blood for use in paternity analyses. To collect blood, we punctured the alar vein with a 26-gauge needle and collected blood with heparinized microcapillary tubes. Immediately after collection, blood was frozen at -20˚C before being moved to storage at -80 ˚C.

(f) ***Paternity analysis***

We determined genetic paternity using six microsatellite markers previously developed for the zebra finch (Forstmeier et al. 2007). We genotyped all parents from the breeding experiment (F1 males and untreated mothers) and nestlings (F2) at each of the six loci. We performed PCR amplifications using the QIAGEN Multiplex PCR kit (QIAGEN). Each 10 µl PCR contained 1-2ng of DNA, 5µl of QIAGEN Multiplex PCR Master Mix (containing 5 mM $MgCl_2$), 3 µl of ddH_20, and 1 µl of one of the following primer mixes. Primer mix one contained 1 µM of forward and reverse primer of Tgu1, Tgu 8, and Tgu 10. Primer mix two contained 1 µM of forward and reverse primer of Tgu5, Tgu 9, and Tgu 12. The cycling conditions for both primer mixes were: a 2-min initial denaturation step at 94°C, 35 cycles of 30-s denaturation at 94°C, 30-s of annealing at 60°C, and a 20-s extension at 72°, followed by a final four-min annealing step at 72°C. One µl of primer product was mixed GS-500(-250) size standard and analyzed on a Blank Genetic Analyser under standard conditions. We analyzed raw data using Gene Mapper 3.7 (Applied BioSystems, 2004).

All six microsatellite loci were highly polymorphic with the number of alleles per locus ranging from 11 to 18 (mean=14.67, s.d.=2.50). Paternity was initial assigned using exclusion analysis. We excluded paternity if loci showed multiple mismatches

between the putative father and offspring. The results were confirmed using exclusion analysis in Cervus 3.0.3. Two nestlings died before we were able to obtain blood samples for genetic analysis. We were unable to obtain DNA from three samples. Therefore, our sample sizes for social and genetic paternity are 100 and 95 respectively.

(g) ***Beak color analysis***

We quantified spectral components of F1 male beak coloration by taking photographs of each bird and analyzing the resulting images in Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA). We photographed the right and left side of each bird using a Cannon Powershot digital camera. We used a white reference to standardize measurements across images (The Tiffen Company, Hauppauge, New York). Using the magic wand tool (tolerance = 30), we selected the beak and used the mean values of red, green, and blue channels displayed in the histogram window to calculate hue, saturation, and brightness using the color picker function (methods as per Behbahaninia et al. 2012, Butler and McGraw 2009). Hue values ranges from 359 to 6. We changed hue values of 359 to -1 in order to make all values numerically consecutive for statistical analyses. We subtracted the mode hue, saturation, and brightness measurements for the standard from the corresponding measurements for each sample. We then averaged the value for the right and left picture and used the resulting values in all statistical analyses.

(g) ***Statistical analyses***

We analyzed all data using PASW Statistics18.0. All measurements of nestling provisioning were non-normally distributed (Shapiro-Wilk, $p<0.04$) except for the

paternal nest attendance and maternal nest association ($p<0.12$). We log transformed non-normal data and used the resulting values in all statistical analyses. We analyzed nestling provisioning data using multivariate general linear models with paternal treatment as a fixed factor. We used generalized linear models (GLM) to analyze the effect of paternal treatment on nestling morphology and condition with paternal treatment as a fixed factor and nest identity as a random factor to account for multiple measurements per nest (as per Saino et al. 2003).

The numbers of nestlings reared by social fathers and the number of offspring sired by males were non-normally distributed (Shapiro Wilk, $p<0.001$). We analyzed the effects of developmental treatment on social and genetic paternity using generalized linear models using a Poisson distribution with treatment as a fixed factor. We included experimental replicate as a fixed factor and male age (days post-hatch) as a covariate in initial model. These factors were non-significant and removed from final analyses. We further explored the effects of developmental treatment on EPCs using a chi-square analysis to evaluate if treatment affected the success of achieving EPCs and the likelihood that a male would raise non-genetic offspring.

Hue, saturation, and brightness measurements were non-normally distributed (Shapiro Wilk, $P=0.003$, 0.04, 0.06 respectively for all). Log transformation failed to normalize data ($p<0.05$ for all). Age had a non-significant effect on hue values with older birds having less red beaks ($P=0.055$, Spearmans $rho=0.29$, $n=45$). Age affected brightness measures with older birds having brighter beaks ($P=0.03$, Spearmans $rho=0.32$, $n=45$). To account for age effects, we used a generalized linear mixed model

with a Poisson distribution and treatment as a fixed effect, age as a covariate. The sample sizes were n=24 and 21 for control and CORT-fed males respectively.

3. RESULTS

(a) Nestling provisioning

CORT treatment during development increased the total amount of combined time that males and females spent in nest attendance and nest association (figure 1, $F_{1,13}$=5.22, p=0.04). However, there was no difference between treatment groups in the number of paternal nest association bouts or the time spent in nest association ($F_{1,13}$<0.550, p>0.49). Likewise, CORT treatment during development did not affect the amount of time males spent feeding nestlings ($F_{1,13}$=1.426, p=.29). The number of nest attendance trips made by males exposed to CORT during development was in the predicted direction but the relationship was not significant ($F_{1,13}$=3.01, p=0.14). Paternal treatment group during development did not affect any measure of maternal behavior ($F_{1,13}$<1.40, p>0.26).

(b) Nestling body size and condition

Fathers exposed to CORT during development reared nestling in higher condition at 12 and 28 day post-hatch compared to control males (figure 2, table 1, Wald statistic=7.71, 9.09, d.f.=1, 1, p=0.006, 0.003 respectively). There were no differences between treatment groups in any nestling morphological measurement at 12 or 28 days post-hatch (Table 1). However, there was a non-significant trend for nestlings reared by control

fathers to have longer wing chords at 12 days post-hatch (Wald statistic 3.57, d.f.=1, p=0.059).

(c) Offspring sired and extra-pair offspring

Males exposed to CORT during development reared similar numbers of social offspring compared to control males (figure 3, Wald chi-square= 1.96, p=0.16, d.f.=1,38). Of 95 nestlings produced across four replicates, 39 (30.5%) were sired through extra-pair fertilizations (EPFs). This EPF percentage is similar to what has previously been described in captive zebra finches (Bolund et al. 2009). Males exposed to CORT during developmental sired more genetic offspring compared to control males (Wald chi-sqaure=5.15, p=0.02, d.f.=1,38). CORT-fed and control males sired similar numbers of offspring through EPFs (n=15 and 14 respectively, p=0.85, $\chi 2$=0.03). However, control males were more likely to rear non-genetic offspring compared to males exposed to CORT during development (n=20 and 9 respectively, p=0.04, $\chi 2$=4.17). CORT-fed and control males were equally likely to be chosen as social mates (p>0.9, Pearson's χ^2=0.13, d.f.=1) and to sire at least one nestling (p=0.50, Pearson's χ^2=1.03, d.f.=1).

(d) Beak coloration

Developmental treatment did not have an effect on hue, saturation, or brightness measurements of adult beak coloration (P=0.39, 0.18, and 0.77 for all).

4. CONCLUSIONS

Stress exposure (i.e. elevated GCs) experienced during development has long-term effects on phenotype with the potential to influence fitness. For example, developmental stress reduces the quality of sexually selected traits in birds, and so is thought to reduce reproductive success. However, males with poor quality sexually selected traits may choose alternative reproductive tactics in order to maximize reproductive success. Specifically, males exposed to stress during development may invest in parental care and raise higher quality nestlings compared to males from unstressed developments. This leads to the conclusion that control males will invest in offspring quantity, while developmentally stressed males would invest in offspring quantity. However, in our study, male zebra finches exposed to elevated GCs during development had higher quality and quantity of nestlings than control males. Nestlings reared by social fathers exposed to developmental stress received more combined parental care from mothers and fathers and were in better condition compared to nestlings reared by control social fathers and their mates. Additionally, males exposed to developmental stress sired more offspring than control males and were less likely to rear non-genetic offspring compared to control males. Overall, males exposed to development stress had greater reproductive success than control males in both the *quality* and *quantity* of nestlings produced.

Maximizing offspring quality could increase fitness of males living in stressful environments. The transition from nestling to fledging is marked by high mortality in free-living birds. Fledglings in better condition are more likely to survive to adulthood (Losdat et al. 2013). Therefore, by investing in parental care and increasing nestling quality, males living in stressful environments could maximize the survival of their nestlings and, hence, their fitness. The idea is that the environment experienced by the

male parent during development indirectly affects his offspring's phenotype by shaping his parental behavior. In this way, the developmental environment shapes the adult phenotype to optimize fitness in a given environment (Monaghan 2008). This phenotype matching may be especially relevant for animals living in stable environments where the natal environment is predictive of the adult environment.

Developmental stress has well-known suppressive effects on the expression and quality of sexually selected traits and is therefore predicted to decrease reproductive success (Nowicki et al. 1998). However, we found no effect of developmental GC treatment on adult bill coloration, a sexually selected trait in zebra finches. The reproductive success data were also contrary to predictions.We found that males exposed to developmental stress sired *more* offspring compared to control males and were *less* likely to raise non-genetic offspring. Additionally, males from both treatment groups were equally as likely to be chosen as a social mate and achieve EPFs. These results do not support the predictions of the developmental stress hypothesis.

CORT-fed males sired more offspring and were cuckolded less. One possible explanation could lie in differences in aggression between treatment groups. There is increasing evidence that developmental stress can affect animal personality in ways that could potentially affect fitness (reviewed in Schoech et al. 2011). For example, zebra finches fed CORT during the nestling period were less socially dominant at two months of age compared to control birds (Spencer and Verhulst 2007). In our experiment, it is possible that CORT treatment resulted in comparatively reduced social dominance and intrasexual aggression. Control males may have engaged in more aggressive social

encounters than CORT-fed males reducing their available time to pursue EPCs and provision nestlings.

The effect of early environment on animal phenotype is fundamentally important to the study of evolution because the variation caused by early environment creates variation for which natural selection to act upon (Mousseau and Fox 1998). Developmental stress has been associated with a range of seemingly negative phenotypic changes. However, phenotypic changes that occur as a consequence of development could maximize fitness in harsh or stressful environments. Here, we show that males exposed to stress during development invest more in parental care, rear offspring in better condition, sire more offspring, and are less likely to rear non-genetic offspring. Contrary to the predications of the developmental stress hypothesis, developmental stress appears to modulate parental strategy in a way that maximizes reproductive success.

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Variable	Day 12						Day 28					
	Control (n=25)		CORT (n=26)		Difference		Control (n=25)		CORT (n=26)		Difference	
	Mean	St. Dev.	Mean	St. Dev.	P	Wald stat.	Mean	St. Dev.	Mean	St. Dev.	P	Wald stat.
Tarsus (mm)	13.67	1.12	13.32	0.83	0.190	1.70	14.07	0.69	13.89	0.64	0.330	0.97
Wing (mm)	34.98	4.90	32.98	2.28	0.059	3.57	55.18	1.59	54.54	1.84	0.18	1.82
Mass (g)	11.16	1.44	11.29	1.33	0.74	0.11	13.08	1.01	13.37	0.77	0.25	1.34
Condition (g)	10.84	0.88	11.66	1.22	**0.006**	7.71	12.96	0.52	13.51	0.79	**0.003**	9.09

Table 1. Paternal developmental treatment had no effect on nestling body size (tarsus, wing chord, and body mass) at 12 or 28 days post-hatch. However, nestlings reared by fathers fed CORT during development were in better condition than nestlings reared by control fathers at 12 and 28 days post-hatch.

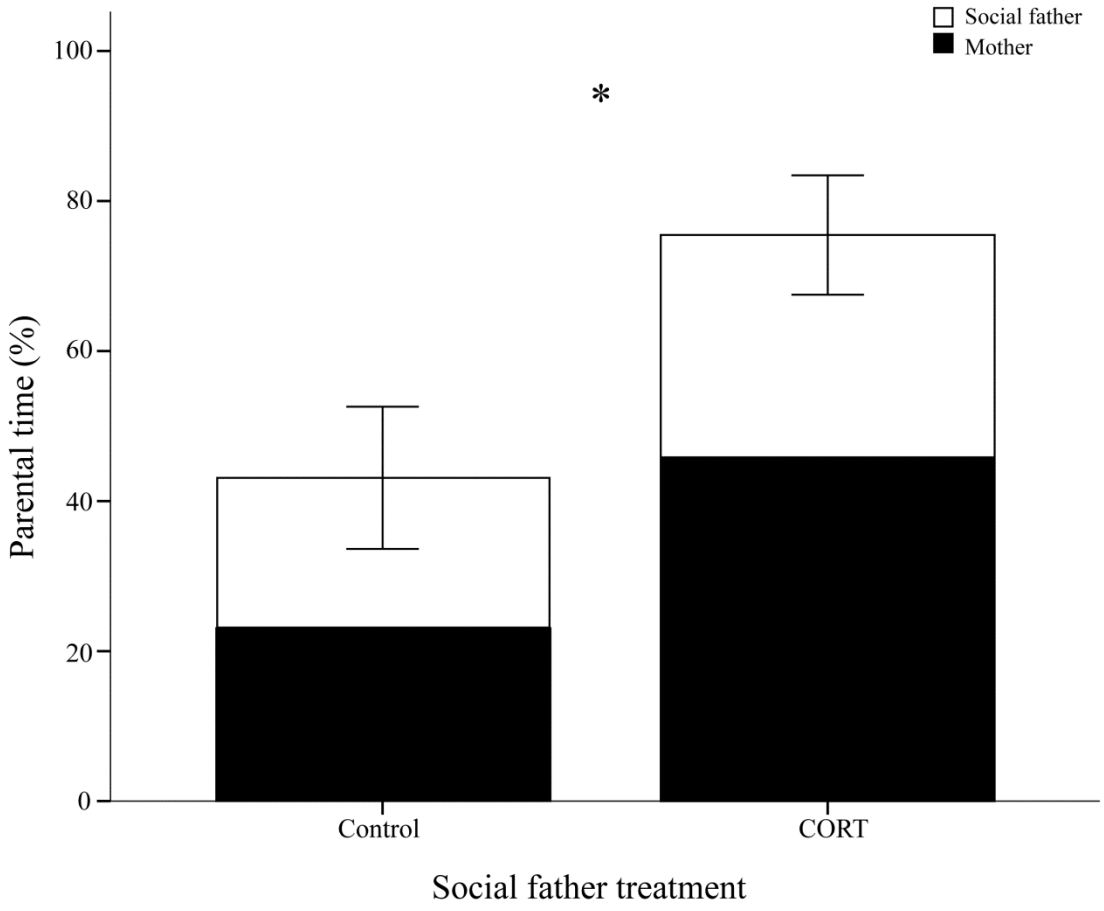

Figure 1. CORT-fed males and their mates invested more time in parental care compared to control males and their mates.

