# EARLY LIFE ENVIRONMENTAL STRESS EFFECTS ON ADULT BEHAVIOR AND BRAIN MORPHOLOGY IN ZEBRA FINCHES

By

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**Summary of Group Work**

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**Katie:** Conceived of and designed study, performed and scored behavior tests, performed MRI scans, performed skullstripping of MRI results, ran statistical analysis of MRI and stress results, wrote the MRI-related methods and results sections, made figures 3 and 4, and provided valuable feedback throughout the paper

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**Abstract**

Early developmental stress can influence brain development and ultimately shape behavioral variation in humans and other animals. While laboratory studies have repeatedly shown that exposure to developmental stressors affects brain morphology and behavioral variation across vertebrates, these studies often use direct application of severe stressors in experimental contexts to induce a response. Less well known is how naturally occurring environmental stressors impact brain and behavioral development and whether distinct stressors have distinct impacts. On the one hand, offspring development may be well buffered from natural environmental stressors, limiting their impact on behavioral traits. On the other hand, natural stressors during development may serve as cues to offspring of environmental conditions and induce changes that enable them to perform better in challenging environments later in life. In the latter case, impacts on behavior might be tuned to specific stressors. We investigate these possibilities in a semi-natural outdoor colony of the zebra finches (*Taeniopygia guttata*). We assessed individual exposure to three distinct environmental stressors during development, measured activity, aggression and anxiety-related behaviors in adulthood, and used magnetic resonance imaging (MRI) to investigate the impact of stressors on structural variation in regions of the brain associated with these behaviors. Overall, we found that early life environmental stressors impacted the development of brain and behavior, with particularly strong effects on aspects of fearfulness. However, individual stressors impacted behaviors differently, suggesting that, rather than generally inhibiting normal brain development, the natural stressors assessed here may instead act as cues of environmental conditions that individuals will experience later in life.

## Introduction

Developmental environment plays a pivotal role in shaping the brain and behavior (Bremner and Vermetten, 2001; Kofman, 2002; Spencer, 2017; Duckworth et al. 2018). Organisms from stressful environments often develop distinct behaviors (Eyck et al., 2019), such as increased fearfulness (Bertin et al., 2018; Boissy, 1995) or decreased aggression (Koolhas et al., 2010; Veneema et al., 2003; Koolhas et al., 1999), compared to individuals from less stressful environments. Environments are considered stressful when external forces disrupt an organism's homeostasis, often triggering adaptive stress responses (Stott, 1981). Many studies showing long lasting effects of stress on the brain and behavior focus on severe developmental stressors or chronic stress on adult organisms, often in experimental contexts (Atwell et al., 2012; Lormont et al., 2020; Nikolakopoulou et al., 2006). Meanwhile, the long term effects of naturally occurring environmental stressors during early development remain poorly understood. Naturally occurring stressors might include mild to moderate nutritional stress that may occur during periods of inclement weather or from having inexperienced parents or access to low quality resources during development. More recently, studies have found that exposure to suboptimal ambient temperatures during development can also be a naturally occurring stressor. Naturally occurring developmental stressors may not impose severe direct effects on brain development, however, they can potentially serve as a cue to induce adaptive changes in brain and behavior (Bateson et al., 2014). Most importantly, exposure to a wide range of environmental stressors is likely to be frequently encountered in natural populations and assessing how they impact offspring development is essential to understanding how behavioral responses to developmental stressors evolve.

One hypothesis is that developmental stressors evoke adaptive responses in organisms that prepare them for the unique challenges in their environment (Bateson et al., 2014; Chaby, 2016; Gluckman et al., 2005; Nettle and Bateson, 2015). This hypothesis is supported by the abundance of studies that show animals demonstrating specific adaptive responses, such as birds developing unique foraging and feeding behaviors after experiencing nutritional stress during early development (Careau et al., 2014; Farine et al., 2015; Krause et al., 2017). This hypothesis predicts that natural developmental stressors may act as a cue of environmental conditions and evoke specific adaptive changes in the brain and behavior that prepare an organism for unique threats in their environment. An alternative hypothesis is that the exposure to stressors impacts brain development directly (e.g. by restricting nutrient availability or corticosteroids negatively impacting growth). Given that brain development is often buffered from mild perturbations (Dobbings, 1964; Starck and Ricklefs, 1998), this hypothesis predicts that we should only see a response with extreme stressors or with an accumulation of stressors. Under this scenario, we may not observe responses to natural environmental stressors unless they are compounded by exposure to multiple distinct stressors at once and reach a threshold of severity. We aim to test these predictions by observing whether distinct and naturally occurring developmental stressors impact different brain regions and associated behaviors in source-specific ways, or whether, alternatively, environmental stressors have cumulative effects that impact brain development and behavioral variation in a generalized way.

In this study, we use a semi-enclosed, outdoor, captive colony of zebra finches and coupled controlled behavioral measures with magnetic resonance imaging (MRI) of structural variation in the brain to assess the mechanism by which early life stress exposure during development shapes brain structure and behavior in adulthood. Environmental stressors have

been well characterized in this passerine model of neurogenesis and behavioral variation, making it an ideal system to test these ideas (Schmidt, 2010). In particular, poor nutrition, predation, competition, and severe abiotic conditions, such as heat, are all known sources of stress in zebra finches (Bowen et al., 2014; Harvey et al. 1984). We focus on ambient heat stress and two indicators of nutritional stress: brood size and chick condition. Prior work in zebra finches and other avian species have shown that heat is an especially prominent abiotic stressor that causes chicks born in hot environments to be significantly smaller than those born in cold environments (Andrew et al., 2017; Andrew et al., 2018; Rodriguez and Barba, 2016; Suave et al., 2021). Large brood sizes are also important because chicks in large broods experience increased competition for resources among siblings and often show nutritional stress effects (Nilsson and Svensson, 1996). Finally, we use residual body mass as a direct measure of chick condition and an overall indicator of the quality of the nutritional environment that they were reared in. This measure captures not just variation that might be due to competition with siblings, but also variation in parental quality and experience that can frequently have important impacts on offspring condition during development (Peig and Green, 2010).

We assess how these early life, naturally occurring stressors impact behaviors related to activity, aggression, and fear. Studies have shown that adult activity levels are strongly related to the developmental environment, with nestling body mass having a positive relationship with nestling and adult activity (Brust et al., 2013; McCowan et al., 2014). Early life environments have also been shown to impact adult aggression (Haller et al., 2014; Walker et al., 2018), with stress-induced maternal effects and early life social environments having profound long term effects on aggression in passerine males (e.g. Potticary and Duckworth, 2020; Ruploh et al., 2018; Spencer, 2017). Finally, the developmental environment also has effects on fear-related

behaviors (Hau et al., 2016; Henriksen et al., 2011). Environmental and prenatal stress can both heighten and quicken emergence of fearful behaviors in rats (Dickerson et al., 2005; Macri and Wurbel, 2006). Moreover, zebra finches exposed to elevated glucocorticoid levels or social stressors in their early life environments had increased neophobia, often measured as an aversion to novel objects, in adulthood (Emmerson et al., 2017; Schielzeth et al., 2010). In our study, we assess whether both the intensity and type of developmental stressor impacts these behaviors in zebra finches. If cumulative effects of stress are most important, then we expect that only measures of overall stress (defined below) will impact these behaviors; however, if impacts are stressor-specific, then we expect that individual stressors will have unique impacts on behavioral variation. For example, nutritional stress effects, measured by chick condition, may be most likely to impact activity levels; whereas, early social environments, such as heightened competition among brood mates, may be most likely to impact aggression.

