

Analysis of stress responses in *Astyanax* larvae reveals heterogeneity among different populations

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Abstract

Stress responses are conserved physiological and behavioral outcomes as a result of facing potentially harmful stimuli, yet in pathological states, stress becomes debilitating. Stress responses vary considerably throughout the animal kingdom, but how these responses are shaped evolutionarily is unknown. The Mexican cavefish has emerged as a powerful system for examining genetic principles underlying behavioral evolution. Here, we demonstrate that cave *Astyanax* have reduced behavioral and physiological measures of stress when examined at larval stages. We also find increased expression of the glucocorticoid receptor, a repressible element of the neuroendocrine stress pathway. Additionally, we examine stress in three different cave populations, and find that some, but not all, show reduced stress measures. Together, these results reveal a mechanistic system by which cave-dwelling fish reduced stress, presumably to compensate for a predator poor environment.

KEYWORDS

Astyanax, cavefish, cortisol, glucocorticoid receptor, novel tank assay, stress

1 | INTRODUCTION

Many physiological and behavioral responses to stressful stimuli are evolutionarily conserved throughout the animal kingdom, and function to increase alertness and promote survival in times of adversity (Campos, Fogaça, Aguiar, & Guimarães, 2013; Cockrem, 2013). Among diverse species, acute stress can be defined by changes in behavior, including reduced exploration and freezing, as well as an increase in circulating levels of catecholamines and glucocorticoids, the primary stress hormones found among vertebrates (Collier, Kalueff, & Echevarria, 2017).

While behavioral and physiological responses to stress are widely thought to be adaptive (Lacey & Lacey, 1958; Schneiderman, Ironson, & Siegel, 2005), pathological stress becomes debilitating and can result in various stress and anxiety-related disorders (Hadany,

Beker, Eshel, & Feldman, 2006). Moreover, it is not clear what predisposes some individuals to stress and anxiety-related disorders, though epidemiological studies point to a complex relationship between genetic and environmental components (Cornelis & Nugent, 2010; Etkin & Wager, 2007; Koenen, Nugent, & Amstadter, 2008). Despite these findings, the environmental factors that shape stress and how naturally occurring genetic variation contributes to physiology and the susceptibility of related diseases states remains poorly understood. A central impediment to addressing this question has been the lack of a model in which stress can be examined, and the responses correlated with naturally occurring genetic variation and a well-established ecological and evolutionary history.

The Mexican tetra, *Astyanax mexicanus*, is a small fresh-water fish native to northeast Mexico and regions of southern Texas, which provides a powerful system for studying behavioral evolution

(Duboué, Keene, & Borowsky, 2011; Elipot, Hinaux, Callebert, & Rétaux, 2013; J. B. Jaggard et al., 2018; J. E. Kowalko, Rohner, Rompani, et al., 2013; Yoshizawa, Gorički, Soares, & Jeffery, 2010). This species exists as an eyed, river-dwelling form, and at least 29 populations of cave-dwelling morphs (Borowsky, 2008a; Jeffery, 2009; Mitchell, Russell, Elliott, & Elliot, 1977). Cave and surface populations are interfertile, allowing for genetic analysis of the complex multilocus traits that distinguish the different surface- and cave-dwelling populations (M. E. Protas et al., 2006; M. Protas et al., 2008; Wilkens, 1971; Yoshizawa et al., 2015). The ecology and environmental setting of the caves and rivers have been well-described (Mitchell et al., 1977). Additionally, an exhaustive phylogenetic history of the species (Bradic, Beerli, García-De León, Esquivel-Bobadilla, & Borowsky, 2012; Bradic, Teotónio, & Borowsky, 2013; Mitchell et al., 1977; Ornelas-García, Domínguez-Domínguez, & Doadrio, 2008), combined with the ability to compare between independently evolved populations provides the opportunity to compare evolutionarily-derived changes in behavior with ecology (Keene, Yoshizawa, & McGaugh, 2015). Lastly, because cavefish are derived from wild-caught populations, these animals represent a genetic model system with intact naturally occurring genetic variation (Borowsky, 2008b; Bradic et al., 2012; Dowling, Martasian, & Jeffery, 2002; Gross, 2016; Rohner, 2018). In addition to the number of morphological traits that differ between the cave and surface morphs, several behaviors, including sleep, schooling, foraging, and aggression have been shown to differ significantly among epigeal and subterranean forms (Aspiras, Rohner, Marineau, Borowsky, & Tabin, 2015; Duboué et al., 2011; Elipot et al., 2013; J. J. Jaggard et al., 2017; J. B. Jaggard et al., 2018; J. Kowalko, Rohner, Linden, et al., 2013; J. E. Kowalko, Rohner, Rompani, et al., 2013; Patton, Windsor, & Coombs, 2010; Yoshizawa et al., 2010).

The zebrafish, *Danio rerio*, and a number of other teleost species, have emerged as powerful models for addressing neuronal and genetic principles underlying the regulation of stress (Cachat et al., 2010; Laberge, Yin-Liao, & Bernier, 2019; Maximino et al., 2010, 2014; Sakamoto, Yoshiki, & Sakamoto, 2017; Schreck, Tort, Farrell, & Brauner, 2016). Studying stress in fish not only has implications in aquaculture and farming (Sneddon, Wolfenden, & Thomson, 2016), but the principles, neuronal circuitry, and neuroendocrine systems that modulate these behaviors are largely analogous with mammalian systems (Biran, Tahor, Wircer, & Levkowitz, 2015; Mueller, Dong, Berberoglu, & Guo, 2011). Moreover, more than 80% of disease-causing genes are conserved (Varga et al., 2018). The neuroendocrine stress axis and the primary neuroendocrine stress hormone, cortisol, are conserved between fish and humans (Collier et al., 2017). Standardized assays have been developed for studying stress responses in both adult and larval fish (Cachat et al., 2010; Maximino et al., 2010). For example, in response to a mild electric stimulus, fish, like mammals, undergo a characteristic stress response, which involves prolonged freezing followed by recovery to baseline (Duboué, Hong, Eldred, & Halpern, 2017). These powerful high-throughput assays, combined with genetic tools for manipulating gene and neuronal function, have made these fish systems an invaluable asset to the study of stress biology.