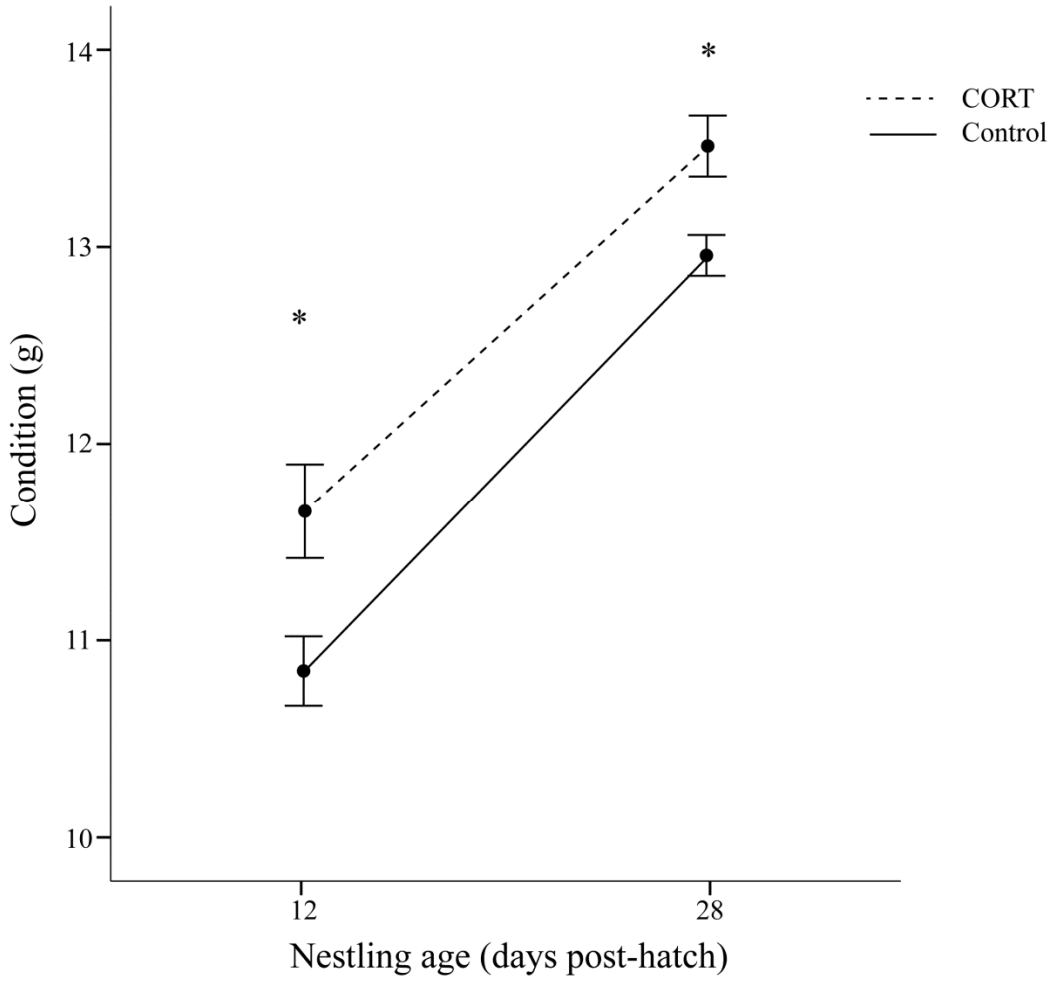

Figure 2. Nestlings reared by fathers fed CORT during development were in better condition compared to nestlings reared by control fathers at 12 and 28 days post-hatch.

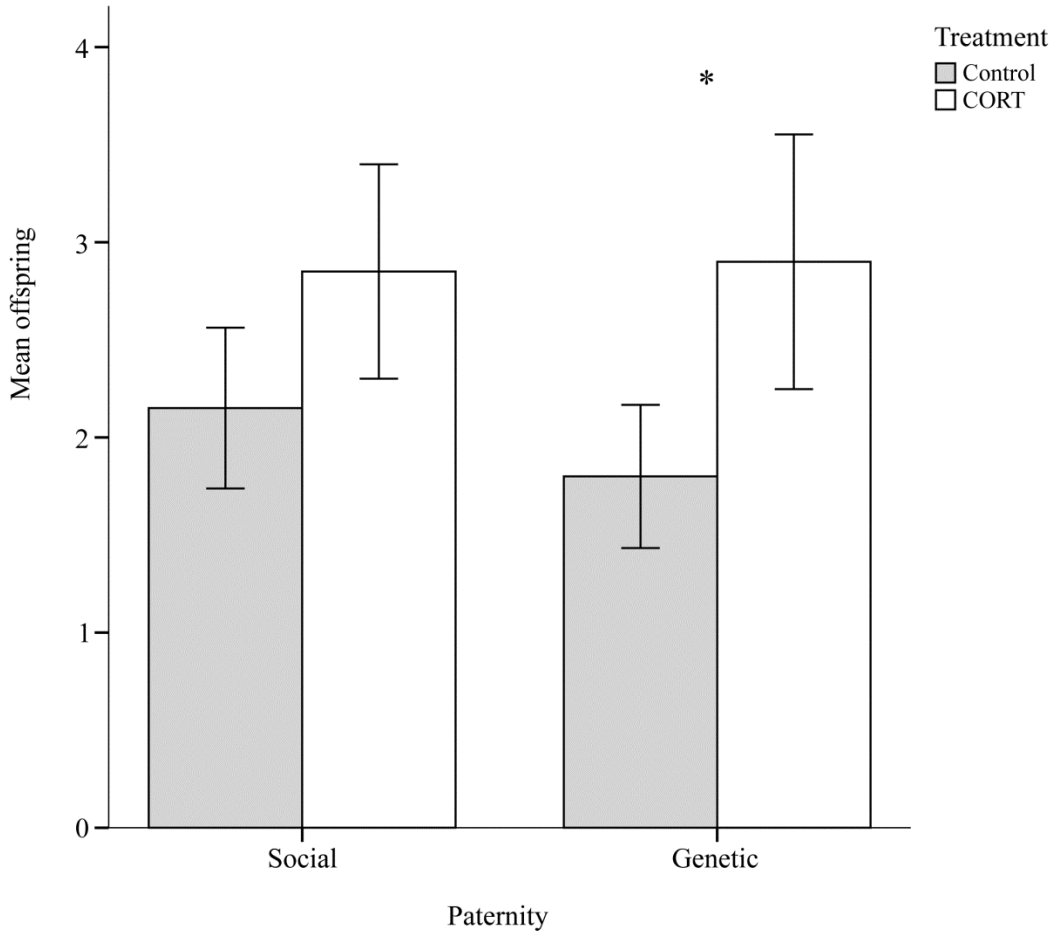

Figure 3. CORT-fed males sired more offspring compared to control males. There was no difference in the number number of social offspring fathers reared.

Chapter 4: Proximity to a high traffic road: glucocorticoid and life history consequences for nestling white-crowned sparrows

Abstract

Roads have been associated with decreased reproductive success and biodiversity in avian communities and increased physiological stress in adult birds. Alternatively, roads may also increase food availability and reduce predator pressure. Previous studies have focused on adult birds, but nestlings may also be susceptible to the detrimental impacts of roads. We examined the effects of proximity to a road on nestling glucocorticoid activity and growth in the mountain white-crowned sparrow (*Zonotrichia leucophrys oriantha*). Additionally, we examined several possible indirect factors that may influence nestling corticosterone (CORT) activity secretion in relation to roads. These indirect effects include parental CORT activity, nest-site characteristics, and parental provisioning. And finally, we assessed possible fitness consequences of roads through measures of fledging success. Nestlings near roads had increased CORT activity, elevated at both baseline and stress-induced levels. Surprisingly, these nestlings were also bigger. Generally, greater corticosterone activity is associated with reduced growth. However, the hypothalamic-pituitary-adrenal axis matures through the nestling period (as nestlings get larger, HPA-activation is greater). Although much of the variance in CORT responses was explained by body size, nestling CORT responses were higher close to roads after controlling for developmental differences. Indirect effects of roads may be mediated through paternal care. Nestling CORT responses were correlated with paternal CORT responses and paternal provisioning increased near roads. Hence, nestlings near roads may be larger due to increased paternal attentiveness. And finally, nest predation was higher for nests close to the road. Roads have apparent costs for white-crowned sparrow nestlings--increased predation, and apparent benefits --increased size. The elevation in CORT activity seems to reflect both increased size (benefit) and elevation due to road proximity (cost). Whether or not roads are good or bad for nestlings remains equivocal. However, it is clear that roads affect nestlings; how or if these effects influence adult survival or reproduction remains to be elucidated.

1. Introduction

A growing body of evidence indicates that roads have important effects on biological communities. Roads impact ecosystems by fragmenting habitat, increasing land use by people, causing road mortality of wildlife, and altering the composition of native plant and animal communities [19, 20, 52]. Consequently, areas with high road density are associated with decreased population densities, genetic diversity, and biodiversity in many taxa including plants, mammals, reptiles, amphibians, and birds [6, 18, 28]. Although roads have detrimental effects on some species, other species appear unaffected or even benefit from their presence [e.g. 38, 42, 46]. Roads can increase the availability of some food resources such as seeds, insects, and carrion, facilitate foraging by clumping resources along edge habitat, facilitate predator vigilance behavior allowing birds to feed more, and create corridors that facilitate wildlife dispersal [38, 51, 52, 56]. Although the direction and degree of road effects appears to be highly species- and context-specific, roads clearly alter biological communities. The continual increase in both roads and human population make understanding these effects increasingly important for management and conservation.

One way to assess the impacts of roads on wildlife is to examine the effects roads have on physiological stress. The vertebrate stress response includes the release of cytokines and catecholamines, and the release of glucocorticoids via activation of the hypothalamic-pituitary-adrenal (HPA) axis. The acute response promotes survival, but chronic activation leads to suppression of growth, digestion, reproduction, and immune function [47, 66]. Glucocorticoids become elevated in response to human disturbances in wildlife including spotted hyenas (*Crocuta crocuta*), European pine martens (*Martes martes*), yellow-eyed penguins (*Megadyptes antipodes*), gray wolves (*Canus lupus*), and elk (*Cervus elaphus*) [4, 17, 32, 37, 55, but see Romero and Wilkelski 45]. In birds, proximity to roads increased glucocorticoids in the northern spotted owl (*Strix occidentalis caurina*) and the mountain white-crowned sparrow (*Zonotrichia leucophrys oriantha*) [15, 26, 64]. Additionally, traffic noise has been shown to increase fecal corticosterone (CORT; the dominant avian glucocorticoid) in sage grouse (*Centrocercus urophasianus*) and increase behavioral stress and decrease immune function in domesticated chickens [Blickley et al. *in prep*, 11].

Previous studies have focused on the effects of roads on adult birds; however nestlings are also susceptible to anthropogenic disturbances. Nestling Magellanic penguins (*Spheniscus magellanicus)* living in tourist-exposed sites had elevated stress responses compared to nestlings from undisturbed populations [62]. Similarly, juvenile Hoatzins *(Opisthocomus hoazin)* living in areas with tourists secreted more CORT in response to a stressor and had lower body weights than individuals in areas without tourists [40]. Nestlings may be particularly susceptible to stressors associated with roads because they are confined to nests during early development and are unable to move away from stressful stimuli [53]. Additionally, adult animals can habituate to stressors over time by reducing HPA activity, thus reducing the negative effects of elevated CORT [22, 45, 63]. In contrast, nestlings may not habituate to novel stressors over the short nestling period and thus anthropogenic disturbances may elevate stress responses in developing animals, but not in adults [9, 63, but see 41].

In addition to being more susceptible to anthropogenic disturbances, nestlings may also suffer greater consequences from their exposure. Elevated CORT during development has immediate effects, such as inhibition of growth and immune function [54, 59]. However, this brief elevation during development can have life-long consequences for physiology, morphology, and behavior [12, 24, 57]. Stressed neonates can be hypersensitive to stress as adults and can transmit alterations in stress physiology to future offspring [e.g. 27, 36]. Hence, exposure to stress can be detrimental to nestlings during development, but can also have lifelong effects on reproductive success and survival. As such, exposure to even short periods of stress during development could translate to community-level effects. Examining the effects of roads on nestling adrenocortical activity will provide a more comprehensive assessment of the impact of roads on avian communities.