Developmental stressors often impact behavioral variation through long term impacts on brain morphology (Duckworth, 2015), especially with regard to the limbic structures of the brian, which are associated with emotion and memory (McLaughlin et al., 2009). Stress response studies on rats found that rats who perceive stressors in their environment develop a significant increase in their amygdala volumes (Bourgin et al., 2015; Butler and Cotterill, 2005). A study on acute early life stress effects on the brain found that chicks who were exposed to an aversive gustatory stimulus developed heightened cortisol levels and decreased cell proliferation in the hippocampus during adulthood (Nikolakopoulou et al., 2006). Numerous studies have also shown that varied forms of stress and stress related diseases are related to increased hypothalamic volumes (Bains et al., 2015; Schindler et al., 2018; Tognin et al., 2012). Thus, we predict that zebra finches who experienced high developmental stress will exhibit smaller

relative hippocampus volumes and larger relative hypothalamus and amygdala volumes in adulthood.

## Methods

*Environmental Stress Measures*

We followed 78 zebra finches (45 males and 33 females) that were raised in a semi-natural outdoor colony on the University of Arizona campus from 2018 to 2023 (Chenard et al., 2023; Chenard, 2023). Zebra finches breed all year round and Tucson, where ambient daily maximum temperatures vary from -14.4℃ to 47.2℃ (US Department of Commerce, 2024), has a similar climate to Australia, where zebra finches naturally reside. Early life environmental stressors were monitored and recorded during daily nest checks. To measure early life heat stress, we recorded the highest and lowest in-aviary daily ambient temperatures. From these daily temperature measurements, the average high and low temperature was calculated for the incubation stage (10 days before hatching), early nestling stage (10 days after hatching), and late nestling stage (11-20 days after hatching), enabling us to assess extreme temperature conditions during these crucial developmental stages. For each nest, we monitored them closely and recorded brood size and hatch order of nestlings. Brood size was measured as the number of siblings that hatched, including those that died as hatchlings/nestlings. Nestlings were measured once during the nestling period between day 8 and 20. We measured bill length, depth, and width, wing length, right and left tarsus length, and tail length in mm, as well as the mass in grams. A subset of these individuals were also measured as adults and this allowed us to assess whether there was long-term stability of condition over an individual's life.

We used chick relative body mass as our measure of condition during development (hereafter "chick condition"). To calculate this measure, we used a regression with mean tarsus length as the independent variable and body mass as the dependent variable. This showed a strong linear relationship between the two ($F = 40.94$; $\beta = 0.62$; $P < 0.0001$, $N = 69$) and there was no effect of sex on either body mass (GLM: $F = 1.46$, $t = 1.21$, $P = 0.23$) or the relationship between body mass and tarsus length (interaction term with sex: GLM: $F = 1.35$, $t = 1.35$, $P = 0.25$). Chicks were measured at different ages, but including chick age did not substantially change the regression results ($\beta = 0.52$; $P < 0.0001$), so we used only the residuals from the simpler model to assess chick condition. We were unable to assess whether adult condition at the time of various behavioral trials was related to behavior because not all birds had body measurements available that coincided with the time of the trial. However, we were able to assess whether individuals showed consistency in condition over their life. For this analysis, we only used males because female body mass was not related to tarsus length ($F = 3.31$; $\beta = -0.34$; $P = 0.08$, $N = 28$). This was likely because females in our colony were frequently gravid and their mass was more likely to reflect this rather than their current condition. Male body mass showed the expected positive association with tarsus length ($F = 4.58$; $\beta = 0.34$; $P = 0.03$, $N = 40$) and so we used the residuals from this regression as a measure of adult male body mass. Using this, we found that, at least for males, there was a nonsignificant positive relationship between condition during development and condition during adulthood ($F = 3.54$; $\beta = 0.30$; $P = 0.07$, $N = 38$).

*Behavior Testing*

We measured adult behavior through a series of controlled behavioral tests. We identified birds that qualified for the study as birds where we had data on behavioral measures as well as developmental stressors. Because of this, birds varied in age during the behavior testing, ranging from 122 days to 1991 days. During the behavior testing period, we conducted one behavior test per day per bird between 05.30 and 15.00. Up to four birds were kept together in testing groups during this period. Test order was decided with a Latin square schedule, which arranged the four birds and four individual tests in a square grid, designed to reduce confounding variables, such as test order and day. Birds were habituated in the home cage (76.2L x 45.72W x 45.72H cm) in the testing area at least one day before testing began, where they had food and water available at all times before testing. The home cage consisted of a perch near the top of the cage, a perch just above the floor of the cage, and filled seed and water dishes at the bottom left and right, respectively. On testing days, all birds were removed from the home cage and placed in a visually, but not acoustically, isolated waiting cage. To record trials, we used a Panasonic HC-V180K Full HD Camcorder and placed the entire testing area behind a partition so they were unable to see the tester or other birds. Variables with potential for impacting behavior, such as age during testing, time of day when testing began, order of testing, were recorded for all tests.

*General Activity*

We measured the intrinsic activity level of each bird in both individual and group environments by counting their movements during ten minute trials in the home cage. To quantify activity, the home cage was organized into a grid, made up of 9 areas (3 areas encompassing the top perch, 3 areas encompassing the low perch, and the seed dish, the cage floor, and the water dish).

Movements were counted when the bird moved between the areas, as well as when the bird rose from their position and flew around the cage to return to their original position. Seed and water were available in the cage throughout the test. Both the group and solo tests involved a 5 minute acclimation period, followed by a 10 minute testing period where all movements were measured. Group general activity was assessed on the fourth and final day of testing for all birds in the home cage. The novel object test occurred directly after the solo general activity test ended..