We have demonstrated recently that stress in adult cavefish differs significantly from their surface counterparts (Chin et al., 2018). Adult cavefish have reduced behavioral measures of stress, but the response of larvae is unknown. In this study, we examine stress responses in larval stages of *A. mexicanus*. Our results reveal that diminished stress in cavefish is likely driven by an upregulation of the glucocorticoid receptor. We also show a divergence in ontogeny of stress states, where some cavefish populations have dampened behavioral measures of stress in both adult and larval stages, whereas other populations have dampened stress in adult forms, but not in larval stages. Together, our data reveal that upregulation of a negative regulator of stress may underlie reduced stress states in *A. mexicanus* cavefish, and suggest that the cavefish system may be a powerful tool for examining how naturally occurring genetic variation leads to susceptibility of stress-related disorders.

2 | METHODS

2.1 | Animal husbandry

All animals used in this study were maintained on a dedicated recirculating aquatics facility (Aquaneering Inc., San Diego, CA, USA) at Florida Atlantic University, using methods previously described (Borowsky, 2008c; Stahl, Jaggard, et al., 2019). All fish stocks originated from either the Borowsky laboratory (New York University) or the Jeffery laboratory (University of Maryland, College Park). Fish were housed in standard 10–37 L glass tanks, and the water temperature held constant at $21 \pm 1^\circ\text{C}$. The aquatics facility was equipped with overhead lights (25–40 lux), and the light cycle was set to 14:10 light:dark. Adult fish were fed twice a day at zeitgeber times ZT2 and ZT12 with a mixture of fish flakes (TetraMin) and black worms (Aquatic Foods, Fresno, CA, USA) as previously described (J. B. Jaggard et al., 2018; Stahl, Jaggard, et al., 2019).

Larvae used in this study were generated by natural spawning of adult breeder lines, as described previously (Borowsky, 2008c). Briefly, on the night before spawning, a heater was placed in a clean tank containing male and female breeders, and the temperature was raised by $4\text{--}5^\circ\text{C}$. Eggs were collected between 00:00 h (midnight) and 04:00 h and transferred to clean glass bowls (Pyrex). All debris and unfertilized eggs were removed from the glass bowls, and dirty water was replaced with clean water. Eggs were then transferred to a temperature and humidity-controlled incubator set to $21 \pm 1^\circ\text{C}$. From 0 to 6 days postfertilization (6 dpf), water in all bowls was replaced two to three times a day. Beginning at 6 dpf, larvae were transferred from the incubator to the aquatics facility. Larvae were then fed twice a day with live *Artemia* (brine shrimp). At 9 dpf, larvae were tested for stress. Tracking in the transparent cavefish is challenging, and thus 9 dpf was chosen because this was the youngest stage for which we could still track animals reliably. All methods and experiments in this study were approved by the Institutional Animal Care and Usage Committee (IACUC) at Florida Atlantic University (protocol number A17–21).

2.2 | Behavioral measures of stress using shock

The shock assay was carried out as previously described in zebrafish (Duboué et al., 2017), with minor modifications to use in *A. mexicanus* fry. A 6 cm³ chamber was constructed by placing custom-printed four-walled outer barrier (MakerBot Replicator 2) atop a 0.5 cm clear acrylic bottom. A pair of stainless-steel electrodes was placed on either side of the box, and the electrodes were attached to a square pulse electrical stimulator (Grass S48D; Natas Neurology, RI, USA). An elevated platform was mounted in the center of the chamber, and a 40 µm cell strainer (Corning, Durham, NC, USA; Inner diameter = 20 mm, height of water = 6 mm) was positioned on the platform. The entire chamber was positioned on top of a custom-designed infrared light source (880 nm), and a high speed charged couple device (CCD) camera (Grasshopper 3; Point Gray) fitted with a fixed focus lens (75 mm DG Series fixed focus lens; Edmond Optics) was positioned on top. The camera was connected to a computer (Dell XPS, core i7) and videos were recorded using FlyCap2 software (Point Gray) at 60 frames per second. To examine their response to shock, a single 9 dpf *A. mexicanus* fry was placed centrally in the cell strainer and allowed to acclimate for 1-min before the video camera was activated. Fish were recorded for 1 min, and then five electric shocks were delivered (28 V, 1 pulse per second, 200 ms pulse duration). Locomotor activity was then recorded for an additional minute. The protocol for assessing fish behavior were taken from those originally described for zebrafish (Duboué et al., 2017).

Videos were analyzed offline by first tracking individual larvae using Ethovision XT software (v13; Noldus). Tracking data was then inspected by a trained experimenter to ensure that tracking data accurately reflected the locomotor behavior of the larva, and when discrepancies were found, the tracks were modified to accurately reflect the (x,y) position of the fish using the “track editing” function in Ethovision XT. All (x,y) pixel coordinates per frame were exported from Ethovision and imported into MATLAB (MathWorks). Using custom-written scripts in MATLAB, we determined locomotor activity over time as well as total freezing. Total freezing duration was determined as any period of inactivity equal to or longer than 1.99 sec (Duboué et al., 2017).