Roads could affect nestlings directly through traffic noise, the visual stimuli of cars, pollution, increased human presence, altered food availability, and/or changes in vegetation [16, 21, 42, 50, reviewed in 18]. However, roads could also have indirect effects by modifying parental behavior. For example, male Florida scrub-jays (*Aphelocoma coerulescens)* living in roadside habitat provisioned their nestlings more than jays living in habitat far from the road [38]. Excluding roads, other anthropogenic

disturbances have been shown to affect avian nesting behavior. For example, McGowan and Simons [36] describe a negative association between all-terrain vehicle traffic and time spent incubating by nesting American oystercatchers (*Haematopus palliates*). Roads could therefore indirectly affect nestling growth and development by altering parental behaviors such as provisioning or nest attendance.

Here, we examine the effects of a high-traffic road on nestling mountain white-crowned sparrow stress physiology, body size, and fledging success. Our approach was three-fold: First, we investigated associations between road proximity and nestling CORT levels and body size. We predicted that road stimuli would elevate nestling CORT levels, resulting in smaller body size. Second, we examined several possible indirect factors that may influence nestling CORT secretion in relation to roads. To examine covariates that could affect nestlings and vary with road proximity (indirect effects of roads), we measured nest-site vegetation, parental stress physiology, and parental feeding rates. Roads can alter local habitat by affecting hydrology, segmenting land, and increasing erosion [20]. These alterations could potentially affect nestling HPA activity and growth by changing plant communities, food availability, and the vegetation cover for nests. And finally, we examined the fitness consequences of roads by measuring relationships between road proximity and nest abandonment, nest predation, and nestling fledging success.

2. **Methods**

2.1. Study area and birds

Our study was conducted on the population of white-crowned sparrows located in Tioga Pass Meadow, directly outside the eastern entrance to Yosemite National Park, CA (37˚54′N, 119˚15˚′W). This population of sparrows has been studied extensively since 1967, and a substantial proportion of adults in this population are marked with a unique combination of colored bands for identification. Mountain white-crowned sparrows are migratory songbirds that arrive at Tioga Pass in May and breed from late May to early August [39]. The principal nest predator on white-crowned sparrow nests in this area are Belding's ground squirrels (*Spermophilus beldingi*) although long-tailed weasels

(*Mustela frenata*), coyotes (*Canis latrans*), Clark's nutcrackers (*Nucifraga columbiana*), and common ravens (*Corvus corax*) are also important predators [39]. Over a 22-year period, an average of 47% of nests at this site successfully fledged nestlings. Nest predation was the primary cause of nest failure (57%). The remaining nest failures were due to nest abandonment (22.3%), inclement weather (16.2%), and other factors (4.5%) including death of attending parents and nests falling apart due to poor construction [39].

Highway 120 runs through this study site to the entrance of Yosemite National Park (Fig. 1). Once the entrance into Yosemite opens (late May), traffic on HWY 120 is heavy during daylight hours. The California Department of Transportation recorded an average traffic rate of 2971 cars/day along this section of Highway 120 from June-August in 2009 [10]. Although this traffic load is low compared to larger highways and interstates, traffic-induced increases in adult bird stress responses have been demonstrated on smaller roads [15]. This traffic load exists throughout the white-crowned sparrow nesting and fledgling periods. The closest nests to Highway 120 were 5 m from it and the farthest over 300 m away (Fig. 1).

Nestling provisioning data was collected from 5-21 July of 2010. All other data were collected between 28 May and 18 July of 2009.

2.2. Nest searching and GIS analyses

Nests were located by direct search as well as observation of adult behaviors. If nests were found prior to clutch completion, we checked them daily until the clutch was complete (number of nest checks did not influence predation rates, data not shown). Females begin to incubate the day before the last egg is laid and nestlings hatch an average of 12.3 days after incubation commences [39]. Using this information, we estimated hatch date for clutches and checked nests daily starting 1-4 days before hatching to determine hatch date. If nests were found with a complete clutch, we monitored them daily until hatch date. We did not check nests after nestlings hatched to ensure that our presence would not influence nestling glucocorticoid physiology.

Nests were considered successful if at least one nestling fledged. We confirmed fledging success by observing or trapping fledglings, or by watching parents carry food or feed fledglings. Nests were considered predated if eggs or nestlings vanished before

fledging and were considered abandoned if parents stopped building nests, feeding nestlings, or laying eggs.

We took GPS coordinates of each nest using a Garmin eTrex GPS device in WGS 84 datum. The distance between each nest and Highway 120 was calculated using the Nearest Features 3.8 extension in ArcView Geographic Information System (GIS).

2.3. Stress protocol and morphological measurements - nestlings

White-crowned sparrow nestlings respond to stressors with increased CORT secretion in as few as five days after hatching and reach peak levels of CORT by 30 minutes of stress exposure [60]. Seven days after hatching, the largest nestling in each nest was exposed to a standardized capture and handling protocol as described by Wingfield (1994) [65]. Although plasma CORT typically increases within three minutes of stress exposure [44], Nuttall's white-crowned sparrows (*Zonotrichia leucophrys nuttalli*) do not show an increase in CORT until four minutes following stress exposure [60]. For this reason, we used blood samples obtained within four minutes of disturbing a nest as baseline samples. Samples obtained within four minutes show no significant increase in corticosterone (CORT; the dominant avian glucocorticoid; $P=0.56$; $r=0.14$; $df=1,19$). After initial blood samples were obtained, we placed nestlings in cloth bags and collected two more samples 15 and 30 minutes after initial disturbance. To collect blood, we punctured the alar vein with a 26-gauge needle and collected 25µl of blood with heparinized microcapillary tubes. Immediately after collection, blood was kept on ice (<2hours) until it could be centrifuged to separate the plasma from red blood cells (3000 rpm for seven minutes). After separation, the plasma was isolated and stored at -20˚C.

After blood sample collection, we weighed nestlings to the nearest 0.1 g and measured tarsus length (posterior to anterior tarsus) to the 0.1 mm. All nestlings were measured once by one person (O.C.). Each nestling was banded with a USGS service band on the right leg prior to release back into the nest.

2.4. Stress protocol– parents

To examine correlations between parental and nestling stress responses, we captured parents after fledging and exposed them to a standard capture and handling protocol. Parents were captured using Potter traps baited with millet seed between one and nine days after at least one nestling had fledged. We determined parentage by noting which sparrows reacted defensively (chipping, swooping flights, etc.) when we disturbed nestlings for our study. Maternity could also be verified by noting the band combination of females if they flushed from their nests.

We collected 100 µl of blood from adults for a baseline sample within three minutes of approaching a trap (there is no significant increase in CORT within three minutes in this population, C. Breuner, unpublished data). Following the initial sample, birds were placed in cloth bags and two additional 50 µl blood samples were obtained after 15 and 30 minutes of restraint. Blood was collected as per described for nestlings (see above). We sampled 16 males for baseline CORT, but were unable to obtain post-disturbance samples for three males. Therefore, n=16 for paternal baseline and n=13 for stress induced levels of CORT.

2.5. Corticosterone assays

Corticosterone was quantified with Enzyme Immunoassay (EIA) kits (Cat No. 901-097, Assay Designs), previously optimized for white-crowned sparrow nestlings [60]. Following the protocol used by Wada et al. (2007) [60], we used a raw plasma dilution of 1:40 to determine CORT levels. Briefly, we added 10 µl of 1:100 steroid displacement buffer (SDB) to 10 µl of plasma. After 15 minutes, we added 380 µl of assay buffer for a total dilution of 1:40. Samples were vortexed and 100 µl of a sample was added to individual wells in triplicate. These samples were compared to a standard curve with six samples run in triplicate ranging from 20,000 to 15.53 pg/ml (100 µl/well). An external standard of 500 pg/ml was run on every plate in triplicate and used to calculate inter-plate variation. Plates were read on a Multiskan Ascent microplate reader at 405 nm corrected at 595 nm. Intra- and inter-plate variation was 6.04 and 11.77% respectively.

From the plasma samples, we were able to calculate baseline CORT (the amount of endogenous CORT prior to disturbance) and total integrated CORT (the total amount of CORT secreted over the 30-minute period of restraint).

2.86. Nest-site vegetation analysis

To examine the effects of nest site habitat on nestling stress responses, we quantified the composition of the dominant vegetation in a 12-m^2 area around each nest using a qualitative ranking system [adapted from 25]. Briefly, a one-meter square divided into four equal quadrats was placed at each compass direction surrounding a nest (e.g. west, north, etc) covering a total of 4 m^2. Two additional 1-m^2 plots were placed on each side of the 4-m^2 block creating a cross of plots that covered a total of 12 m^2. Within each 1-m^2 plot, the density of two dominant willow species diamondleaf willow (*Salix planifolia)* and Sierra willow (*S. orestera*), lodgepole pine (*Pinus contorta*), herbaceous plants (grasses and forbes), and dirt/rock cover was rated on a scale from 0-5 using estimates of percent cover as follows: 0=0%, 1=0-20%, 2=20-40%, 3=40-60%, 60-80%, and 5=80-100%. We also measured the highest stem of each vegetation type for each quadrat. The values for the quadrats were averaged for each 1-m^2 plots and then all 12 plots were averaged for mean total density and mean height scores for each type of vegetation at each nest site.

2.10. Parental provisioning rates

We measured nestling provisioning by recording nests for ≈1.5 hours for three consecutive days starting four days after hatching. We recorded nests using Veho Muvi microDV camcorders that were zip-tied to vegetation surrounding nests or attached to stakes planted in the ground. Parental feeding rates were recorded starting on average between 9-10 AM. Video footage was downloaded and analyzed by one single observe (E.J.) using VideoLAN VLC media player. Videos were scored for male and female behaviors including number of feeding trips, time spent feeding nestlings, nest attendance, and, for females, incubation bouts and time spent incubating. We defined nest attendance as the total time spent provision nestlings, removing fecal sacs, and maintaining the nest. We divided the amount and duration of behaviors observed by the

total length of video footage which varied slightly between cameras depending on battery life. We used the subsequent behaviors/hour in all statistical analyses. We were able to distinguish between individuals because parents were color banded with a unique combination of color bands. We excluded one nest from analyses because we were unable to obtain videos for three consecutive days so n=11 for analyses of provisioning and incubation. Additionally, we were unable to obtain a blood sample from one nestling so n=10 for analyses involving parental provisioning and nestling stress physiology. The nests used in these analyses were located 17 – 279 m from the road.

2.11. Statistical Analyses

Tarsus length and mass were highly associated ($P<0.01$; $r^2=0.79$). For this reason, we used a principal component analysis (PCA) to reduce morphological traits to one component score for size. Both traits loaded highly and positively on component one (hereafter: body size) which explained 89.7% of the variation. The size component was used for all analyses.

CORT data for nestlings were normally distributed (Shapiro-Wilk test of normality, $p>0.05$ for all). For this reason, raw data were used in all analyses for nestlings. Hormonal data for adults were not normally distributed (Shapiro-Wilk, $p<0.05$ for all). However, because rank-order statistics were used to analyze parental stress responses, we did not transform CORT data (see below).

Baseline and total integrated CORT for nestlings were highly associated ($p<0.01$, $r^2 > 0.58$). For this reason, we used simple linear regressions to examine relations between proximity to road and both nestling adrenocortical activity and body size. To analyze nest site characteristics, we used principal components analysis (PCA) with varimax rotation to reduce nine nest-site variables to four component scores that explained 92% of cumulative variation (Table 1). We examined associations between each PCA score and nestling adrenocorticoid activity and body size using a general linear model (GLM). We also used GLM analysis to examine changes in nest-site vegetation in relation to proximity to the road.

3. Results

3.1. Nest searching and GIS analyses

Between 28 May to 7 July, we located 50 nests. Of these, 27 were predated or abandoned. We obtained stress series on nestlings from the remaining 23 nests. Three nests were later excluded from analyses because either the nestlings died before fledging (n=1) or we were uncertain about hatching date (n=2). We did not obtain wing measurements for two nestlings therefore n=18 for analyses with body measurements. Nests ranged between 5-300 m from the road.

3.2. Nestling morphological measurements and CORT responses

Nestlings were larger near roads (Fig. 3, N=18, $F_{1,18}$=7.12, P=0.02). Nestling HPA activity increased with proximity to road (Fig. 4). This was observed for baseline CORT (N=20, $F_{1,19}$=5.343, P=0.033) and total integrated CORT (N=20, $F_{1,19}$=6.99, P=0.017) As they age, nestlings mount stronger stress responses during standardized handling [58]. Because of the positive correlation between proximity to road and body size, it is possible that slight differences in age or size explain the positive correlation between stress response and proximity to road. However, 1) nestling body size was not correlated with nestling baseline or total integrated CORT (P>0.25 for all); 2) when corrected for body size, total integrated CORT remained significantly elevated with proximity to road (two-stage least squares regression, Fig. 5, N=18, d.f.=17, F=4.72, P=0.045); and finally, 3) road proximity did not affect days until fledging (N=18, d.f.=17, F=0.307, P=0.587).

3.3. Parental CORT response

Nestling CORT responses were positively correlated with paternal CORT responses (Fig. 6; paternal integrated CORT: N=13, r_s= 0.516, P=0.036 one-tailed, P=0.071 two-tailed). There was no association between nestling CORT responses and maternal CORT responses (N=17, r_s=0.027, P=0.918). Likewise, there was no relationship between nestling baseline CORT and CORT level in either parent (paternal: N=16, r_s= 0.091, P=0.86; maternal: N=17, r_s=-0.076, P=0.772).