*Novel Object Test*

Approximately 5 minutes after the end of the solo general activity test, a novel object (randomly selected white, black, or zebra-patterned wooden block) was randomly placed on either the right or left side of the cage. Because side placement and novel object type did not influence any of the measured behaviors (all $P > 0.15$), we combined these groups for analysis. After placement of the block and the experimenter left visual proximity, the bird's behavior towards the novel object was recorded for 20 minutes. Similar to the activity test, the home cage was organized into a grid, with 11 areas, using a scoring system that accounted for closeness to the block. All 11 areas, as well as the novel object itself, were assigned neophobia/neophilia scores ranging from 1 to 7, based on the area's distance from the novel object (novel object contact = 1 and cage area furthest from novel object = 7). Seed and water were available at the bottom left and right of the cage, respectively, throughout all tests. Millet seed was also present in the upper right and left areas of the cage, with one spray always located near the novel object, to motivate birds to approach. Measuring neophilia/neophobia has often proven difficult because tests tend to measure movement as well as include the presence of food and novel objects and even novel environments, which can make it unclear if tests are adequately testing neophobia or

neophilia (Greggor et al., 2015). We attempted to overcome this problem by including food sources throughout the cage, with millet on the right and left sides of the top perch and food and water on the bottom left and right, and by ensuring that all birds are familiar with the home cage in which the test is performed. The number of movements between each area was tallied and compared to each area's associated neophobia/neophilia score to determine two NO scores. First, we recorded 'closest approach,' which was the 'neophobia' score of the area closest to the novel object that the test bird entered throughout the entire test. We call this measure 'neophobia' because the millet spray serves as a motivation for the birds to approach the object, and so, birds that do not get close to the novel object likely have a higher fear of it (Greggor et al., 2015). We also scored the area where the bird spent the most time during the trial and called this measure 'neophilia' because birds could approach the object closely (i.e. score low on neophobia), but then mostly avoid it which can indicate their interest in exploring or being near the object. When a bird frequently moved between multiple areas during the test (high activity), neophilia was calculated as the assigned score of the area with the most tallied movements, or an average of the assigned scores for the multiple areas that all shared the most tallied movements. For birds with lower activity, neophilia also considered the time spent in each area to account for birds staying in one area for most of the test, but then moving around other areas during a short portion of the test. A higher number represents higher neophobia or lower neophilia, depending on the measure.

*Novel Environment Test*

We measured how birds respond to novel environments by physically isolating the test bird in a small box, with a removable door that is attached to a string. After 10 minutes of acclimation in

this box, the door is remotely pulled open from behind a screen and the novel environment cage becomes visible. The novel environment cage is a custom-designed cage with white sheets on all sides except the top and 9 potential areas to explore at different locations and heights, including various branches and the cage floor. An overhead camera was used to capture the bird's behavior in their new environment, where there is no food or water available. Exploratory behavior was measured as the number of different areas (1 as the minimum and 9 as the maximum) that birds explored in their first five minutes after leaving the box. Latency, or how long the bird stays in the box once the door opens, was also recorded as a measure of boldness.This latency measure is similar to commonly used open field test (Burns, 2008; Walsh and Cummins, 1976). The test was stopped at 45 minutes and birds that never left the box had maximized latency results of 45 minutes. In cases of maximized latency, exploratory behavior was not recorded.

*Tonic Immobility Test*

Fearfulness towards predators was measured using a tonic immobility test following the methods of Wuerz and Kruger (2015). For this test, the test bird was separated physically and acoustically from the other birds in both the test group and colony. The test bird was then held by the experimenter in a small netted cage that was especially designed for this test. The bird was placed in this cage and held so that its back was against a flat surface and its wings were pressed firmly to its sides. The bird's head was gently pushed back for approximately 5 seconds to induce tonic immobility. Tonic immobility was considered a success if a bird remained immobile for at least 5 seconds after the tester had completely withdrawn their hand. A timer was used to measure the length of tonic immobility, starting from the moment of induction. The duration of tonic immobility was recorded for a maximum of 10 minutes. For birds that failed to enter tonic

immobility on the first attempt, the procedure was repeated. For birds that failed to enter tonic immobility after 10 attempts, testing ended and duration time was recorded as 0 seconds.

*Mirror Aggression Test*

To assess aggressive behavior, we measured how birds responded to their reflection in a mirror (Wuertz and Kruger, 2015). The aggression test cage (76.2L x 45.72W x 45.72H cm) included a (76.2L x 45.72H cm) mirror on one side that was covered by a curtain with a food dish directly in front of it. Birds were placed in the cage for one of two acclimation periods. In the first, an egg treat was placed in the dish and birds were allowed 5 minutes with it before the curtain was slowly pulled back through a remote pulley system to reveal the 'unknown competitor' in the mirror. The second acclimation method was placing the bird in an empty aggression cage for 30 minutes, adding the egg dish after this acclimation period, and then pulling the curtain to reveal the mirror 5 minutes after the egg placement. Acclimation method did not affect aggression scores ($t$ = -0.20; $P$ = 0.84), so we combined data for analyses. After the mirror was revealed, the bird's interaction with the mirror was recorded for approximately 15 minutes, with tests ranging from 12-18 minutes. Aggressive behaviors included pecking at the mirror, flying at the mirror, and breast contact against the mirror. Every individual contact with the mirror (or second of contact with the mirror with breast contact) was tallied as an aggressive behavior. The overall aggression score was calculated as the number of aggressive behaviors displayed per second, to account for the variable trial time.

*MRI Procedure*

Birds were captured from the communal aviary and placed in a temporary housing cage (76.2L x

45.72W x 45.72H cm) within visual and auditory contact of the colony the day before scanning, and provided with the same daily care resources as described above. On the scanning day birds were weighed, and food was removed from focal individuals one hour before anesthesia began, so the crop and proventriculus would be empty. This was a precautionary measure to prevent regurgitation and asphyxiation due to the effects of the anesthesia.

We followed a procedure similar to the protocols used in prior MRI studies of this species (Hamaide et al. 2018). Animals were anesthetized using isoflurane (3% to induce, 1.4-1.6% to maintain after setup was complete, with an $O_2$ flow rate of 1 L/min). Anesthesia induction was performed at a mobile station induction chamber placed on a heated blanket and covered with a second blanket to reduce light and keep the bird calm. Once fully under anesthesia, birds were transferred to the scanner room, and restrained in a prone position on the scanner cradle using custom 3D-printed flat-topped ear bars and beak cone, and a soft cloth jacket to secure the wings against the body.

Body temperature was monitored using a fiber optic anal thermometer sized for mice, set up using medical water-based lubricant. Body temperatures were maintained throughout the scan within 40 ±0.5 °C using an adjustable hot water jacket. In addition, a small towel was placed over the hot water jacket at setup to keep the area warm and prevent loss of heat. Breathing was monitored using a respiratory pillow, placed under the belly and kept between 40 and 100 breaths per minute. If vital signs deviated from these parameters the scan was aborted. Total anesthesia time including set up and scan was not longer than 2 hours. After scanning, birds were removed from the MRI and allowed to recover in a cage with ad libitum seed and water. Recovery (eyes open and able to perch and move normally) occurred within 5 minutes for all birds.

*MRI Image Acquisition*

Focal birds were imaged between 8 months and 3.5 years old (mature adulthood for all birds). Data acquisition was performed using a 7T small-bore (Bruker BioSpec 70/20) MR scanner located at the University of Arizona's Biosciences Research Center. The scanner was fitted with an 86 mm excitation coil, and 2x2 receiver coil sized for mouse brains. MRI data were collected using (ParaVision 360 V2.0) to generate T2-weighted 3D TurboRARE Isotropic images at 150 um resolution using the following imaging parameters: 2 NEX, TE 30.0 ms, TA 46:04, TR 1800 ms, FOV 14.40x14.40 mm, acquisition matrix 96x96x64, SL 0.15/0.15 mm. FOV saturation bands were used to suppress signals outside of the skull area to limit the chance of wraparound effects and other unwanted artifacts from affecting the image. Acquisition time was 52 minutes.