2.3 | Measuring whole body cortisol levels

Whole body cortisol was measured using previously described protocols modified for *A. mexicanus* larvae (Cachat et al., 2010; Duboué et al., 2017). Briefly, 9 dpf larval *A. mexicanus* were grouped in pools of 10 larvae per pool. Pools of larvae were transferred to 40 µm cell strainer baskets (Corning; inner diameter = 20 mm, height of water = 6 mm). The baskets containing the larvae were transferred to the shock apparatus described above, and larvae were allowed 60 min to acclimate to the environment. After acclimation, larvae were shocked five times using the same Grass stimulator described above (28 V, 1 pulse per second, 200 ms pulse duration). After stimulation, larvae were given 15 min recovery period, as this is the

time that it takes for maximum activation of the cortisol pathway (Facchin, Duboué, & Halpern, 2015). A control group of larvae were handled similarly, except no stimulation was delivered. Larvae were then euthanized using an overdose of MS222 (cat. number A5040; Sigma), and transferred to 1.5 ml Eppendorf tubes. Water was removed from the tubes, fish were flash frozen using liquid nitrogen, and the tubes were placed in a −20°C freezer overnight. The following day, fish were homogenized in 1 ml of a 1× phosphate-buffered solution (PBS; BP3994; Fisher) using a pestle. Fifty microliters of the homogenate was taken from each and stored for protein analysis (see below). The remaining homogenate was transferred to 4 ml glass scintillation vials. Two milliliters of diethyl ether was added to the tube, and tubes were vortexed at high frequency for 1 min. Samples were then centrifuged at 5000 rpm at 4°C for 10 min. The top organic layer was removed using a glass pipette and transferred into a second 4 ml glass vial and kept under the fume hood. This process was repeated two additional times. To expedite the rate at which diethyl ether evaporates, a small tube supplying nitrogen gas was fitted to each tube, and nitrogen gas applied until the ether was evaporated. Following complete evaporation, the glucocorticoid homogenate was resuspended in 1 ml 1× PBS, and the vials were placed in a 4°C refrigerator overnight. On the following day, samples were assayed using a human salivary enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (cat. Number 1–3002; Salimetrics, State College, PA, USA). ELISA plates were then read on a spectrophotometer plate reader (FLOUstar Omega; BMG Labtech). The ELISA protocol was originally described for its use in zebrafish (Cachat et al., 2010), and we maintained those specifications including standards for *Astyanax*. Runs were accepted when intra-assay coefficients of variation were below 20%. Inter-assay coefficients were ~30% since daily fluctuations in temperatures and extraction procedures might differ slightly.

Whole body cortisol measures were normalized using total protein. Total protein was measured from the 50 µl sample of homogenate (described above) using a Quick Start Bradford assay (cat. number 5000205; Bio-Rad). Bovine serum albumin (BSA) with concentrations 0.125–2.0 mg/ml from a standard set served as protein standards (cat. number 5000207; Bio-Rad). Then, 250 µl of a 1× Bradford dye was added to 5 µl homogenate taken from each sample and mixed well in a 96-well plate (cat. number CLS3997; Corning). Samples were incubated for 5 min, and absorbance was determined using a spectrophotometer plate reader (FLOUstar Omega). All samples were converted from total absorbance to total protein concentration by relating absorbance to the BSA standards. Finally, total cortisol was normalized to total protein.

2.4 | Quantitative real-time polymerase chain reaction of crf, nr3c1, and nr3c2

Expression analysis of *corticotropin releasing factor (crf)*, the *glucocorticoid receptor (nuclear receptor subfamily 3 group C member 1; nr3c1)*, and the *mineralocorticoid receptor (nuclear receptor subfamily*

3 group C member 2; *nr3c2*) were performed using quantitative real-time polymerase chain reaction (qRT-PCR). BLAST/BLAT (Ensembl; Altschul, Gish, Miller, Myers, & Lipman, 1990) was first performed on the genes to ensure that there are no gene duplications and only a single gene isoform exist. Then, primers were designed for all three genes using Primer-BLAST (NCBI), and the regions amplified were ensured to be conserved in both surface and Pachon populations. Primer sequences were as follows: *crf*—forward primer, CCATCTC TCTGGACCTGACC; reverse primer, TCCATCATTCTGCGGTGCT; *nr3c1*—forward primer, CGCCGAAATCATCAGCAACC; reverse primer, TAAGGCATGGTGTCCCGTTG; *nr3c2*—forward primer, TTCTT CAAGAGAGCGGTGGAA; reverse primer, GCCTGGAGACACTTCC GTA; β -actin—GCTCTCTTCCAGCCTTCCTT; reverse, GCACTGTG TTGGCATACAGG. Larvae grown in glass bowls (Pyrex, 470 ml) were sampled for analysis. RNA was extracted from pools of twenty larvae at 9 dpf with TRIzol (cat. number 15596018; Fischer) and chloroform, and purified using the QIAGEN RNeasy mini kit. Then, RNA was reverse-transcribed to complementary DNA (cDNA) using iScript Reverse Transcription Supermix (cat. number 1708840; Bio-Rad), and the cDNA was used in the qRT-PCR. The detection of fluorophore (SsoAdvanced Universal SYBR Green Supermix, cat. number 172570; Bio-Rad) and thermal cycling were performed in the CFX96 Real-Time System (Bio-Rad) that is connected to the C1000 Touch Thermal Cycler (Bio-Rad). All genes analyzed were normalized to β -actin.

2.5 | Statistics

All statistics were performed using Prism software (Graphpad, version 8) or Excel (Microsoft version 16). In the cases where pairwise comparisons of before shock and after shock were performed, a Wilcoxon paired *t* test was used. In cases where more than two comparisons were examined, we used a Kruskal-Wallis test, and when differences were reported, this was followed by a Dunn test correcting for multiple comparisons. Correlational analysis was performed using Spearman's rank order correlation. For all statistical test, significance was determined as *p* being ≤ 0.05 . All data will be made available upon request.