3.4. Nest-site characteristics

PCA produced four factor scores that explained 92% of the variation in nest site characteristics (Table 1). Nest height and lodgepole pine height and density loaded highly and positively on factor 1. Nests in pine trees are higher from the ground than nests in other vegetation. That nests that were high from the ground were found at sites with high pine tree abundance. In this way, factor 1 seems to be a measure of *pine cover*. The mean height and density of diamondleaf willow loaded highly and positively on factor 2 (hereafter: diamondleaf willow cover) and the mean height and density of Sierra willow loaded highly and positively on factor 3 (hereafter: Sierra willow cover). Factor 4 (hereafter: ground cover) captured variation in mean bare ground (positively) and mean herbaceous plant density (negatively).

Nestlings from nests with low ground cover (more open ground, less herbaceous plants) had elevated baseline CORT compared to nestlings from nests with low ground cover (N=17, $F_{1,16}$=5.447, P=0.04). No other factor score explained variation in nestling baseline CORT (P> 0.69 for all). There were no association between nestling total integrated CORT and body size with any factor score (P>0.31 for all). Analysis of variance (MANOVA) revealed no difference in nest-site characteristics between nests that fledged and those that failed (N=35, P>0.53 for all factors). Of nests that failed, there was no difference in nest-site characteristics between nests that were found by predators and those that were abandoned (N=13, P>0.144 for all factors).

Willow type tended to change with proximity to the road. Diamondleaf willow cover increased close to the road (N=17, $F_{1,16}$=3.905, P=0.07), while Sierra willow cover decreased (N=17, $F_{1,16}$=4.332, P=0.06). Examining the direct interactions between raw variables and road proximity, diamondleaf willow density increased significantly close to roads (N=17, d.f.=16, F=7.439, P=0.015) and diamondleaf willow height had a non-significant trend to increase close to road (N=17, $F_{1,16}$=3.953, P=0.064). In contrast, Sierra willow density and height decreased significantly close to the road (F=5.132, 7.052, P=0.038, 0.017).

3.5. Parental feeding rate

Fathers with nests close to the road were more attentive (Fig. 7a; N=11, $F_{1,10}$=8.334, P=0.018) and tended to engage in more feeding trips per hour than fathers with nests far from the road (Fig. 7b; N=11, $F_{1,10}$=4.252, P=0.069). There was no association between road proximity and other measures of male provisioning behavior, female provisioning behavior, or incubation (P>0.233 for all). A paired samples t-test revealed no difference between males and females in the number of feeding trips per hour (N=11, $t_{1, 10}$=-0.243, P=0.813). Linear regression revealed no significant association between nestling adrenocorticoid activity and any measure of parental provisioning or incubation (P>0.120 for all). Finally, there were no associations between average nestling mass, tarsus length, or wing length with adrenocorticoid activity or any measure of parental provisioning or incubation (P>0.587 for all).

3.6. Nest Success and distance from road

There was no relationship between nest success and distance from the road (Fig. 2a; N=50, $F_{1,49}$=0.19, P=0.67). However, of the nests that failed, those near the road were more likely to be predated whereas those far away were more likely to be abandoned (Fig. 2b; N=27, $F_{1,26}$=5.16, P=0.03).

4. **Discussion**

4.1 Nestling HPA activity, growth, and road proximity

Nestling sparrows reared close to a road had elevated HPA activity and were larger than nestlings reared far from the road (Fig. 3, 4). In developing birds, exposure to elevated levels of glucocorticoids decreases growth and development [e.g. 27, reviewed in 58]. For this reason we expected HPA activity to be negatively associated with body size in nestling white-crowned sparrows. However, altricial nestlings (including white-crowned sparrows), can have dampened HPA responses during early development that increase over the nestling and fledgling periods [7, 8, 60, 61]. A positive association between nestling body size and HPA activity could result from maturation of the HPA axis. Therefore, elevated HPA activity in nestlings in relation to road proximity may

simply indicate that nestlings close to the road develop faster, not that they are more stressed. In this study, body size accounts for a significant amount of variation in CORT secretion. However, when body size was accounted for, nestling stress-induced CORT still increased with proximity to the road (Fig. 5). Additionally, proximity to the road did not shorten the nestling period suggesting that developmental rate was not influenced by the road. Hence, even though nestling body size explains some of the variation in nestling HPA activity, it appears that nestling HPA activity and growth are affected by independent mechanisms that are both associated with road proximity. To our knowledge, this is the first study to demonstrate that proximity to roads affects nestling body size/growth.

4.2. Indirect parental effects

Fathers closer to the road had greater nest attendance and tended to provision more than fathers further away from the road (Fig.7a, b). Nest attendance combines several behaviors and could indicate the amount of food delivered to nestlings. Fathers close to roads may provision nestlings more often and with greater amounts of food because road proximity increases food availability, facilitates foraging, or decreases vigilance behavior. The primary foods for nestling white-crowned sparrows are arthropods, adult insects, and larvae [39]. The amount of insects killed by cars on roadways increases with increased traffic load [49]. Male white-crowned sparrows provisioning nests close to the road may increase provisioning effort because insect prey is more abundant and easier to obtain. Morgan et al. [38]) describe similar results with male Florida scrub-jays living close to roads increasing energy intake and nestling provisioning despite lower foraging rates, suggesting that food is more plentiful or easier to obtain close to the road.

Although male sparrows increase nest attendance and provisioning close to the road, there was no association between maternal provisioning and road proximity. In tree swallows (*Tachycineta bicolor*), males modulate feeding behavior more than females in response to artificial brood-size manipulations [3]. Additionally, male tree swallows provision nestlings more in areas with higher food abundance, while food availability has no effect on female provisioning [3]. In many passerines, females provision nestlings

more than males suggesting that sexes differ in foraging costs and/or the benefits [3, 29, 31, 39]. In our study system, female sparrows may already maximally provision nestlings, and therefore changes in food availability associated with the road have no effect on their provisioning rate.

We observed a correlation between paternal and nestling stress physiology. Such correlations between parents and offspring are not surprising because there is thought to be a strong genetic component to the stress response [48]. However, in our study, we assessed social and not genetic paternity. A high rate of extra-pair fertilizations (EPFs) occur in this population of white-crowned sparrows, so that 30-56% of nestlings are not genetically related to attending males [33]. Therefore, it is likely that correlations between paternal and nestling stress responses arise through paternal behavior or epigenetic effects and not from genetic relatedness. Elevated CORT levels increase foraging behavior in birds such as the Adélie penguin (*Pygoscelis adeliae*), red-eyed vireos (*Vireo olivaceus*), and black-legged kittiwakes (*Rissa tridactyla*) [1, 2, 30]. Dietz [15] describes elevated levels of CORT in male mountain white-crowned sparrows trapped close to a road. In our system, proximity to the road could elevate paternal CORT which elevates foraging and, thus, nestling provisioning (as per Angelier et al. [1]). Increased resources could accelerate nestling growth and HPA axis development. Therefore, the positive correlation between paternal and nestling CORT physiology would result from increased provisioning mediated by proximity to the road. Conversely, there was no association between maternal and nestling stress physiology. Assuming that provisioning rate is important for nestling growth and HPA axis development, this lack of association is not surprising because females did not provision nestlings more close to the road.

4.3. Nest-site vegetation and nestling stress

We observed a negative association between ground cover and nestling baseline CORT (PCA factor 4). Reduced ground cover could increase nestling exposure to wind and/or temperature changes. Nestling white-crowned sparrows are unable to thermoregulate until three to four days after hatching [39]. Therefore, until they transition to endothermy, nestlings are dependent on their mother to minimize

fluctuations in temperature that can occur at night or during the day when the nest is exposed to direct sunlight [39]. Fluctuations in temperature increase CORT responses in some birds and nestlings are potentially susceptible to these effects [13, 14, 23]. Female sparrows could compensate for reduced ground cover (hence greater temperature fluctuations) by increasing incubation or shading. However, we found no association between incubation bouts/duration and proximity to the road. Alternatively, reduced ground cover could increase nestling CORT physiology by increasing exposure to predators. However, nests surrounded by low ground cover were equally likely to fail as to succeed and were no more likely to fail from predation compared to nest abandonment. The amount of ground cover surrounding a nest is predictive of nestling baseline CORT, but how or if this relates to road proximity is unclear.

Roads can affect the composition of plant communities which could affect food abundance for white-crowned sparrows. The height and density of diamondleaf willow increased close to the road while the height and density of Sierra willow increased far from the road. Parents feed nestlings bits of plants including leaves, buds, and flowers and arthropods including caterpillars [39]. Although, the specific components of nestling diet are unknown at this site, it is possible that changes in the abundance of willows affects food availability which could influence both nestling growth and CORT physiology. Even though there were no associations between diamondleaf willow and Sierra willow cover surrounding nests and any measure of nestling HPA physiology or growth, large-scale changes in this vegetation (i.e. along a gradient from the road) may be more important determinants of food availability than the vegetation closely surrounding nest sites.

4.4. Nest success and proximity to road

Nest success has previously been shown to decrease with proximity to road [5, 15]. In contrast to these findings, we found no association between nest success and road proximity (Fig. 2a). However, of nests that failed, nests close to the road were more likely to fail from predation than abandonment (Fig. 2b). In Tioga Pass Meadow, Belding's ground squirrels are thought to be the principal predator on white-crowned sparrow nests [39]. Other nest predators in this area such as long-tailed weasels and

coyotes may avoid areas close to the road because of traffic or human activity [43]. A decline in predators that prey on nests and small mammals could make roadside habitat safer for Belding's ground squirrels and indirectly increase nest predation by increasing ground squirrel populations. Alternatively, nest predation may be higher close to roads because increased provisioning by males attracts predators to nests. In a study of ten open-nesting passerines, Martin et al. [34] showed that large increases in parental activity around the nest during the nestling period increase nest predation. In our system, we describe increases in both nest attendance and nestling provisioning by fathers in relation to road proximity. Potentially, increased paternal activity around nests explains increased nest predation close to the road.

4.5. Conclusions

Like adults, nestling passerines are affected by roads: road proximity increases nestling stress and body size. However, the majority of variance in nestling stress physiology was explained by differences in body size indicating elevated CORT levels result from HPA axis development and not necessarily from stressors. Nestlings may grow and develop faster close to the road because paternal provisioning rate increased with road proximity. Overall, it appears that roads have consequences for nestling white-crowned sparrow stress physiology and life-history. However, these effects do not appear to be directly influenced by the road, but rather appear to be indirectly mediated through interactions with social fathers. Whether or not roads are bad for nestlings remains unclear. Nestlings close to the roads were larger which could increase survival after fledging, but these nestlings have a greater chance of dying from predation while in the nest. While not explored in the current study, future work should examine whether these influences of road proximity during early development influence later life history stages and the population dynamics of avian communities. Finally, this study examined just one population; future studies should examine the influence of roads across populations of birds to assess whether the findings described here are representative of the impact of roads on avian communities.

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Figure 1. GIS map of white-crowned sparrow nests in Tioga Meadow, CA.

Figure 2. On average, nests in close proximity to Hwy 120 experienced a) no difference in fledging success and b) a greater chance of failing from predation than nest abandonment.

Figure 3. Nestling body size (PCA component score 1) decreases with distance from road.

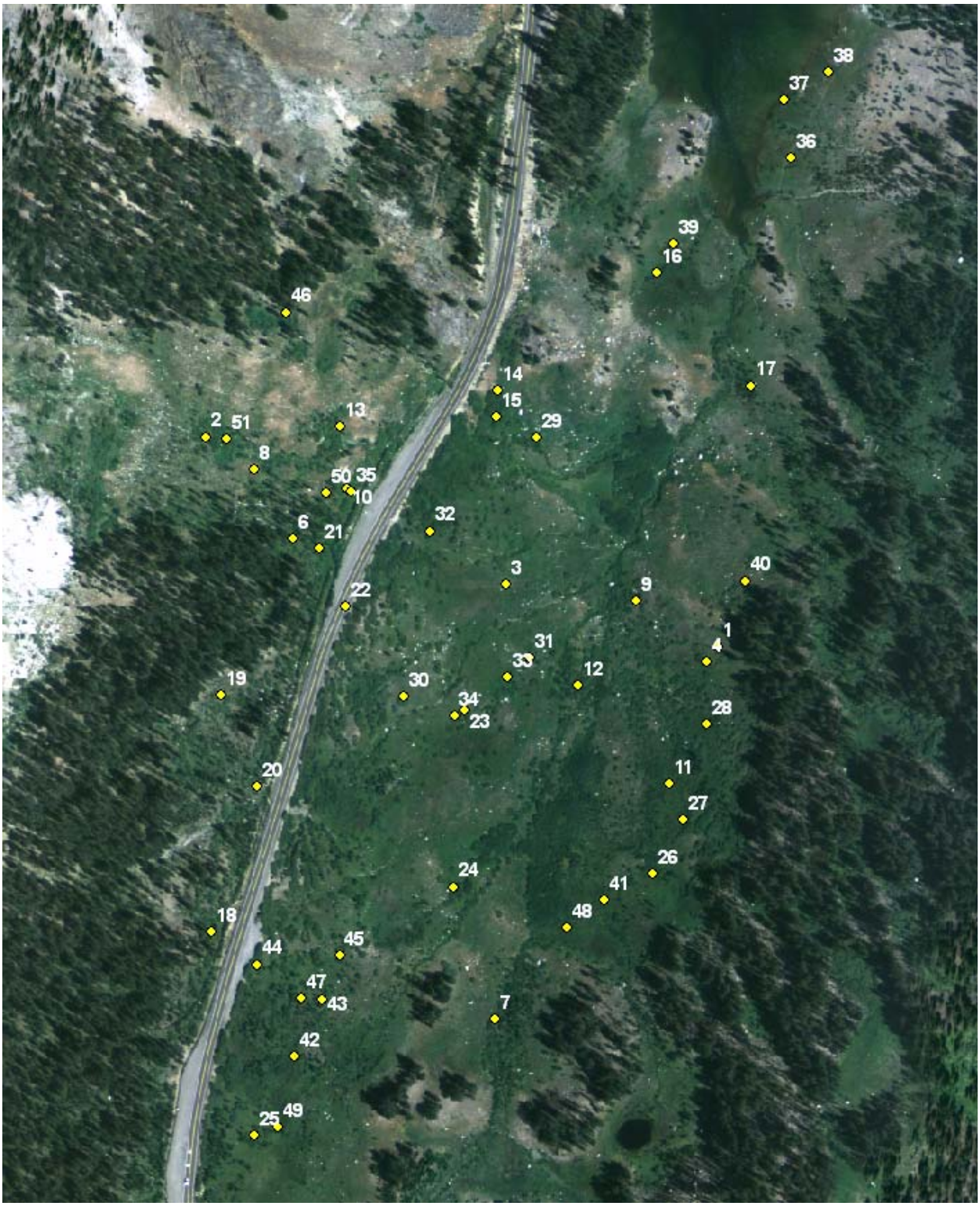
Figure 4. Nestling baseline (A) and total integrated (B) CORT increase with proximity to road.