*MRI Analysis: pre-processing*

To compare impact of early life stressors on variation in volumes across the brain, we used tensor-based morphometry. Post-scan, images were corrected to remove noise (ANTs 'DenoiseImage';Rician noise model, search radius: 1, patch radius: 1; Avants et al. 2011) and Gibb's ring artifacts (Mrtrix3, 'mrdegibbs' function set for 3D volumes; Kellner 2016). The images were manually masked from all three viewing angles to remove non-brain tissues using the manual mask editor in BrainSuite (version 21a). To align population scans to this template, images were flipped on the x-axis in mrtrix ('mrtransform' -flip 0), and then registered using manual rigid body translation and rotation followed by a rigid body rescale by 10.41 in SPM12 (Statistical Parametric Mapping, Wellcome Trust Center for NeuroImaging; MATLAB version R2022B). This step was necessary as the orientation was different between the template and study images, and rotation without first flipping across the x-axis in mrtrix meant that the right

hemisphere of our study scans became aligned with the left hemisphere of the template. Next, image bias field was corrected (light bias field regularization), and images were segmented into 'gray matter', 'white matter', and cerebrospinal fluid using the 'Old Segment' batch in SPM12, with tissue class probability maps obtained from Hamaide et al. (2018). This process is necessary as existing procedures are adapted from methods used on humans and other mammals, however birds do not have separation between gray and white matter as is found in mammals (Reiner et al. 2004). Segmentation in this case instead separates images based on variation in cytoarchitectural feature variation best delineated through T2-weighted imaging. Segmentation files (seg_sn.mat) were imported into DARTEL (Ashburner 2007) and used to create a population average template. The flow fields also generated from this process (which encode the linear and nonlinear spatial transformations necessary to warp the individual images to our population average template) were used to create maps of the Jacobian determinant of the deformation matrix of each individual. These Jacobian determinant maps were then spatially normalized and smoothed (Gaussian FWHM of 3x3x3), as well as modulated to preserve volume information (Ashburner and Friston 2000). To generate estimates of whole brain volume to modulate the images, we used the 'get_totals' function (Pew Research Center, 2020, "pewmethods" Available at: github.com/pewresearch/pewmethods) on the segmented images generated from this process to obtain and combine volumes from tissue classes.

*Statistical Analysis*

All statistical analyses of stressors on behavior were performed in SAS v9.4 (SAS Institute). Temperature environments were highly correlated across incubation, early and late nestling periods. Therefore, we first standardized the values to mean zero and standard deviation of one

and used principal components analysis to create a single "heat stress" variable. Only the first eigenvalue was greater than 1 ($\lambda$=5.28) and it explained 88.0% of the variance (see Table S1). Both of our measures of environmental stress were negatively correlated with chick condition (heat stress: $r$ = -0.46, $P < 0.0001$; brood size: $r$ = -0.28, $P < 0.03$), so to test for overall exposure to developmental stress we created a single measure using principal component analysis. Only the first eigenvalue was greater than 1 ($\lambda$=1.77) and it explained 58.9% of the variance (see Table S2). Thus, we used PC1 as our general measure of stress during development as individuals with higher values generally had higher exposure to all three stressors. Higher PC1 values indicated that individuals had experienced greater ambient heat during development, came from a nest with a greater brood size and overall had lower condition compared to individuals with low PC1 values. Because we were interested in the impacts of both overall stress and the relative importance of each stressor, we first related this measure of overall stress to behavioral traits and then carried out subsequent analyses to assess whether behavioral variation was related to particular stressors individually.

Before assessing the relationship between behavioral traits and stressors, we first determined whether any of the behaviors were influenced by age, trial day or trial time to assess whether they should be included as covariates. The only important covariates were age for exploration latency as older birds had higher latencies ($\chi^2$ = 7.80, $P < 0.005$), and time of day for the novel object measure as birds measured later in the day spent more time farther from the novel object ($r$ = 0.29, $P$ = 0.01). All other behavioral measures were unaffected by these covariates (all $P > 0.10$) and were not included in further analysis.

Many individuals had zero scores for both aggression and exploration latency (no aggression displayed or individuals that left the acclimation box immediately). Given this, we

dichotomized these data. For aggression, we categorized individuals that showed no response to the mirror as 'nonaggressive' ($N = 59$) and individuals that showed a response as 'aggressive' ($N = 11$). For exploration latency (hereafter 'boldness'), we categorized individuals that left the acclimation box within 10 seconds ($N = 32$) as 'bold' and individuals that left the box in more than 10 seconds ($N = 32$) as 'shy'. We then used these categorical variables to assess the relationship between developmental stressors and behavior using logistic regression with a negative binomial distribution and a log-link function. For all other behaviors, we used general linear models, linear regression or partial regressions. For all analyses, we first fit a model assessing the relationship between our overall measure of either overall developmental stress (PC1) or each individual stressor and included sex and interaction with sex. These latter two terms were dropped from subsequent analyses if nonsignificant. Plots of the data and the residuals were assessed to ensure these tests met assumptions of normality of residuals, homoscedasticity, linearity and there were no influential outliers. Outliers with Cook's $D > 4/N$ were removed from analysis, but are included with labels in data plots.

All statistical tests of MRI images were conducted in SPM 12. The degree of local transformation of individual brains relative to the template was compared voxel-by voxel (the '3D pixels' which make up MRI images) using statistical parametric mapping on the smoothed, normalized, and modulated Jacobian determinant maps, which uses a series of t-tests to determine if voxels are significantly different between groups. After local transformation of individual scans to match the population-average template, the Jacobian determinant maps used in this analysis capture the local changes necessary to align individuals to the template. Combined with modulation to preserve volume information, it enables measurement of how local volumes differ among individuals.

Global normalization was used to factor out variation in volumes of local regions due to scaling with whole brain volume (*i.e.,* larger brains are likely to have larger structures in general), and so the results presented are relative volumes. To investigate effects of stressors on variation in brain structure, we used a series of 1-tailed hypothesis-driven t-tests, testing both the contributions of each stressor individually (heat, chick condition, and brood size), as well as the overall effect of the first principal component score of general stress on brain structure. Individual stressors were tested using a combined dataset of both males and females. However, as we found variation in the impact of our principal component stress score on behavioral response on males and females, we analyzed the impact of PC1 on brain variation for males and females separately. To control for false-positives due to the multiple comparisons necessary to test for variation across the whole brain, we used a family-wise error (FWE) corrected cluster p-value threshold of P = 0.05, and present the FWE corrected p-value for each cluster as well as the t-values of the peak voxel of the clusters.