3 | RESULTS

3.1 | Behavioral stress responses are reduced in Pachón cavefish

Electric shock has been used extensively in adult and larval zebrafish (Agetsuma et al., 2010; Kenney, Scott, Josselyn, & Frankland, 2017; Tabor et al., 2014; Valente, Huang, Portugues, & Engert, 2012). To determine whether larval cavefish have reduced responsiveness to stress compared with surface conspecifics, we assessed stress by applying mild electric shock and examining differences in freezing times pre- and post-stimulation. We subjected 9-day-old surface fish

and compared their responses to shock to age-matched Pachón fry. Briefly, larvae were placed individually in a chamber fitted with electrodes, and their locomotor activity recorded before and after electric stimulation (Figure 1a). Similar to zebrafish, larval surface fish show an increase in freezing duration in the minute following stimulation (Figure 1b, blue bars). By contrast, exposure to electric shock did not induce significant differences in freezing in Pachón cavefish (Figure 1b, black bars). These data reveal that reduced stress in Pachón cavefish previously described in *A. mexicanus* adults, is also present in the larval stage.

3.2 | Diminished activation of the hypothalamic-pituitary-interrenal (HPI) axis in cavefish

A hallmark of stress is elevated levels of glucocorticoid stress hormones, such as cortisol in humans and fish, and corticosterone in rodents (Alsop & Vijayan, 2008, 2009). At the presentation of a stressful cue, hypothalamic neurons expressing *corticotropin releasing factor* (*crf*) direct the head kidney to produce and release glucocorticoids into the blood stream (Collier et al., 2017), and glucocorticoids signal back to the brain by binding to glucocorticoid and mineralocorticoid receptors (Collier et al., 2017). To determine the response of the HPI axis to a stressful stimulus we measured cortisol levels in 9 dpf surface fish and Pachón cavefish larvae following exposure to shock. We found that, compared with unshocked controls, surface animals had a significant increase in cortisol 15-min following stimulation (Figure 2a, blue bars). Conversely, Pachón cavefish had a small increase in cortisol, which was not significantly different than unshocked controls (Figure 2a, black bars). Analysis of cortisol levels at 30-min or 1-h post-shock did not indicate a delayed response in Pachón cavefish, and in surface larvae, cortisol levels were comparable to before shock by 30-min (Figure S1). These data demonstrate that, in addition to diminished behavioral responses to stress, Pachón cavefish have diminished physiological markers of stress as well.

We next quantified differences in mRNA expression of genes in the HPI axis in surface and Pachón cavefish. We first examined the expression of *corticotropin releasing factor a* (*crfa*), a gene that is upstream of cortisol and predominantly responsible for cortisol activation, and found no significant differences (Figure 2b). We next examined the expression level of cortisol receptors. The mineralocorticoid receptor (*nr3c2*) is a high-affinity receptor that is considered to be saturated with low levels of cortisol. By contrast, the low-affinity glucocorticoid receptor (*nr3c1*) becomes activated by high levels of cortisol (Reul & De Kloet, 1985; Reul, Van Den Bosch, & De Kloet, 1987). The glucocorticoid receptor (*nr3c1*) is a nuclear receptor, which suppresses the HPI axis through a negative feedback loop mechanism. We found that expression of glucocorticoid receptor was significantly higher in Pachón cavefish than in surface fish, but no significant differences in the mineralocorticoid receptor were found. Together, these data suggest elevated levels of glucocorticoid receptor suppress cortisol levels in cavefish.

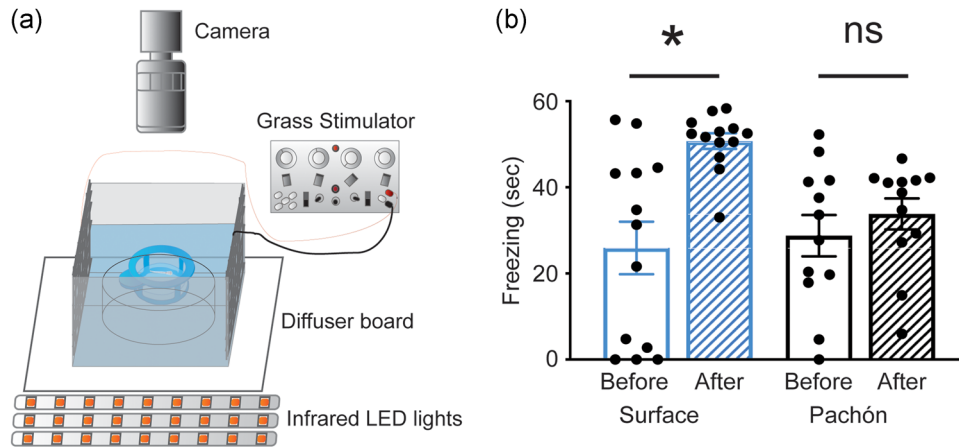


FIGURE 1 Pachón cavefish have dampened behavioral response to stress. (a) Diagram of shock assay. (b) Pachón cavefish freeze for less time relative to surface fish. Surface fish (blue) have increased freezing post-shock (hatched bar) relative to pre-shock freezing durations (solid bar; Wilcoxon's signed-rank test, $W = 71.0$, $p = .012$). By contrast, freezing levels pre- (solid bars) and post-shock (hatched bars) were not significantly different for Pachón cavefish larvae (black bars; Wilcoxon's signed-rank test, $W = 22.0$, $p = .421$). Error bars show $\pm$ SEM. ns, no significance. *Significance below $p = .05$ [Color figure can be viewed at wileyonlinelibrary.com]