Figure 5. Nestling total integrated CORT (ng/ml) increases with road proximity after controlling for increased body size (PCA component 1).

Figure 6. Rank correlations for nestling and paternal (A) baseline CORT and (B) total integrated CORT, and nestling and maternal (C) baseline CORT and (D) total integrated CORT.

Figure 7. Paternal nest attendance increases with proximity to the road (A). Paternal provisioning trips per hour non-significantly increased with proximity to the road.

	Pine tree cover **1**	*S. planifolia* cover **2**	*S. orestera* cover **3**	Ground cover **4**
Nest height (m)	**0.77**	0.26	-0.15	0.06
Mean *S. planifolia* height (m)	0.17	**0.96**	-0.12	-0.01
Mean *S. planifolia* density (m)	-0.41	**0.93**	-0.28	0.15
Mean *S. orestera* height (m)	0.14	-0.11	**0.96**	-0.10
Mean *S. orestera* density (m)	-0.08	-0.21	**0.96**	-0.03
Mean *P. contorta* height (m)	**0.96**	-0.02	0.12	0.05
Mean *P. contorta* density (m)	**0.97**	-0.06	0.07	0.07
Mean annual grass density	-0.13	-0.42	-0.06	**-0.86**
Mean bare ground density	0.06	-0.49	-0.35	**0.74**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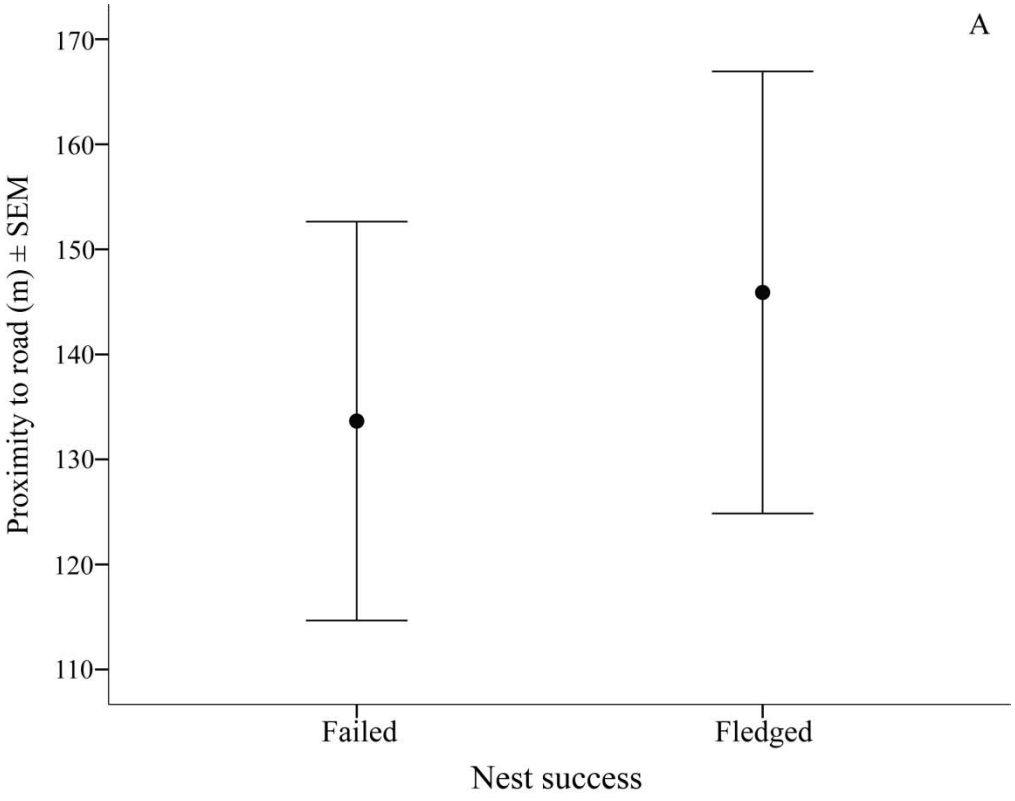
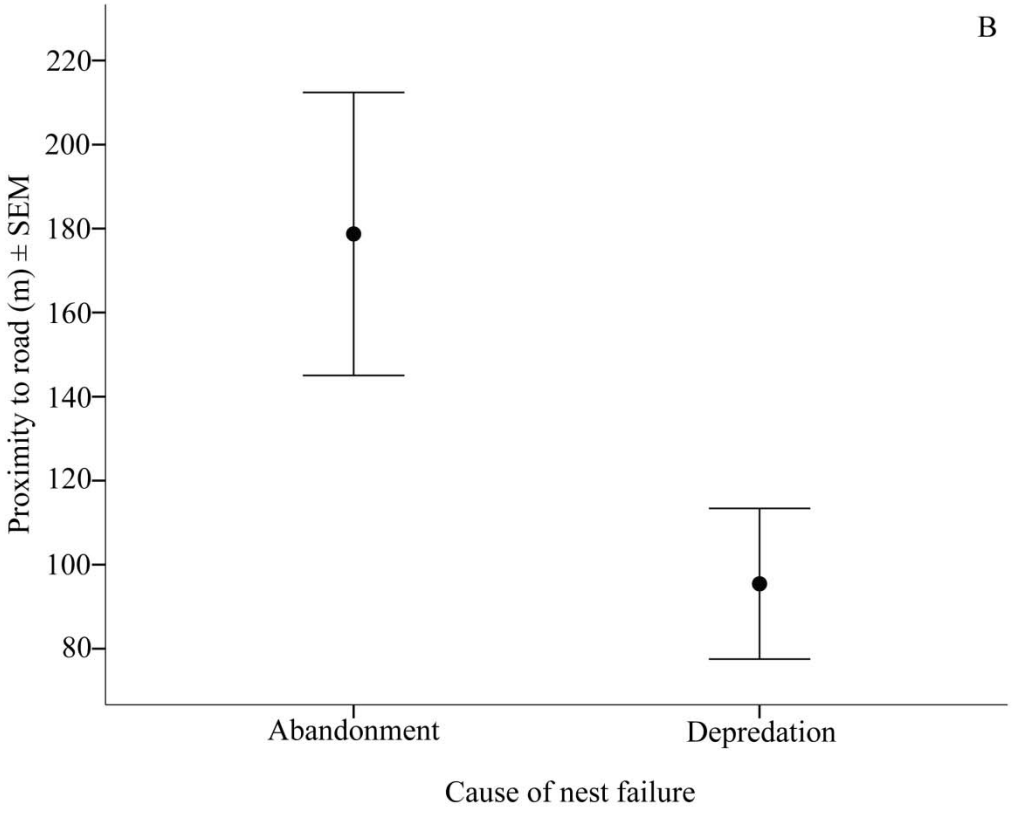

A
170
160
150
140
130
120
110
Proximity to road (m) ± SEM
Failed
Fledged
Nest success


B
220
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180
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120
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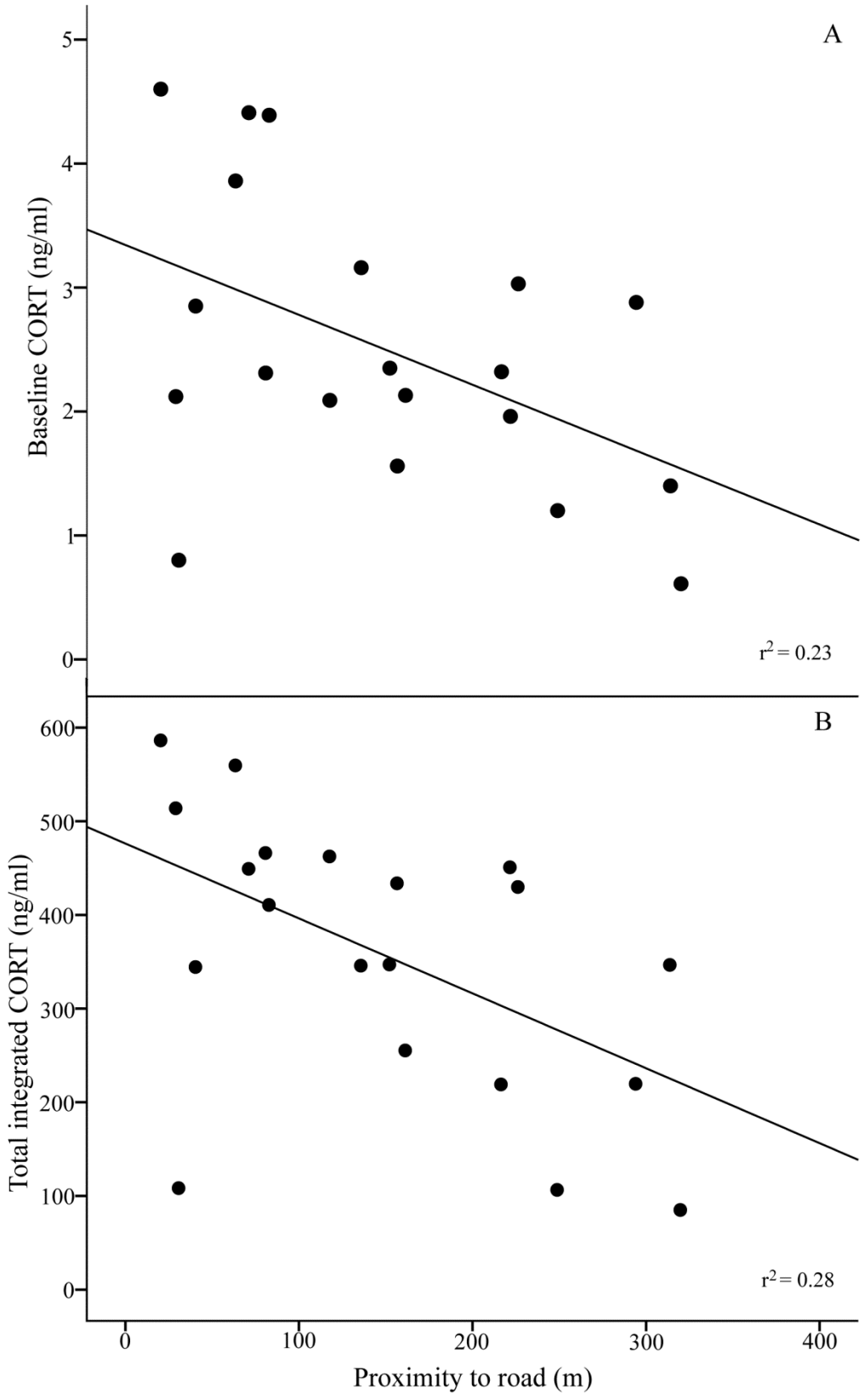
80
Proximity to road (m) ± SEM
Abandonment
Depredation
Cause of nest failure


A
Baseline CORT (ng/ml)
5
4
3
2
1
0
r2 = 0.23
B
Total integrated CORT (ng/ml)
600
500
400
300
200
100
0
r2 = 0.28
0
100
200
300
400
Proximity to road (m)

Chapter 5: The effects of experimentally elevated traffic noise on nestling white-crowned sparrow stress physiology, immune function, and life-history

Keywords: traffic noise, stress physiology, nestling, development

SUMMARY

Roads have been associated with behavioral and physiological changes in wildlife. In birds, roads decrease reproductive success and biodiversity and increase physiological stress. Although the consequences of roads on individuals and communities have been well described, the mechanisms through which roads affect birds remain largely unexplored. Here, we examine one mechanism through which roads could affect birds: traffic noise. We exposed nestling mountain white-crowned sparrows (*Zonotrichia leucophrys oriantha*) to experimentally elevated traffic noise for five days during the nestling period. Following exposure to traffic noise we measured nestling stress physiology, immune function, body size, condition, and survival. Based on prior studies, we expected the traffic noise treatment to result in elevated stress hormones (glucocorticoids), and declines in immune function, body size, condition and survival. Surprisingly, nestlings exposed to traffic noise had lower glucocorticoid levels and improved condition relative to control nests. These results indicate that traffic noise does affect physiology and development in white-crowned sparrows, but not at all as predicted. Therefore, when evaluating the mechanisms through which roads affect avian populations other factors (e.g. edge effects, pollution, and mechanical vibration) may be more important than traffic noise in explaining elevated nestling stress responses in this species.