**Results**

*Activity and developmental stressors*

There was a tendency for females to be more active in the solo context (F = 3.03, $t = 1.74$, $P = 0.09$). Activity measured in the solo context was not related to exposure to developmental stressors overall (F = 2.31, $t = -1.52$, $P = 0.13$) and was also not influenced by the interaction between sex and developmental stress (F = 0.73, $t = 0.85$, $P = 0.40$); however, post-hoc analysis of specific stressors showed individuals that were in better condition as chicks were more active as adults (F = 7.37, $\beta = 0.32$, $P < 0.01$; Fig 1A). There were two outliers that were removed from this analysis (shown in Fig. 1A), however, their inclusion did not substantially change the results

(F = 5.10, β = 0.27, $P < 0.03$). Neither brood size nor heat stress influenced solo activity level (β = 0.05, $P = 0.69$ and β = 0.04, $P = 0.79$, respectively). In the group context, activity was not related to overall developmental stress (F = 0.15, $t = -0.37$, $P = 0.71$), sex (F = 0.40, $t = -0.63$, $P = 0.53$) or the interaction with sex and developmental stress (F = 0.71, $t = 0.84$, $P = 0.40$). Nor was it related to any of the stressors independently (chick condition: β = 0.02, $P = 0.92$; brood size: β < 0.01, $P = 0.98$; heat stress: β = 0.05, $P = 0.76$).

*Fearfulness and boldness and developmental stressors*

Overall, developmental stress was unrelated to either neophobia (F = 0.23, $t = 0.41$, $P = 0.68$) or neophilia (F = 0.66, $t = -1.00$, $P = 0.32$), but there was a difference in neophilia between the sexes (F = 3.81, $t = 1.95$, $P = 0.056$) with females being less neophilic (spending more time farther from from object) than males (mean ± SE female = 4.90 ± 1.08 versus male = 4.69 ± 1.14; Fig. 1B). There were no sex differences in neophobia (F = 0.00, $t = -0.04$, $P = 0.97$). There was no significant interaction between sex and stress for either neophobia (F = 0.00, $t = -0.05$, $P = 0.96$) or neophilia (F = 0.22, $t = 0.46$, $P = 0.64$). In the analysis of individual stressors, none were related to neophobia (chick condition: F = 0.07, $t = 0.27$, $P = 0.79$; brood size: F = 0.01, $t = -0.09$, $P = 0.93$; heat stress: F = 0.67, $t = 0.82$, $P = 0.42$); however, individuals in better condition during development were less neophilic (spent more time far from the object) as adults compared to individuals that were in poorer condition (F = 5.95, $t = 2.44$, $P = 0.017$; Fig. 1B). Brood size and heat stress did not impact neophilia (brood size: F = 1.03, $t = 1.02$, $P = 0.31$; heat stress F = 0.00, $t = 0.05$, $P = 0.96$).

In the novel environment test, individuals that experienced lower levels of overall developmental stress were bolder as adults (left the start box quicker) compared to individuals

that did not (estimate = -1.10 [-2.01, -0.20], $\chi^2 = 5.70$, $P < 0.02$). There was also an interaction between developmental stress and sex on boldness (estimate = 1.41 [0.30, 2.53], $\chi^2 = 6.17$, $P = 0.01$) with stressors impacting boldness of males (estimate = -1.11 [-2.00, -0.22], $\chi^2 = 6.00$, $P = 0.01$, $N = 38$) more strongly than females (estimate = 0.39 [-0.36, 1.13], $\chi^2 = 1.04$, $P = 0.31$; Fig. 2A). This was mainly driven by the impact of both brood size and heat stress on male boldness as males that were hesitant to leave the box came from larger broods (estimate = -0.93 [-1.78, -0.09], $\chi^2 = 4.68$, $P = 0.03$) and had experienced greater heat stress (estimate = -0.56 [-1.00, -0.12], $\chi^2 = .12$, $P = 0.01$) in ontogeny. There was no influence of chick condition on boldness (estimate = 0.07 [-0.51, 0.65], $\chi^2 = 0.05$, $P = 0.82$). Moreover, similar to the measure of overall stress, there was no influence of either brood size or heat stress on female boldness (brood size: estimate = 0.12 [-0.32, 0.5], $\chi^2 = 0.30$, $P = 0.59$; heat stress: estimate = 0.20 [-0.15, 0.55], $\chi^2 = 1.20$, $P = 0.27$).

Tonic immobility was not influenced by developmental stress ($F = 0.18$, $t = 0.21$, $P = 0.71$) and there was also no effect of sex ($F = 0.03$, $t = 0.16$, $P = 0.87$) or the interaction between developmental stress and sex ($F = 0.42$, $t = -0.65$, $P = 0.52$). Moreover, there were no independent effects of specific stressors on this behavior (chick condition: $F = 0.01$, $t = 0.11$, $P = 0.91$; brood size: $F = 0.12$, $t = -0.35$, $P = 0.73$; heat stress: $F = 0.01$, $t = 0.08$, $P = 0.93$).

*Aggression and developmental stressors*

Aggression did not differ between the sexes (estimate = -0.26 [-1.90, 1.39], $\chi^2 = 0.09$, $P = 0.76$), nor was it influenced by an interaction between sex and developmental stress (estimate = 0.19 [-1.03, 1.41], $\chi^2 = 0.09$, $P = 0.76$). However, there was a nonsignificant tendency for individuals that experienced greater overall stress during ontogeny to be less aggressive as adults (estimate =

-0.51 [-1.10, 0.0], $\chi^2 = 2.97$, $P = 0.08$). Post-hoc analysis revealed that this was almost entirely driven by the impact of heat stress (estimate = -0.41 [-0.84, 0.01], $\chi^2 = 3.57$, $P = 0.059$) as aggression was unrelated to both chick condition (estimate = -0.13 [-0.91, 0.65], $\chi^2 = 0.11$, $P = 0.74$) and brood size (estimate = -0.12 [-0.70, 0.46], $\chi^2 = 0.16$, $P = 0.68$) in adulthood.

*MRI Results*

The direction of the relationship between brain volumes and overall developmental stress (PC1) was opposite between males and females. Male finches with a higher PC1 stress score score had larger relative volumes in the left and right arcopallium (left hemisphere cluster: $t = 5.86$; *P(FWE-corr)* = 0.001; right hemisphere cluster: $t = 4.75$; *P(FWE-corr)* = 0.003; $N = 18$; Figure 4A). In contrast, females that experienced high stress conditions as nestlings had relatively smaller volumes overlapping left and right arcopallium, as well as portions of the caudal nidopallium, mesopallium, left hippocampus, thalamus, and cerebellum ($t = 10.46$; *P(FWE-corr)* < 0.001, $N = 8$; Figure 4B).

Comparing the contribution of individual stressors (both sexes combined), finches with a lower condition score as nestlings had decreased relative volume as adults in an area that was largely dominated by the cerebellum, that also included parts of the thalamus and midbrain ($t = 3.87$; *P(FWE-corr)* = 0.014, $N = 30$; Figure 3A). Heat, in contrast, was correlated with larger volumes ($t = 4.02$; *P(FWE-corr)* = 0.044, $N = 35$) in the left optic tectum (Figure 3B). Finally, chicks from larger broods had smaller the right and left arcopallium volumes, as well as smaller volumes of portions of the nidopallium, mesopallium, left hippocampus, and cerebellum (($t = 5.20$; *P(FWE-corr)* < 0.001, $N = 31$; Figure 3C).