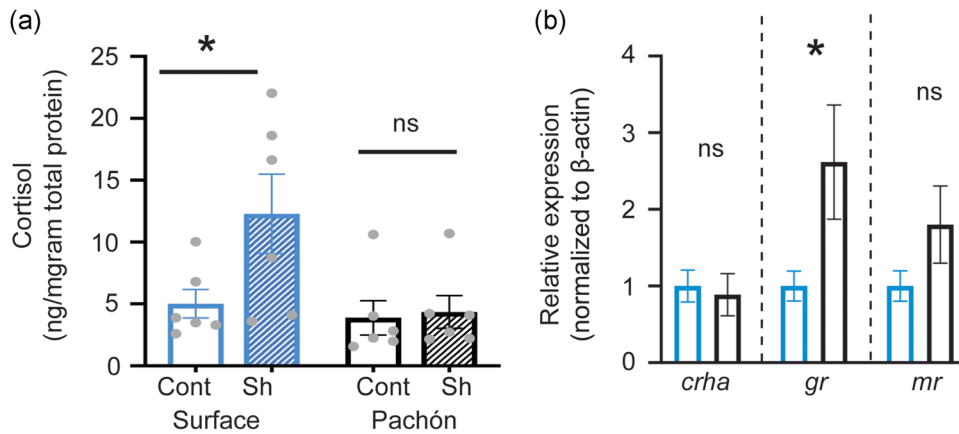


FIGURE 2 Pachón cavefish have reduced activity of the neuroendocrine stress axis. (a) In response to stress, surface larvae exhibit an increase in the stress hormone cortisol (Wilcoxon's signed-rank test, $W = 30.0$, $p = .0078$); however, in response to the same stimulus, Pachón larvae show no significant changes in whole body cortisol levels (Wilcoxon's signed-rank test, $W = 18.0$, $p = .25$). (b) Quantification of genes in the hypothalamic–pituitary–interrenal axis using quantitative polymerase chain reaction. Measures of corticotrophic releasing hormone (*crha*) were not significantly different between cave and surface fish (Student's t test, $t = 0.5386$, $p = .602$). Expression of the glucocorticoid receptor (*nr3c1*) was higher in cavefish relative to surface fish (Student's t test, $t = 2.559$, $p = .028$). No significant differences were observed in the expression of the mineralocorticoid receptor (*nr3c2*; Student's t test, $t = 1.201$, $p = .257$). For all plots, blue bars represent surface fish and black bars represent cavefish. Error bars show $\pm$ SEM. Cont, controls; ns, no significance; Sh, shocked larvae. *Significance below $p = .05$ [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | F₂ analysis reveals independence of stress and morphological traits

The ability to breed surface/cave and cave/cave hybrids allows for examining how morphological and behavioral traits segregate. To explore whether the diminished stress response in cave animals is dominant or recessive, we crossed a pure-breeding surface fish to a pure-breeding Pachón fish to produce a brood of F₁ progeny. F₁ fish were tested in the shock assay. The mean total freezing duration in F₁ animals was not significantly different than freezing pre-shock, though total times were

more variable (Figure 3a, purple bars), suggesting a dominant cave trait. Next, we incrossed F₁ fish and examined the F₂ progeny in the shock assay. Total pre- and post-shock freezing times were significantly different in F₂ animals (Figure 3a, magenta bars). Importantly, the range of freezing times in F₂ animals was significantly greater than either of the pure-breeding stocks (surface $\sigma^2 = 42.97$; Pachón $\sigma^2 = 154.0$; F₂ $\sigma^2 = 283.92$), and the range of post-shock freezing times spanned the range of surface to cave animals. These data reveal that diminished stress in Pachón animals is genetically encoded, and the genes in cavefish are mostly dominant to those in surface conspecifics.

To determine whether stress responses are related to morphological phenotypes, for example, their ability to see, we tested the relationship between stress and eye size and body size in F_2 hybrid fish. We first looked at both eye size, since visual cues could affect stress responses, and body size, as the size of the body could alter stimulus sensation. Consistent with previous reports, body size is smaller in age-matched surface animals compared with cavefish, whereas eye size is smaller in cavefish relative to surface. We measured both body and eye size in the F_2 fish that we tested for stress, and found that these values were variable (Figure 3b). We next asked whether stress was correlated with either morphological traits. A linear correlation of body size to freezing revealed a negative slope ($m = -20.18$, Figure 3c), whereas a linear correlation of eye size to freezing revealed a positive slope ($m = 52.29$, Figure 3d). However, statistical correlation analysis revealed that neither body size nor eye size was significantly correlated with freezing times (body size ~ freezing: Spearman's $\rho^2 = -0.27$, $p > .05$; eye size ~ freezing: Spearman's $\rho^2 = 0.006$, $p > .05$), suggesting that reduced stress in cave animals is a novel trait, which is likely independent of these two morphological attributes.

3.4 | Convergence of stress in some, but not all larval *A. mexicanus* populations

The multiple independently evolved cavefish populations of *A. mexicanus* provide a unique opportunity to address whether evolution repeatedly has altered different traits, or whether the evolved differences are unique to some cavefish populations and not others. To assess whether or not cavefish larvae from other localities also have dampened stress responses, we raised larvae from the Tinaja and Molino caves. Previous studies suggest that Tinaja are more closely related to fish from the Pachón cave and other populations from the El Abra region, whereas individuals from the Molino cave are evolved from "newer" colonization of surface fish in the Sierra de Guatemala region and are more divergent (Brdic et al., 2012). Surface fish subjected to shock exhibit a significant increase in freezing times, consistent with them having a characteristic stress response (Figure 4, blue bars). When larvae derived from the Tinaja cave were tested, they showed little freezing post-shock (Figure 4, pink bars), similar to our observations with Pachón larvae. By contrast, larvae