INTRODUCTION

Roads are a ubiquitous component of human-altered landscapes. Although roads have many positive effects for people, they can engender negative effects for biological communities. For example, areas near roads are associated with decreased population densities, genetic diversity, and biodiversity in many taxa including plants, mammals, reptiles, amphibians, and birds (e.g., Benítez-López et al., 2010; Fahrig and Rytwinski, 2009; Holderegger and Di Giulio, 2010). Proximity to roads increases physiological stress in birds including the northern spotted owl (*Strix occidentalis caurina*) and themountain white-crowned sparrow (*Zonotrichia leucophrys oriantha*; Crino et al., 2011; Hayward and Wasser 2006, Wasser et al. 1997). Additionally, proximity to off-trail vehicles such as snowmobiles has been associated with elevated levels of stress hormones in the gray wolf (*Canis lupus)* and elk (*Cervus canadensis*; Creel et al., 2002). Although the individual- and population-level effects of roads on wildlife have been well-documented, comparatively little is known about the mechanisms by which roads affect biological communities.

Of factors that alter ecosystems, there is increasing evidence that anthropogenic noise is prominent force which affects the ecology and evolution of many species (Francis et al., 2009; Kight and Swaddle, 2011; Slabbekoorn and Ripmeester, 2008). Anthropogenic sounds are ubiquitous in human-altered landscapes and, as such, are often described as 'noise pollution' (Francis et al., 2009). Unlike natural noise sources, noise pollution is typically loud, low in frequency, and may be constant in duration (Forman and Alexander, 1998; Francis et al., 2009). Because it is different from natural sounds, noise pollution is an evolutionarily unique selection pressure which can affect wildlife at both the individual and population levels (Slabbekoorn and Ripmeester, 2008).

Hence, roads can alter landscapes in ways that affect an individual's ability to survive and reproduce, and the associated traffic noise itself is a likely cause of at least some of these impacts. For example, traffic noise can interfere with acoustic signals animals use to communicate (Barber et al., 2009). The southern brown tree frog (*Litoria ewingii*)

calls at a higher pitch in the presence of traffic noise (Parris et al., 2009). Similarly, birds exposed to traffic noise have been shown to sing louder and at higher pitches (Brumm, 2004; Slabbekoorn & Peet, 2003). Traffic noise may also alter habitat use by organisms. In laboratory experiments, greater mouse-eared bats (*Myotis myotis*) avoided foraging in areas exposed to traffic noise (Schaub et al., 2008). Greater mouse-eared bats are gleaning predators that rely on acoustic cues to detect and capture insect prey. By disrupting cues used by bats for foraging, traffic noise could decrease the suitability of habitat close to roads in natural environments (Schaub et al., 2008). And finally, experimental evidence demonstrates that traffic noise increases stress in animals. In greater sage-grouse (*Centrocercus urophasianus*), males avoided breeding display grounds (leks) with experimental playback of noise from traffic and industrial activity, and males that remained on noise-playback leks had higher levels of corticosterone metabolites in fecal samples (CORT; the dominant glucocorticoid stress hormone in birds) compared to males on control leks (Blickley et al., 2012; Blickley et al. 2012). In domesticated chickens, traffic noise exposure in the laboratory increased behavioral stress and decreased immune function (Campo et al., 2005). Unlike other sources of anthropogenic noise, traffic noise is intermittent which could startle animals and explain increased stress responses (Dooling and Popper, 2007). Cumulatively, these studies demonstrate the disruptive effects of traffic noise on animal behavior and physiology, and suggest that anthropogenic noise can disrupt community structure and potentially exclude sensitive species from otherwise suitable habitat.

Although many studies have examined the effects of traffic noise on adult animals no study to date has addressed the effects of traffic noise on developing animals. Developing animals are susceptible to anthropogenic disturbances and respond with elevated levels of CORT. For example, nestling mountain white-crowned sparrows (hereafter: MWCS) reared close to a high-traffic road had elevated levels of CORT compared to nestlings reared far from the road (Crino et al., 2011). Developing animals such as nestlings may be particularly susceptible to stressors associated with roads such as traffic noise because they are confined to nests during early development and are unable to move away from stressful stimuli. In addition to being more susceptible to

anthropogenic disturbances, developing animals may also suffer greater consequences from their exposure. Elevated CORT during development has immediate effects, such as inhibition of growth and immune function (Butler et al., 2010; Yorty et al., 2004). However, brief elevations of stress during development can also have life-long consequences for physiology, morphology, and behavior (Catalani et al., 2000; Gluckman and Hanson, 2004; Seckl and Meaney, 2004). For example, developmental stress can sensitize the hypothalamic-pituitary-adrenal axis (HPA axis; the neuroendocrine pathway which releases glucocorticoids) such that stressed neonates are hypersensitive to stress as adults (Francis et al., 1999). These developmental alterations can be transmitted to future offspring by modifying parental behavior (Francis et al., 1999). Hence, exposure to stress can be detrimental to nestlings during development, but can also have lifelong and transgenerational effects on reproductive success and survival. As such, exposure to even short periods of stress during development could translate to large-scale effects. Thus, to fully understand the impact of traffic noise on wildlife, it is necessary to examine how developing animals are affected by such disturbances.

We tested the hypothesis that exposure to traffic noise increases physiological stress (i.e. CORT) in free-living nestling MWCS. We played back traffic noise to nestlings for five days and measured baseline and stress-induced CORT. Glucocorticoid hormones have well-known immunosuppressive effects and can inhibit growth in developing animals and condition in adult animals (Boonstra et al., 1998; Rubolinni et al., 2005; Sapolsky et al., 2000). For this reason, we also measured immunocompetence using the phytohaemagglutinin (PHA) skin test, nestling body size, and condition. We predicted that nestlings exposed to traffic noise would have higher stress-induced CORT, lower immunocompetence, and be of smaller body size and in lower condition compared to control nestlings. Finally, we examined the effects that traffic noise exposure had on nestling survival and fledging success.

MATERIALS AND METHODS

Animals – study site and nest searching

This study was conducted from June 27th to August 10th, 2010 using MWCS nestlings from a population located in Tioga Pass Meadow located directly outside the eastern most entrance to Yosemite National Park, CA (37˚54´N, 119˚15˚´W). Highway 120 runs through part of this study site to the entrance of Yosemite National Park and nestlings close to the road have greater physiological stress responses than nestlings far from the road (Crino et al., 2011). To minimize the effects of ambient traffic noise and other road effects, we used nests located far from HWY 120 (>300m) for this experiment. We took GPS coordinates of each nest using a Garmin eTrex GPS device in WGS 84 datum. The distance between each nest and Highway 120 was calculated using the Nearest Features 3.8 extension in ArcView Geographic Information System (GIS). Mean ambient noise at control sites was 42.74 ± 0.34 d(BA). We found no associations between the distance to HWY 120 from each nest and all measures of nestling stress physiology, immunocompetence, growth, and fledging success (All $p > 0.37$; data not presented here). This indicates that the nests used in this experiment were located far enough from HWY 120 to minimize the effects of ambient noise and other road effects.

Nests were located by searching vegetation and observing parental nesting behaviors. If nests were found with a complete clutch we checked them daily to determine hatch date. Nests that were found with incomplete clutches were checked until clutch completion. Females begin to incubate the day before the last egg is laid and nestlings hatch an average of 12.3 days after incubation commences (Morton, 2002). Using this information, we estimated hatch date for clutches and checked nests daily starting 1-4 days before hatching to determine hatch date.

Sound recording and sound pressure level measurements

We recorded 130 sound clips of cars, motorcycles, trucks, and other vehicles on Hwy 120 onto a Marantz PMD 660 digital flash recorder using a Sennheiser ME 67 shotgun

microphone fitted with a Rycote softie windscreen (sample rate: 16 bit; bit depth: 44.1 KHz). We edited sound files for length and standardized amplitude using Raven Pro 1.3 (Charif et al., 2006). To reduce clipping between the playback files we added 'fade in' and 'fade out' effects using Audacity 1.3 Beta.

We used a hand-held sound pressure level (SPL) meter (Larson-Davis System 824, Depew, NY) to determine the amplitude of traffic along Hwy 120. We collected SPL measurements for 30 minutes five meters from the road. From these measurements we determined the equivalent continuous sound level (Leq) and maximum noise levels (Lmax- maximum RMS amplitude) of traffic noise five meters from the road (the distance of the closest nests in this population). All SPL values are A-weighted (dBA) re 20 μPa.

Experimental set-up - traffic noise playbacks

Nests were randomly assigned to either a control or traffic noise playback treatment (n = 8 for traffic noise, n = 5 for control). Similar to Blickley et al. (2012), we played back traffic noise using a rock-shaped outdoor speaker (300W Outdoor Rock Speakers, TIC Corporation, City of Industry, CA), a car amplifier (Xtant1.1, Xtant Technologies, Phoenix, AZ) and an MP3 player (Sansa m240, SanDisk, Milpitas, CA). The playback system was powered with 12-volt batteries that were changed every day. Control nests were exposed to fake speakers of similar size and color to the speakers (20 gallon Rubbermaid roughneck containers). We visited control nests daily to account for any stress effects caused by experimenter disturbance. Nests were exposed to treatments for five days starting one day after hatching.

Speakers and control containers were placed 5 meters from nests. To set the amplitude for the playbacks, one researcher held the SPL meter over the nest while another played one standardized traffic noise file. The amplitude was adjusted until the Lmax of the playback was 60 dBA ±1.5 at the nest. We then quantified the amplitude of the playback files by recording the Leq at each treatment nest for five standardized traffic noise

recordings which totaled 40 seconds in length. The Leq for playback of five standardized traffic noise files ranged from 58.5 – 60.6 dBA (mean 59.53 ± 0.94). For control nests, we recorded the Leq of ambient noise by holding the SPL meter over the nest for five minutes. We chose to quantify ambient noise for five minutes (compared to 40 seconds on playback nests) since noise levels can be variable over time (e.g. with vehicles passing), so we needed a longer sample interval to characterize a typical noise level. Ambient noise at control nests ranged from a 5-minute Leq of 42.4 – 46.4 dBA (mean 44.53 ± 1.47). In addition, we measured sound output from playback files and ambient noise at all nests for 60 minutes following fledging. These measurements provide a longer sample time of noise levels for both treatments while minimizing disturbance to nestlings.

We played back traffic noise files on noise-treatment nests at a rate the simulated traffic on Hwy 120. In 2009, Yosemite National Park reported an average traffic rate of 1225.67 cars/day from May 1st to August 31st on Hwy 120. Using this information, we calculated that an average of 51.07 cars/hour entered the park. Since the average length of our vehicle noise files was 7.93 seconds, in an average hour there was 6.75 minutes of traffic noise. Therefore, the MP3 players were loaded with 53.25 vehicle noise files (cars, trucks, motorcycles and other vehicles; see above) and 135 silent files, each 60 seconds long; these files were played back on random shuffle 24 hours a day throughout the experiment. We played files continually, rather than mimicking diurnal changes in traffic patterns, in order to decrease investigator disturbance at nests. As a result, simulated traffic levels were lower than actual traffic levels during the day and higher than actual traffic levels at night.

Nestlings – stress, immune, morphological, and condition measurements

Six days after hatching, we measured nestling stress by exposing the two nestlings in each nest to a standardized restraint stress protocol (Wingfield, 1994). Although plasma CORT typically increases within three minutes of stress exposure (Romero and Reed, 2005), Nuttall's white-crowned sparrows (*Zonotrichia leucophrys nuttalli*) do not show

an increase in CORT until four minutes following stress exposure (Wada et al., 2007). For this reason, we used blood samples obtained within four minutes of disturbing a nest as baseline samples. Samples obtained within four minutes show no significant increase in CORT ($P=0.94$, $F<0.01$, $n=22$). After initial blood samples were obtained, we placed nestlings in cloth bags and collected two more samples 15 and 30 minutes after initial disturbance. To collect blood, we punctured the alar vein with a 26-gauge needle and collected 25µl of blood with heparinized microcapillary tubes. Immediately after collection, blood was kept on ice (<2hours) until it could be centrifuged to separate the plasma from red blood cells (3000 rpm for seven minutes). After separation, the plasma was isolated and stored at -20˚C.

We used the phtyohaemagglutinin (PHA) skin test to measure nestling immunocompetence. PHA induced-swelling involves innate and adaptive components of the immune system and is widely used in avian research to assess immunocompetence (reviewed by Martin et al., 2006; Tella et al., 2002). The vertebrate immune system is complex and the PHA test measures only one aspect of immune function. However, PHA induced-swelling has been positively correlated with nestling survival in great tits (*Parus major*; Horak et al., 1999) and house martins (*Delichon urbicum*; Christe et al., 1998). Stress exposure can decrease immune responses in nestlings (e.g. Butler et al., 2010; Saino et al., 2003). For this reason, we only used nestlings in this assay that were not exposed to restraint stress or blood collection.

To measure immune response to PHA we injected nestlings subcutaneously with 0.1mg of PHA-P (SIGMA Chemicals, L9017) dissolved in 0.02ml of sterile phosphate-buffered saline into the center of the left wing-web (patagium). We measured the thickness of the patagium to the nearest 0.01mm using a digital a pressure-sensitive spessimeter (Mitutoyo gauge, MIT-700-118, Brooklyn, NY) prior to injection and 24 ± 2 hours after injection. We collected three measurements at each time point and used the average value of these three measurements in statistical analyses. One person (O.C.) performed all injections and measurements.