**Discussion**

While the impact of experimentally induced acute and chronic stress on behavior and brain development is well studied (Meaney, 2001), the impact of naturally occurring early life stressors is poorly understood. In our investigation, we proposed two hypotheses for why natural stressors may impact the brain and behavior: specific stressors evoke adaptive responses in organisms to prepare them for the unique challenges in their environment, or alternatively, that developmental stressors have broad and direct stress-related effects on the brain and behavior and the cumulative impact of stressors is most important. The results of our study support the former, suggesting that naturally occurring environmental stressors during early development can act as powerful cues that direct specific, long term effects on the brain and behavior.

In our study, we found strong relationships between specific environmental stressors and both adult behavior and brain morphology. Chicks in higher condition during development showed higher activity levels in adulthood, similar to Brust et al. (2023). These behavioral effects are also reflected in our MRI results, where chicks in higher condition developed larger relative cerebellum volumes in adulthood. Since the cerebellum is involved in many motor and coordination functions, this suggests that there may be a functional link between how early nutritional stress influences cerebellum growth and this may explain why these birds also have higher activity levels as adults (Paulin, 1993; Thach, 1998). These results support our primary hypothesis because, if the alternative were true, and stress had broad effects that were independent of source, we would expect to see a general pattern of variable stress effect across behaviors and the brain. Instead, however, we found a clear relationship between chick condition, adult activity levels, and adult cerebellum volumes, indicating that the source of stress plays a significant role in brain and behavior development.

For neophobic/neophilic behavior, we found that chicks in higher condition during development showed decreased neophilia; whereas, neophobia was not related to any stressors. In some ways, this was surprising. Other studies on adolescent stress effects, measured through delivered corticosterone or immune response stimulation during early life, found increased neophobia in adulthood (Emmerson and Spencer, 2017; Grindstaff et al., 2012). These studies, however, measured stress in terms of injected corticosterone and antigen exposure, which are relatively severe stressors. Our neophilia results were also surprising because there are few studies that demonstrate how developmental stressors significantly impact neophilia in adulthood. Our results suggest that nutritional conditions during development may play a role in determining how interested individuals are in exploring novel objects later in life. It is possible that individuals that experienced significant nutritional stress during ontogeny develop heightened interest in novel objects or attractive food sources. If so, then this supports our primary hypothesis that nutritional stress acted as a specific cue that led birds to develop increased neophilia in adulthood. On the other hand, the neophobia results indicate that none of our examined environmental stressors were impactful, despite other studies linking severe developmental stressors to increased neophobia (Emmerson and Spencer, 2017; Grindstaff et al., 2012). This may indicate that the source of early life stress that shapes neophobic behavior exists outside of the scope of our study.

Another fascinating result is that birds who experienced high heat stress tended to show lower aggression in adulthood, as well as higher left optic tectum volumes. Temperature is very important for avian development (Olson et al., 2008, 2006; Rubin et al., 2021; Stier et al., 2020) and developmental stress has been shown to impact aggression (Ruploh et al., 2018; Spencer, 2017), however, we didn't expect that heat stress would be the only developmental stressor that

impacted aggressive behavior in our study. A relationship between stress responsiveness and associated anxiety-related behavior and the optic tectum have been shown in various fish and amphibian studies (Overli et al., 2001; Carr, 2015; Isa et al., 2021). Since the optic tectum's functions involve visual recognition, it is possible that increased environmental heat reflects increased light exposure during development and this is the true cue that produces long term effects on optic tectum volume. After all, ambient temperatures are hottest during parts of the year when days are longest. While the mechanisms underlying these results are not entirely clear, they do support our primary hypothesis since we found that variation in ambient temperature, specifically, was related to optic tectum volume and may also influence aggression.

We also found significant sex-specific effects on both the behavior and brain. Males were more neophilic, on average, than females, and only males showed impacts of early developmental stressors on boldness, measured in terms of novel environment latency, in adulthood. The latter behavioral findings mirror the MRI results, which showed that males that experienced increased overall developmental stress had relatively larger volumes in left and right arcopallium regions. Interestingly, females who experienced increased developmental stress also showed a difference in left and right arcopallium volumes but in the opposite direction from males, with females that experienced higher stress as chicks also having relatively smaller volumes. The arcopallium functions in the processing of fear and boldness (Fujita et al., 2020; Kops et al., 2013) making these impacts of developmental stressors on the brain somewhat consistent with our behavioral results. We expected that sex would impact our results, as sex is known to contribute to significant differences in physiology, brain structure, hormone production and metabolism, and subsequent behavior (Khan, 2013; Schett, 2009). Our found sex effects on boldness are also somewhat surprising because existing literature has shown inconsistent results

when investigating boldness in males and female zebra finches. Previous studies have investigated boldness in zebra finches by measuring male and female behavior in both group and isolated settings. Behavioral results have found that birds in groups, regardless of sex, generally behave less bold, while isolated behavior results have been less consistent: finding that females behave more boldly or that there are no significant sex differences in observed boldness (Mainwaring et al., 2011; Kerman et al., 2018). These results do not match our own, however, these studies did not investigate developmental stress effects across the sexes on observed boldness in adulthood. Our results suggest that males and females have alternative mechanistic pathways by which they process developmental stress, resulting in opposite and long lasting effects on arcopallium volumes in the brain, which ultimately leads to differences in boldness.

Overall, our study shows that naturally occurring developmental stress has prominent and long lasting effects on behavior. Early life environmental stressors, including temperature and chick condition, were shown to have specific impacts on the brain and resulting behavior which may better prepare organisms for environments they experience later in life. Thus, our results support our hypothesis that natural environmental stressors may act as cues that induce adaptive changes in the brain and behavior. These findings suggest that mechanisms underlying stress effects on behavior and brain development are source dependent, with different stressors producing distinct behavioral and morphological effects. These results build on the existing literature by showing the importance of the source and severity of developmental stress in shaping the brain and behavior. These results also raise questions for how mechanisms of stressor-specific developmental effects evolve. Future work comparing such stressor-specific effects  across taxa will shed light on how diverse environments may impact evolution of these effects in a wide variety of species. In studying the relationship between environmental cues and

behavioral development, we may learn how these varying or uniform responses across taxa become linked to such cues in the first place (Duckworth et al. 2023). Finally, future studies are also needed to investigate stress effects on neuron number, neuron density, and how epigenetics factors may be involved in creating the observed effects in the brain and behavior.