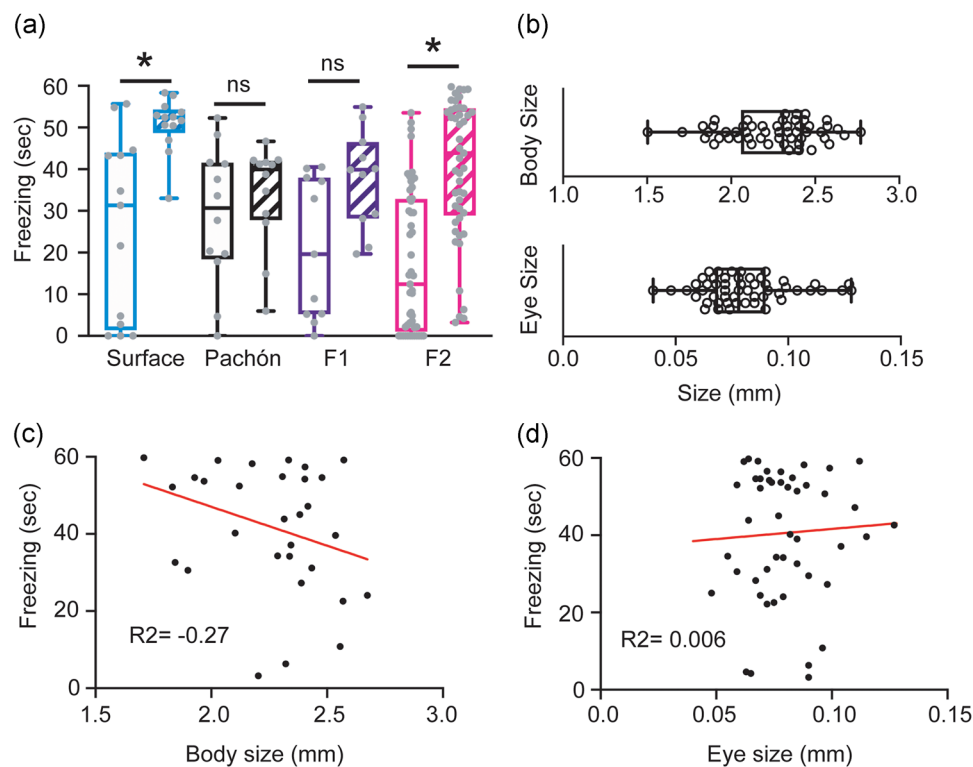


FIGURE 3 Reduced stress in cavefish is genetically determined, and does not segregate with body or eye size. (a) Distribution of freezing duration pre- and post-shock for surface fish (blue), Pachón cavefish (black), surface $\times$ cave F_1 hybrids (purple), and surface $\times$ cave F_2 hybrids (magenta). Freezing was significantly increased post-shock for surface fish (Wilcoxon's signed-rank test, $W = 71.0$, $p = .012$), but not significantly different pre- and post-shock for Pachón cavefish (Wilcoxon's signed-rank test, $W = 22.0$, $p = .421$). Freezing durations pre- and post-shock for F_1 hybrids approached significance (Wilcoxon's signed-rank test, $W = 42.0$, $p = .067$), and was intermediate between surface and cavefish. Freezing times pre- and post-shock for F_2 hybrids was significant (Wilcoxon's signed-rank test, $W = 49.0$, $p < .001$), and spanned the range of surface and cave post-shock freezing. (b) Distribution of body length and eye size in surface $\times$ Pachón cave F_2 hybrids. (c) The length of the body in F_2 hybrids was not correlated significantly with freezing duration post-shock (Spearman's test, $\rho = -0.27$, $p = .14$). (d) Eye size in F_2 hybrids was not correlated significantly with freezing duration post-shock (Spearman's test, $\rho = 0.006$, $p = .96$). Error bars in (a) and (b) depict the max to min range. Red lines in (c) and (d) show the best fit in a linear regression analysis. ns, no significance. *Significance below $p = .05$ [Color figure can be viewed at wileyonlinelibrary.com]

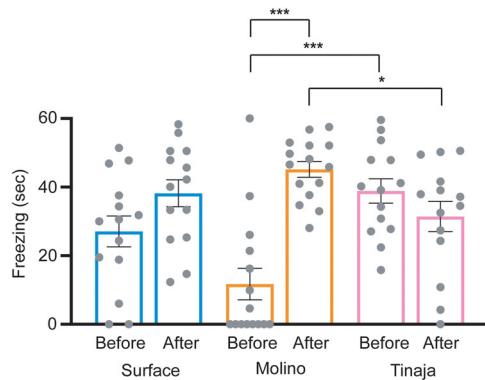


FIGURE 4 Divergence of stress responses in different, independently evolved populations of cavefish. Surface fish (blue bars) show more freezing post-shock compared with before stimulation (Wilcoxon's signed-rank test, $W = 62.0$, $p = .049$). Molino cavefish (orange bars) show significantly higher levels of freezing post-shock relative to pre-stimulation (Wilcoxon's signed-rank test, $W = 116.0$, $p = .0002$), whereas Tinaja cavefish (pink bars) show no significant differences (Wilcoxon's signed-rank test, $W = -51.0$, $p = .12$). Comparing basal activity pre-stimulation, Molino cavefish show significantly reduced levels of freezing compared with Tinaja cavefish (Kruskal-Wallis test, $p = .0004$), yet poststimulus, Tinaja cavefish have reduced freezing compared with Molino cavefish (Kruskal-Wallis test, $p = .0423$) [Color figure can be viewed at wileyonlinelibrary.com]

from the Molino cave showed an increase in freezing similar to what we observed for surface fish (Figure 4, orange bars). Interestingly, analysis of Molino cavefish behavior pre-stimulus revealed significantly diminished basal freezing or immobility compared with Tinaja; and also trended toward diminished stress compared with surface fish, although the differences were not significant ($p = .093$). Conversely, Tinaja cavefish froze significantly less than Molino cavefish after shock, despite having higher basal freezing, suggesting a divergence in stress responses among different cavefish populations at larval stages. Taken together, these data suggest that dampened stress responses are conserved across developmental time points for Pachón and Tinaja populations, and demonstrate a divergence in these dampened responses in Molino, whose baseline activity is higher than both Tinaja and Pachón, and where an increase in freezing following shock is apparent in larval stages.