After blood sample collection or PHA injections, 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. All nestlings were measured once by one person (O.C.). We used these morphological measurements to calculate condition for each nestling. Here, we define condition as the energy capital accumulated by nestlings due to parental feeding. Condition is thought to be indicative of an animal's health and well-being and related to fitness (Peig and Green, 2009). In developing animals, condition could be an important indicator of competitive ability within the nest, maturation, and survival and recruitment following fledging (Miller, 2010; Mock et al., 2009; Searcy et al. 2004). We assessed condition using residual body mass (mass divided by tarsus length) and the scaled mass index (Peig and Green, 2009; 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).

Nestling fledging success

Exposure to experimentally elevated traffic noise could have affected nestling survival directly, by increasing stress, or indirectly, through changes in parental behavior. We quantified fledging success by tracking nestlings from hatching to fledging. We considered a nestling to have successfully fledged if it was missing from the nest after reaching the developmental stage where fledging is possible (at least seven days post-hatch; Morton, 2002). Nestlings that were found to be dead in the nest or nestlings that were missing from the nest less than seven days post-hatch were considered unsuccessful at fledging. Partial nest predation is rare in this population (Morton, 2002). Therefore, our criteria for fledging success provide an accurate measurement of nestling survival.

Corticosterone and corticosteroid binding globulin assays

Corticosterone was quantified with Enzyme Immunoassay (EIA) kits (Cat No. 901-097, Assay Designs), previously optimized for white-crowned sparrow nestlings (Wada et al., 2007). Following the protocol used by Wada et al. (2007), we used a raw plasma dilution of 1:40 to determine CORT levels. Briefly, we added 10 µl of 1:100 steroid displacement buffer (SDB) to 10 µl of plasma. After 15 minutes, we added 380 µl of assay buffer for a total dilution of 1:40. Samples were vortexed and 100 µl of a sample was added to individual wells in triplicate. These samples were compared to a standard curve with six samples run in triplicate ranging from 20,000 to 15.53 pg/ml (100 µl/well). An external standard of 500 pg/ml was run on every plate in triplicate and used to calculate inter-plate variation. Plates were read on a Multiskan Ascent microplate reader at 405 nm corrected at 595 nm. Intra- and inter-plate variation 5.08 and 12.90% 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 and Orchinik, 2002; Malisch and Breuner, 2010). Stress can decrease CBG levels which may increase the amount of CORT available to interact with target tissues. Therefore, measuring free CORT (the portion of CORT not bound to CBG) can provide additional information about how animals respond to stressors. We quantified CBG using a ligand-binding assay with tritiated CORT (as described in Breuner et al., 2003). This assay has been optimized for MWCS adults (Lynn et al., 2003) and used for MWCS nestlings (Wada et al., 2007). We thawed nestling plasma at 4˚C and stripped plasma with deactivated charcoal at a 1:3.5 ratio. Samples were incubated at room temperature for 20 minutes and then vortexed at 4˚C, 10,000rpm for 10 minutes. The stripped plasma supernatant was removed and stored at -20˚C until assayed. We determined total CBG binding using 50µl buffer, 50µl tritiated CORT, and 50µl stripped, diluted plasma (for a 1:1050 final dilution of raw plasma). Non-specific binding (NSB) was determined using 50µl of 1 µM unlabeled CORT, 50µl tritiated CORT, and 50µl stripped plasma. Intra- and inter-filter variation for CBG point samples were 5.7 and 14.32% respectively.

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

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

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. (2007). Individual CBG capacity estimates were approximately 83% of B_{max} so capacity values were increased to 100% for free CORT calculations.

We compared nestling CORT physiology by examining the amount of total and free CORT circulating before restraint stress (baseline CORT) and after 15 and 30 minutes of stress exposure. Additionally, we examined the amount of total and free CORT released during 30 minutes of stress exposure (total integrated CORT). Total integrated CORT represents that total amount of CORT target tissues are exposed to during an acute stressor.

Statistical analyses and sample size

All CORT measurements were normally distributed ($P>0.17$), except for total CORT response ($P=0.048$) and free CORT after 30 minutes of stress exposure ($P=0.02$). We log transformed total CORT response and free CORT after 30 minutes of stress exposure and used the resulting values in all statistical analyses. We used analysis of variance (ANOVA) to statistically evaluate differences between treatment groups. Brood number can affect nestling growth and development in white-crowned sparrows (Morton, 2002). However, we found no statistical difference in brood size between treatment groups ($P=0.90$, $F=0.02$).

We exposed eight nests to traffic noise and five nests to the control treatment. We were unable to use the blood samples from one nest exposed to traffic noise. Therefore, our samples sizes for analyses examining the effects of traffic noise on CORT physiology are

seven and five for traffic noise and control treatments respectively. The sample sizes for all other analyses are eight and five for traffic noise and control treatments respectively. We estimated effect sizes for all analyses by calculating the standardized mean difference between treatment groups using the formula: $d = \frac{m_1 - m_2}{s_{pooled}}$ (Cohen 1988, Nakagawa and Cuthill 2007). In this equation m_1 is the mean of group one, m_2 is the mean of group two and s_{pooled} is the standard deviation of both groups. Effect sizes are reported as positive values.

RESULTS

Experimentally elevated noise

Following fledging, the Leq of one hour of ambient noise at control nests ranged from 42.5-43.3 dBA (mean 42.74 ± 0.34; Lmax 55.06 ± 4.42). The Leq of one hour of traffic noise playback ranged from 45.9 – 50.3 dBA (mean Leq 47.88 ± 1.49; Lmax 65.23 ± 3.61). Noise at nests exposed to traffic noise was higher during one hour of playback compared to one hour of ambient noise at control nests ($F_{1,9}$=43.98, P<0.0001).

Nestlings – stress, immune, and morphological measurements

There was no difference in baseline CORT between treatment groups (Fig.1A, $F_{1,10}$=1.65, P=0.23, d=0.73). However, nestlings exposed to traffic noise released significantly lower levels of CORT 15 minutes after exposure to a standardized stressor compared to control nestlings (Fig. 1A, $F_{1,10}$=8.77, P=0.01, d=1.33). At 30 minutes of stress exposure there was no difference in CORT release between treatment groups (Fig. 1A, $F_{1,10}$=2.72, P=0.13, d=0.90). However, nestlings exposed to traffic noise had lower total CORT release over the entire 30 minute period of stress exposure compared to control nestlings indicating that they are responding with dampened CORT output (Fig. 2A, $F_{1,10}$=6.23, P=0.03, d=1.25). CBG levels did not differ between treatments (F=0.84, P=0.38, d=0.54), nor did calculated free CORT levels after zero, 15, and 30 minutes of stress exposure or the total amount of free CORT available during 30 minutes of stress exposure (total free

integrated; Fig. 1B, P>0.53 for all, d=0.33, 0.12, 0.13, and 0.17 respectively). There was no difference in any of the body size measurements between treatment groups (P>0.29 for all; Table 1). However, nestlings exposed to traffic noise were in better condition compared to nestlings in the control group. This relationship was significant for condition calculated as mass/tarsus ($F_{1,11}$=7.24, P=0.02) and trended toward significance for condition measured using the scaled mass index ($F_{1,11}$=4.62, P=0.055, Fig. 3, Table 1).

Traffic noise exposure did not affect nestling immunocompetence as measured by the PHA test. There was no difference in the amount of wing web swelling between nestlings in the control and traffic noise treatments ($F_{1,7}$=0.54, P=0.82, Table 1).

Nestling fledging success

All nests in both treatments successfully fledged at least one nestling. We recorded nestling mortality up to two nestlings in seven nests. However, treatment had no effect on nestling mortality ($F_{1,11}$=0.006, P=0.938, Table 1) or the proportion of nestlings which successfully fledged ($F_{1,11}$=0.072, P=0.793, Table 1).

DISCUSSION

Proximity to roads has been associated with elevated stress hormones in wildlife including white-crowned sparrow nestlings (Crino et al. 2011). Here we examined one possible cause of this effect, traffic noise, by examining the effects of experimentally elevated traffic noise on nestling CORT physiology, growth, immunity, and fledging success. To our knowledge, this is the first experiment to examine whether traffic noise is responsible for the effects of roads on nestlings. We found that experimentally elevated levels of traffic noise affects nestling stress physiology and condition. However, contrary to our predications, nestlings exposed to traffic noise had lower CORT release and were in better condition compared to control nestlings.

Mass, body size and condition

Experimentally elevated traffic noise did not affect nestling body size. However, nestlings exposed to elevated traffic noise were in better condition compared to control nestlings as estimated by tarsus/mass and marginally better condition as measured by the scaled mass index (Fig. 3, Table 1). Condition provides an estimate of the energy reserves available to an animal and is an important ecological and evolutionary variable because it is widely considered to be an important determinant of fitness (Peig and Green, 2009; 2010). However, in developing animals, indices of condition should be interpreted with caution because developing animals prioritize growth over energy storage. In adults, greater condition indicates a superior ability to obtain and assimilate food. It is possible that greater nestling condition in this study indicates higher parental feeding rates. Crino et al. (2011) describe higher feeding rates of male white-crowned sparrows in close proximity to a high-traffic road. Potentially, traffic noise masks acoustic cues adult sparrows use to detect predators, promoting greater feeding behavior and, hence, increasing condition. However, if traffic noise increases paternal feeding rates, we would expect greater nestling mass, not just greater condition. Hence, it appears that traffic noise is somehow altering energy deposition decisions, with greater energy apportioned to soft tissue than skeletal size.

Corticosterone physiology

We predicted that traffic noise would elevate nestling stress responses. This prediction was based on the knowledge that acute or short-term stressors result in elevated levels of glucocorticoid hormones (Cyr and Romero, 2009; Wingfield et al., 1998). Contrary to this prediction, we found significantly dampened CORT responses compared to control nestlings. Chronic or prolonged stressors which consist of multiple, frequent exposures to a stressor and/or long-term or constant exposure to stressors can decrease baseline and stress-induced CORT levels (Cyr and Romero, 2009; Dallman et al., 2001; Rich and Romero, 2005; but see Dunlap and Schall, 1995; Moore et al., 1991). If experimentally elevated traffic noise chronically stresses nestlings, low levels of CORT secretion may

indicate adrenal exhaustion and suggest that traffic noise has a substantial negative effect on nestlings.

We found that nestlings exposed to traffic noise had significantly lower CORT release after 15 minutes of restraint stress compared to control nestlings. However, we found no differences in baseline CORT between nestlings exposed to traffic noise and control nestlings. This suggests that nestlings exposed to traffic noise are responding less strongly to stress and not that their HPA activity has been down-regulated (as would be expected in response to chronic stressors). Additionally, if nestlings were under conditions of chronic stress, we would expect to detect this effect in other system by observing decreased skeletal growth, weight gain, or immune function (Butler et al., 2010, Martin, 2009, Sapolsky, 2000, Wada and Breuner, 2008). Contrary to this, we found no difference in tarsus or wing length, an increase in condition indices, and no difference in PHA-induced immune response. Finally, we have previously described that nestlings reared close to a high-traffic road have higher CORT responses than nestlings reared from the road (Crino et al., 2011). In this natural system with continuous traffic noise exposure we described the opposite CORT responses as we observed in response to experimentally elevated traffic noise. If traffic noise constituted a chronic stressor in this system we would have observed that nestlings close to the road had dampened CORT responses compared to nestlings far from the road. The fact that we observed the opposite suggest that factors associated with road (such as traffic noise) do not act as a chronic stressor in a natural system (Crino et al., 2011). Although the patterns of CORT responses to elevated traffic noise could suggest chronic stress exposure, the organismal consequences do not match, suggesting that other factors are influencing nestling stress responses. Future studies could determine if traffic noise is acting as a chronic stressor by measuring CORT responses at multiple time points of traffic noise exposure.

In developing animals, dampened stress responses could also result from delayed maturation of the physiological systems that control CORT output. Specifically, traffic noise could indirectly affect CORT output by decreasing development of the HPA axis.

Altricial nestlings such as white-crowned sparrows have dampened HPA responses during early development that increase over the nestling period (Blas et al., 2005; Blas et al., 2006; Wada et al., 2007; Wada et al., 2009). In seven day-old white-crowned sparrow nestlings, up to 28% of the variation in CORT release can be explained by differences in body size (Crino, unpublished data). Factors that decrease growth and development could potentially decrease HPA activity. Therefore, it is possible that the dampened response of nestlings exposed to traffic noise in this study was caused by delayed development rather than by chronic stress exposure. Although we did not observe differences in nestling body size between treatment groups (Fig. 3), it is possible that the duration of our study did not allow sufficient time for body size differences to manifest. American kestrel (*Falco sparverius*) nestlings exposed to elevated levels of CORT via implants displayed no differences in body size (mass, tarsus, and wing length) after one week of exposure (Butler et al., 2010). However, one-week following the removal of the implants, nestlings exposed to elevated levels of CORT had smaller wings (Butler et al., 2010).

Conclusion

Our data suggest that nestling white-crowned sparrows experience phenotypic effects in response to elevated levels of traffic noise. However, contrary to our predictions, nestlings exposed to traffic noise responded with decreased CORT responses and increased condition compared to control nestlings. These results indicate that anthropogenic noise may affect nestling development, but noise alone does not explain the previously observed negative impact of roads on nestling development (Crino et al., 2011). This suggests that factors other than noise, such as chemical pollution, mechanical vibration, dust, increased predation and edge effects, may be more important in causing road impacts in this species. Research addressing noise impacts on adult birds has found wide variation among species in the response to noise, with some species showing increased abundance and others showing decreased abundance in noisy areas (e.g. Francis et al., 2009) as well as wide variation in the degree of behavioral plasticity in response to noise (Francis et al., 2011; Hu and Cardoso 2010). Therefore, further

studies are needed to determine whether the observed lack of (or positive) impact of noise on nestling development is generalizable to other species of birds.