**Figures**

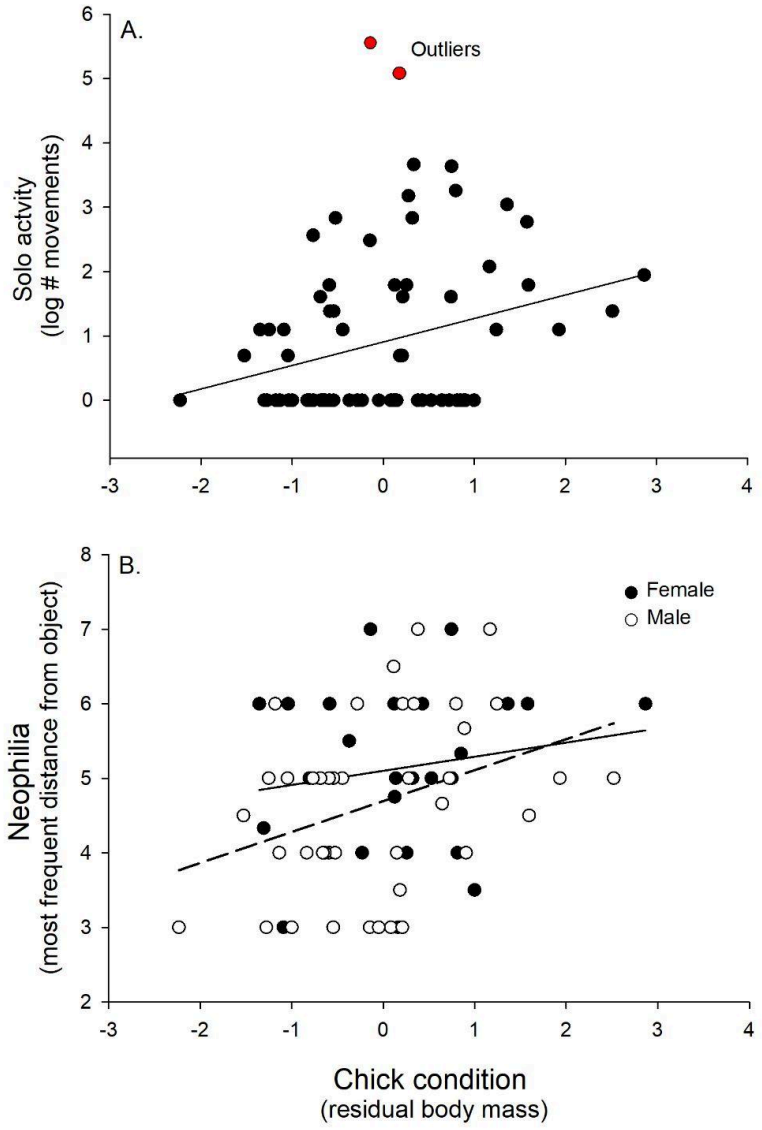


**Figure 1.** Chick condition during development was positively related to adult A) activity levels in the solo context and B) neophilia. Red circles in A indicate two individuals that were exceptionally active and were outliers in analysis. In B, solid points and line indicate females and open points and dashed line indicate males. Chick condition was measured as the residuals of a regression of chick body size on body mass. Positive residuals indicate chicks that were relatively heavy for their size and negative for low condition chicks that were relatively light.

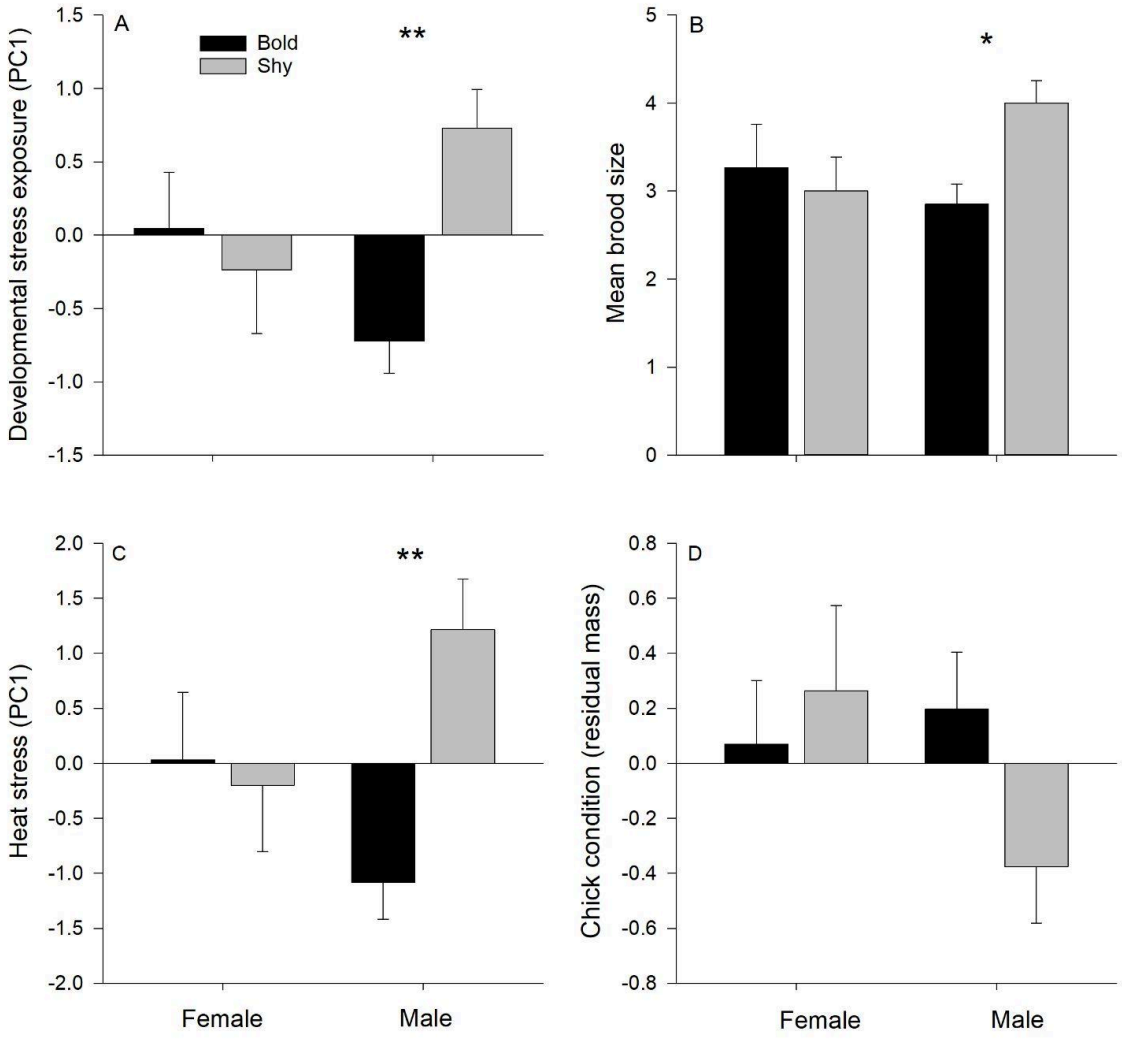


**Figure 2.** Developmental stressors impacted male but not female boldness. A. Males were bolder in adulthood if they experienced greater overall stress (see text for details) during development. This was particularly true if they came from B. larger broods or C. experienced greater exposure to ambient heat stress. D. Chick condition during development did not have a strong impact on the boldness of either males or females. **indicates $P = 0.01$ and *indicates $P < 0.05$.