4 | DISCUSSION

In this study, we have demonstrated that larval cavefish from some but not all caves have evolved a diminished stress response relative to their more ancestral surface fish populations. Moreover, we have found that Pachón cavefish have an elevated expression of the glucocorticoid receptor. These data not only corroborate previous studies demonstrating reduced stress in adult populations (Chin et al., 2018), but they also reveal a change in stress behaviors from larval to adult in Molino populations.

4.1 | Ontogeny of stress in *Astyanax*

In mammals, including rodents and humans, stress responses can vary considerably across development (Adriani & Laviola, 2004; Paus, Keshavan, & Giedd, 2008). Several studies, for example, have demonstrated that young or adolescent individuals have different degrees of stress from their adult counterparts (Hefner & Holmes, 2007; Lynn & Brown, 2010; Paus et al., 2008; Slawewski, 2005). Whereas the developmental reasons for these differences remains poorly understood, one prominent hypothesis is that the neuronal circuits and physiological mechanisms that modulate these behaviors are not fully formed at these early stages of development (McCormick & Mathews, 2007; Zimmermann, Richardson, & Baker, 2019). Our data demonstrate that some cavefish populations such as Pachón and Tinaja have diminished stress at both larval and adult stages, whereas others such as Molino have stress responses similar to surface fish as larvae, but then as adults have a diminished response. These data show clear developmental differences in stress between cavefish populations. For example, differing environmental demands of the Molino cave compared with the caves in which Pachón and Tinaja inhabit could account for these differences (Mitchell et al., 1977). Similarly, differences in genetic variation could also account for the developmental variations. Tinaja and Pachón are derived from an older stock of surface fish, whereas Molino are derived from a separate invasion from a newer stock of fish (Brdic et al., 2012). Moreover, many traits such as eye loss have arisen in new and old stocks through different genetic mechanisms (Borowsky, 2008b). How genetic differences between old- and new-invasion cavefish contribute to differences in the development of stress remain unknown, but the naturally occurring genetic variation in these stocks provides a powerful model to examine how genes can contribute to stress ontogeny.

The demonstration of stress in larval cavefish has considerable implications for establishing cavefish as a model for examining stress. The small size, simplified nervous system, and transparency, together with robust behavioral responses make larval fish especially attractive for evaluating how the brain drives behavior (Ahrens & Engert, 2015; Fero, Yokogawa, & Burgess, 2011; Friedrich, Genoud, & Wanner, 2013; Friedrich, Jacobson, & Zhu, 2010). Moreover, with transgenic technology being introduced recently into the *Astyanax* model (Stahl, Jaggard, et al., 2019; Stahl, Peuß, et al., 2019), the system should be a powerful tool to assess how evolution has impacted the genes and development of neuronal circuits modulating stress in vertebrates.

4.2 | Glucocorticoid receptor mediates reduced stress

The molecular basis for reduced stress in cavefish animals remains unclear, yet our data demonstrating that glucocorticoid receptor is increased points to a role for the modulation of physiological mechanisms of stress. Across vertebrates, stress is modulated by a brain-to-periphery pathway. In mammals, stressful stimuli activate neurons in the hypothalamus that utilize corticotropin releasing factor (crf). crf signals to the anterior pituitary, which then signals to

the adrenal gland to produce and secrete glucocorticoids such as corticosterone or cortisol. Once produced, cortisol feeds back to the brain, and through binding to its glucocorticoid receptor (*nr3c1*), it downregulates the activity of *crf* neurons. Our data reveal that cavefish have diminished stress behaviors, lower cortisol and a higher expression of the glucocorticoid receptor, suggesting that the increase in *nr3c1* expression could, in part account for the diminished stress behaviors. It is not clear what is causing the increased expression of the glucocorticoid receptor, but future work should help to elucidate these mechanisms.

4.3 | Genetic contributions as demonstrated by F₁ and F₂ data, and the F₂ correlation data

A powerful attribute to examining behavioral evolution in blind Mexican cavefish is the ability to cross-breed fish from different populations, including the generation of surface $\times$ cave hybrids as well as crosses of cavefish from different cave localities. This ability allows researchers to examine pleiotropy through co-segregation with other traits, as well as an ability to perform quantitative genetic analyses (J. J. Jaggard et al., 2017; M. Protas et al., 2008; Yoshizawa et al., 2015). For example, differences in stress could be due, partly, to the differences in surface and cavefish's ability to see. Alternatively, altered stress could correlate with differences in metabolism or size of the animal. Our data demonstrate an intermediate stress phenotype in surface cave F₁ hybrids, as well as a distribution of stress responses in F₂ hybrids that spans the range of surface to cave. These data not only point to a strong genetic underpinning of the differences in stress seen between surface and cave animals, but also suggest, given the F₁ data, that cave alleles exhibit either intermediate dominance or a complex relationship with surface alleles.