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ABBREVIATIONS USED

1. **CBG –** corticosteroid binding globulin
2. **CORT –** corticosterone
3. **HPA –** hypothalamic pituitary adrenal
4. **MWCS –** mountain white-crowned sparrow
5. **PHA** - phtyohaemagglutinin

	Traffic noise		Control		Difference between groups		Effect size
Variable	Mean	Std	Mean	Std	F	P	d
Tarsus (mm)	19.41	1.20	20.02	1.91	0.50	0.49	0.41
Wing (mm)	26.79	2.73	28.50	4.69	0.71	0.42	0.48
Mass (g)	16.94	0.98	15.90	2.38	1.24	0.29	0.63
Condition (tarsus/mass)	0.87	0.05	0.79	0.06	7.29	**0.02**	1.14
Condition (scaled mass)	17.62	1.97	15.33	1.69	4.62	0.055	1.08
Wing web swelling (mm)	0.59	0.23	0.55	0.23	0.05	0.82	0.16
Brood reduction	-0.63	0.74	-0.20	0.45	1.31	0.28	0.92
Nestlings fledged	2.63	1.30	3.00	0.71	0.34	0.57	0.34

Table 1. Exposure to traffic noise had no effect on nestling body size, immune function, or fledging success. However, experimentally elevated traffic noise did increase condition as measured as residual mass (mass/tarsus) and the scaled mass index (non-significant trend).

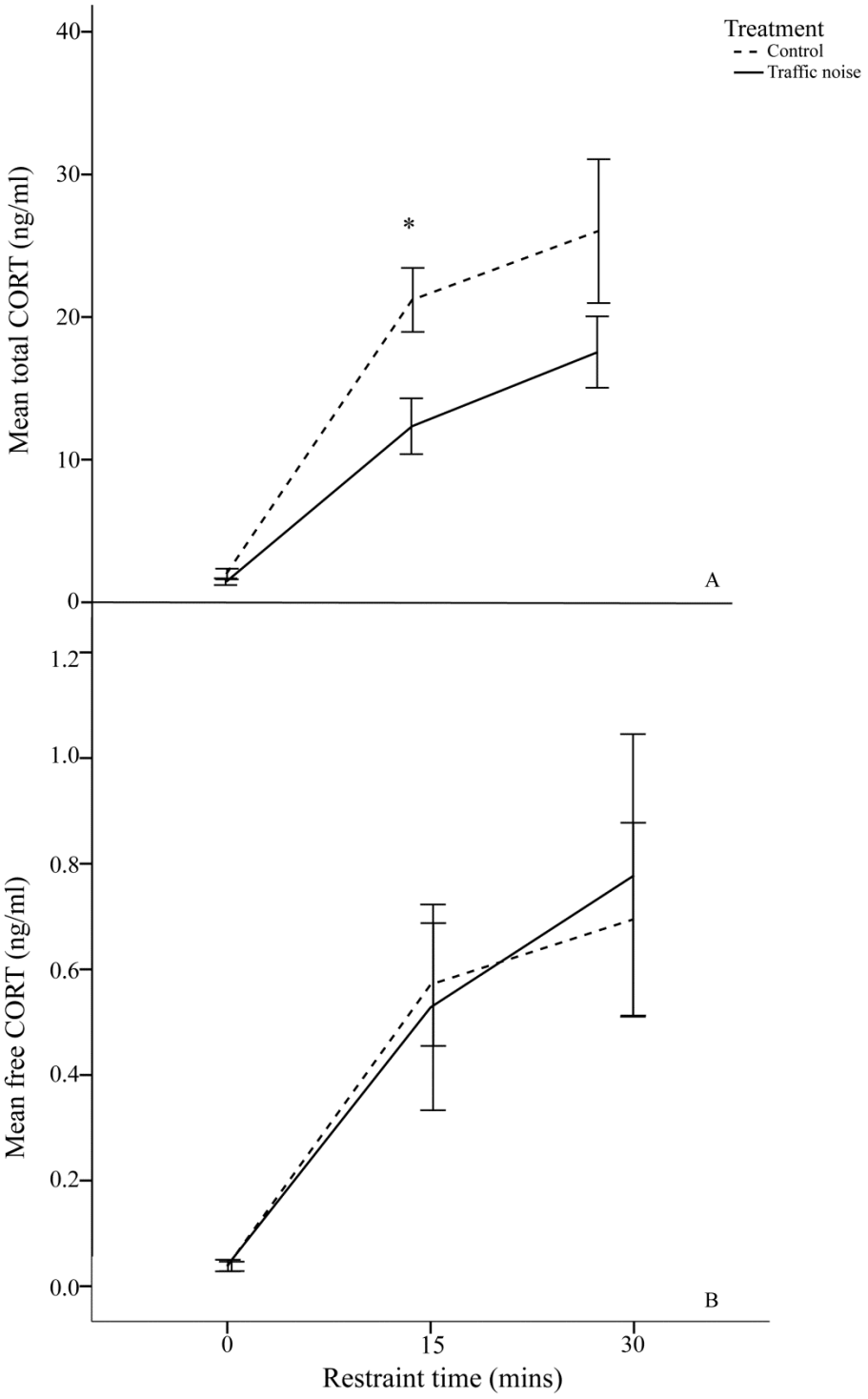

Figure 1. Nestlings exposed to traffic noise released less CORT 15 minutes after exposure to a standardized restraint compared to control nestlings. There was no difference in free CORT release at zero, 15, and 30 minutes after stress exposure.

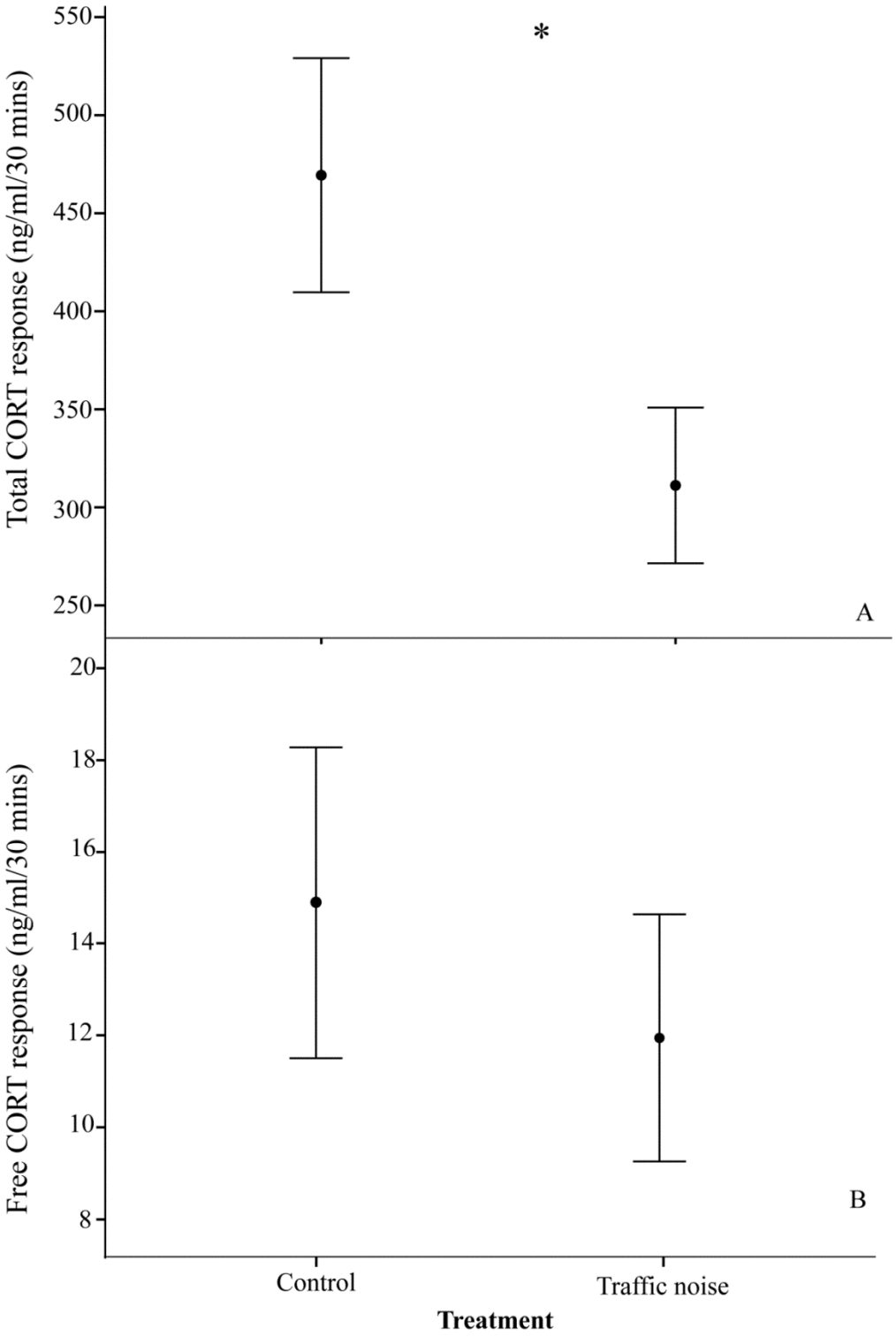

Figure 2. Compared to control nestlings, nestlings exposed to traffic noise released less A) total CORT and B) equivalent free CORT over 30 minutes of restraint stress (*P<0.05).

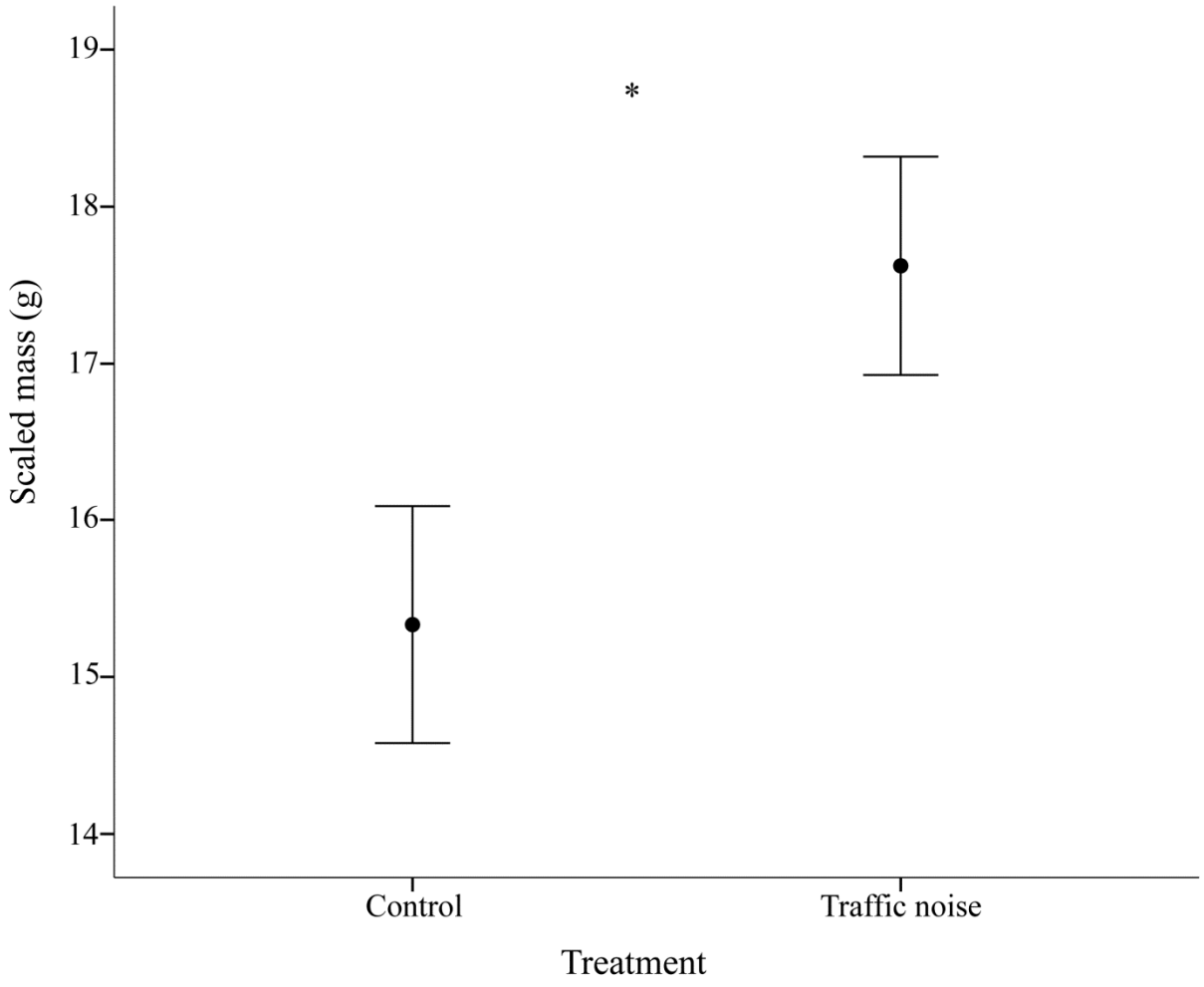

Figure 3. Nestlings exposed to traffic noise were in better condition compared to control nestlings (scaled mass index: *P=0.055).