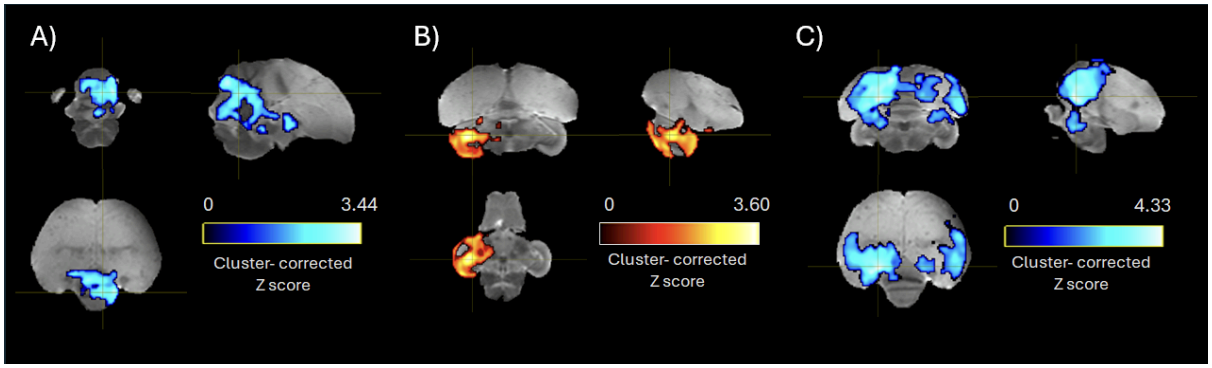


**Figure 3.** Locations where brain volumes varied among individuals in relation to individual stressors. A) Lower chick condition led to decreased relative volume as adults that was largely restricted to the cerebellum, although there was also cluster overlap with parts of the thalamus and midbrain. B) Higher exposure to heat stress, in contrast, was correlated with larger volumes in the left optic tectum. C) Larger brood size was correlated with smaller volumes of right and left arcopallium areas, as well as portions of the nidopallium, mesopallium, left hippocampus, and cerebellum. Left in this figure represents the left hemisphere. Pictured are representative slice images for both hemispheres in three views for each comparison (top left: coronal, top right: sagittal, bottom left: axial) illustrating the location and spatial extent of significant clusters. A warm color represents areas of the brain that are relatively larger, while a cool color represents areas that are relatively smaller (brightness representing Z score of voxels within significant clusters after applying FWE cluster correction threshold of P=0.05). Significant areas are shown overlaying a high-resolution T2 weighted anatomical scan used from Poirier et al. (2008).

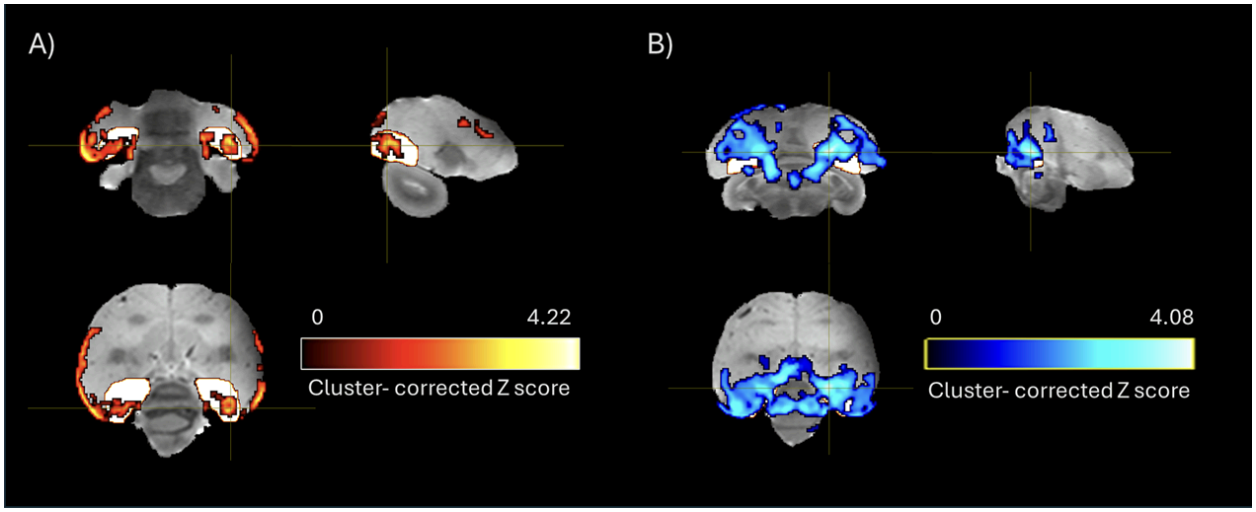


**Figure 4.** Locations where brain volumes varied among individuals in relation to overall developmental stress (PC1). A) Male finches with a higher PC1 stress score score had larger relative volumes in the left and right arcopallium. B) In contrast, females that experienced high stress conditions as nestlings had relatively smaller volumes overlapping left and right arcopallium, as well as portions of the caudal nidopallium, mesopallium, left hippocampus, thalamus, and cerebellum. See Figure 1 for image description details.

**Table 1** Results from principal components analysis for Temperature variables, including eigenvalue.

| Variables | Heat1 | Heat2 | Heat3 | Heat4 | Heat5 | Heat6 |
|---|---|---|---|---|---|---|
| Late Nestling High Temperature | 0.41 | -0.35 | 0.69 | -0.04 | -0.41 | -0.26 |
| Late Nestling Low Temperature | 0.41 | -0.30 | -0.01 | -0.61 | 0.57 | 0.21 |
| Early Nestling High Temperature | 0.41 | -0.37 | -0.18 | 0.69 | 0.08 | 0.42 |
| Early Nesting Low Temperature | 0.42 | -0.07 | -0.61 | -0.02 | -0.13 | -0.42 |
| Incubation High Temperature | 0.39 | 0.67 | 0.31 | 0.28 | 0.44 | -0.21 |
| Incubation Low Temperature | 0.41 | 0.45 | -0.16 | -0.27 | -0.53 | 0.49 |
| Eigenvalue | 5.28 | 0.38 | 0.15 | 0.12 | 0.04 | 0.03 |
| Difference | 4.90 | 0.23 | 0.03 | 0.08 | 0.01 | |
| Proportion | 0.88 | 0.06 | 0.02 | 0.02 | 0.01 | 0.004 |
| Cumulative | 0.88 | 0.94 | 0.97 | 0.99 | 1.00 | 1.00 |

**Table 2** Results from principal components analysis for stress variables, including eigenvalue.

| Variables | str1 | str2 | str3 |
|---|---|---|---|
| Heat1 | 0.62 | -0.20 | 0.76 |
| Residual Mass | -0.59 | 0.52 | 0.62 |
| Brood Size | 0.51 | 0.83 | -0.21 |
| Eigenvalue | 1.77 | 0.74 | 0.49 |
| Difference | 1.04 | 0.25 | |
| Proportion | 0.59 | 0.25 | 0.16 |
| Cumulative | 0.59 | 0.84 | 1.00 |

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