In human populations, stress and other anxiety-related disorders, are caused by both genetic and environmental factors (Dick, 2011; Sharma, Powers, Bradley, & Ressler, 2016). Several genome-wide association studies (GWAS) have been conducted in search of genetic variants that predispose patients to anxiety and stress-related disorders, and the heritability of these disorders has been calculated to be between 30% and 50% (Sartor et al., 2012; Stein, Jang, Taylor, Vernon, & Livesley, 2002; True et al., 1993). However, it remains unclear how standing genetic variation contributes to the differences in susceptibility of anxiety and stress-related disorders. Because the cavefish are a rare model where standing genetic variation is largely unimpeded, this model could be powerful in establishing how genetics lead to susceptibility of these and other psychological diseases.

4.4 | Neuroanatomical regions that modulate stress may be different between surface and cave morphs

Stress is modulated by myriad neuroanatomical loci, including the hypothalamus, the amygdala, hippocampus, preoptic area, and habenulae (Tovote, Fadok, & Lüthi, 2015), and many of these regions

have been shown to differ significantly between cave and surface morphs in neural activity and/or neuroanatomy (Alié et al., 2018; J. B. Jaggard et al., 2018; Loomis et al., 2019; Menuet et al., 2007). For example, in fish and mammals, the hypothalamus has a critical role in the modulation and induction of stress (Cachat et al., 2010; Callahan et al., 1992; Spiess, Rivier, Rivier, & Vale, 1981; Steenbergen, Metz, Flik, Richardson, & Champagne, 2012; Wamsteeker Cusulin, Füzesi, Watts, & Bains, 2013; Yeh, Glöck, & Ryu, 2013). A recently published neuroanatomical brain atlas in cavefish shows that various subnuclei within the hypothalamus are morphometrically different (Loomis et al., 2019). Similarly, these data have revealed that the forebrain, an area which contains cell populations that are similar to the mammalian amygdala and hippocampus, is significantly expanded in cavefish populations (Alié et al., 2018; Loomis et al., 2019). These data suggest that areas that are essential to the proper modulation of stress are evolutionarily different between cave and epigeal *Astyanax*.

Whereas the molecular mechanisms underlying these differences in neuroanatomy are not fully understood, several studies have pointed to key developmental factors that may play a role. For example, the genes *NK homeobox 2.1a* (*Nkx2.1a*) and *LIM homeobox protein 6* (*lhx6*) have important roles in the development of the forebrain (Alié et al., 2018). In cavefish, the expression of both *nkx2.1a* and *lhx6* are significantly expanded as compared with surface fish, suggesting that these factors may be involved in the evolutionary modification of the telencephalon. Similarly, the hypothalamus is modulated by numerous molecular factors, but one that is particularly important is *sonic hedgehog* (*shh*; Corman, Bergendahl, & Epstein, 2018). Expression of *shh* has been shown to be expanded both along the midline as well as in the hypothalamus (Menuet et al., 2007). Expanded expression of this gene may be important for the resistance to stress that we report in cavefish. Together, these data suggest that modifications in the molecular mechanisms underlying brain development could be important in evolutionarily derived behaviors.

4.5 | Functional implications for diminished stress in cavefish

Many caves, including some in the Sierra de El Abra region of Mexico where cavefish are found, are characterized by perpetual darkness, a near absence of primary producer, low food availability, and a reduction of predators (Culver, 1982; Mitchell et al., 1977; Poulson & White, 1969). In the caves of Northeast Mexico, cave *Astyanax* are thought to be at the top of their food chain and to have little to no predatory threat. Predatory threat has been shown to correlate significantly with stress responses (Clinchy, Sheriff, & Zanette, 2013). For example, in both Belding ground squirrels as well as the mosquito fish, *Gambusia hubbsi*, animals raised in a predator rich environment have been shown to have increased stress responses, compared with siblings that were raised with low predator threat (Heinen-Kay et al., 2016; Mateo, 2007). In Trinidadian guppies, increased predator

threat promotes social relationships, and predator stimuli over time induced population changes toward “shy” behaviors such as increased freezing (Heathcote, Darden, Franks, Ramnarine, & Croft, 2017; Houslay, Vierbuchen, Grimmer, Young, & Wilson, 2018). These data reveal that even in unrelated species, predator threat can cause differences in stress. Our data suggest that both adult (Chin et al., 2018) and some populations of larval fish follow this trend. Moreover, our data reveal that predator threat can not only correlate with differences in stress, but also show that predator threat may be a powerful evolutionary driving force. We hypothesize that the low predator threat of the *Astyanax* caves either resulted in relaxed selection of stress responses, or may be a selective driving force itself. Further experiments will reveal whether this is the case, and if so, whether or not diminished stress in cavefish is selected for, or emerged through neutral drift.

4.6 | Future directions

The blind Mexican cavefish has emerged in recent years as a powerful system to examine how naturally occurring genetic variation contributes to various diseases such as obesity (Aspiras et al., 2015), diabetes (Riddle et al., 2018), insomnia (Duboué et al., 2011; J. B. Jaggard et al., 2018) and heart regeneration (Stockdale et al., 2018). Our data suggest that the cavefish may also be a powerful system to examine how naturally occurring genetic variation contributes to differences in stress, and could be important for determining genetic predispositions to stress and anxiety-related disorders. In addition, understanding stress and its effects in the long term on growth, cardiovascular, metabolism, and musculature is important in aquaculture (Sadoul & Vijayan, 2016). We have previously demonstrated that adult *A. mexicanus* have diminished stress responses (Chin et al., 2018), and these data extend this to larval forms. We expect that future work using powerful approaches unique to larvae, such as whole brain functional imaging and tracing of neural circuits that modulate stress, together with genotype–phenotype associations between genes and stress responses will shed significant light into genetic predisposition to stress disorders.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

All data will be made available upon request.

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SUPPORTING INFORMATION

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

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