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Olfaction and reaction: The role of olfactory and hypothalamic investment in the antipredator responses to chemical alarm cues by northern redbelly dace

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Abstract

Neuroplasticity enables teleosts to promote or downregulate the growth of their brains regionally. To compensate for the effects of predation pressure, teleosts may alter their brain morphology and behavioral responses to mitigate its impact on individual fitness. High-predation environments often promote specific patterns of brain growth and produce bolder and more proactive populations. Owing to the expense of maintaining neural tissue, relative size indicates the regions most relied upon. In northern redbelly dace *Chrosomus eos*, as little as 2 weeks of elevated predation pressure, resulted in increased investment in their olfactory bulbs and optic tecta, while the imposition of captivity produced smaller, less symmetric hypothalami. Taken together, these results suggest that an individual could potentially become better able to detect a threat, and simultaneously less inclined to react to it, making the impact of either change in isolation difficult to discern. Here, we compared interindividual variation in gross brain morphology, risk-taking tactics in a novel arena (shy–bold personality), and responding to olfactory cues (proactive/reactive stress-coping style). We hypothesized that olfactory investment would positively correlate with response intensity to predator cue concentration and respond across a wider range of cue concentrations, while hypothalamus size would correlate with shyness and reactivity. Exposure to heightened risk produced more bold/proactive individuals, with larger olfactory bulbs and smaller hypothalami. However, the direction of the correlation between hypothalamus size and behavior varied by treatment, and olfactory investment only corresponded with response intensity amongst proactive individuals. Our findings illustrate the potential pitfalls of relating gross brain morphology to complex behavior and suggest that stress-coping style is a relevant consideration in future studies.

Key words: boldness, brain morphology, neuroplasticity, predation, proactivity, stress-coping style.

Numerous fish species have been found to exhibit divergent brain morphologies between high- and low-predation populations (Kotrschal et al. 2017). These morphologies likely contribute to various behavioral and physiological adaptations exhibited by populations subject to high predation (Dunlap et al. 2019). However, the influence of predation on patterns of neural investment within teleost brains is not easily generalized and is often sex, ecotype, and/or context specific (Gonda et al. 2011; Herczeg et al. 2015; Axelrod et al. 2018). For instance, female guppies from populations that are sympatric with wolf fish (*Hoplias malabaricus*, an ambush predator), invest in their vision centers (optic tecta), while those sympatric with blue acaras (*Andinoacara pulcher*, a pursuit predator), reduced investment in their olfactory bulbs and hypothalamic lobes (Kotrschal et al. 2017). Interpreting the significance of this predation-driven regional investment typically relies on the principle that a region's relative size reflects its utility to the individual (Kaslin et al. 2008; Iglesias et al. 2018).

Generally, the utility of a brain region is presumed to offset the high energetic cost of investing in the growth and maintenance of neural tissue (Tsuboi et al. 2016). This principle—that investment reflects utility—is seen in the optic tectum size of cave-dwelling shortfin molly *Poecilia sphenops* (Eifert et al. 2015), and 9-spine sticklebacks *Pungitius pungitius*, raised

in water with black dye (Pike et al. 2018); both of which, in the absence of useful visual information, had reduced reliance on visual processing. Similarly, both freshwater and marine 9-spine sticklebacks, exposed to predation risk, developed larger olfactory bulbs, but only the freshwater populations exhibited smaller hypothalami (Gonda et al. 2012). While this principle has been found to apply to sensory regions (Lee et al. 2017), the significance of changes in the size of the hypothalamus, an integrational center (Delgado et al. 2017), is less clear.

The hypothalamic–pituitary–interrenal (HPI) axis in fish influences most of their endocrine systems, playing an important role in feeding and reproductive behaviors (Blanco 2020; Trudeau and Somoza 2020). Importantly, the HPI axis governs the intensity of physiological stress responses which, in turn, can influence behavior (Thomson et al. 2016). In redbelly dace, having larger hypothalami correlated with the latency to explore a novel arena (shyness) (Joyce and Brown 2020b); this experiment also observed significant hypothalamic plasticity in response to captivity. Consequently, we looked at hypothalamic investment as an additional variable that might influence risk-taking behavior. However, this approach required an additional consideration; owing to the observation that some aspects of hypothalamic function have been found to vary with stress-coping style (Wong et al. 2019).

Reactive and proactive fish handle stress differently; proactive fish have lower post-stress cortisol, reduced behavioral flexibility, and a higher proportion of cortisol receptors which provide negative feedback for the stress response (Vindas et al. 2017a, 2017b). Butler et al. (2018) found that, in the African cichlid *Astatotilapia burtoni*, patterns of neuronal activation across much of the brain, including the hypothalamus, varied by stress-coping style. Their results suggested that the hypothalami are more active during exploration among proactive individuals and more active among reactive fish when freezing (an antipredator response [APR]). Based on this, we opted to examine morphological correlates of behavior by background risk and behavioral phenotype.

The morphological and behavioral plasticity previously observed in redbelly dace in response to short-term changes in predation pressure and captivity were significant but subtle, and the actual impact of such changes on antipredator behavior remains to be seen. Here, we used predation cues to generate a greater range of brain morphologies and behavioral responses than might be found in the source population (Bell and Sih 2007), to explore, we test several predictions (Table 1) regarding the potential links between exposure to predation risk, gross brain morphology and risk-taking tactics in redbelly dace. First, we compared relative hypothalamic and olfactory investment with shyness and with the intensity of their APR (reactivity) (Dingemanse et al. 2010; Toms et al. 2010), expecting it to positively correlate with both. Then, we predicted that per the investment-utility principle, within groups exposed to the same background risk and cue concentration, relative olfactory bulb size would correlate with APR intensity.

Materials and Methods

Fish collection and holding

In the summer of 2018, redbelly dace were captured and sorted following the methods described in Joyce and Brown (2020a). In brief, redbelly dace were caught in minnow traps from an isolated spring-fed artificially dug pond (≈0.25 ha) in Ontario, Canada. The pond was initially stocked with brown bullhead catfish (*Ameiurus nebulosus*); redbelly and finescale dace *Chrosomus neogaeus* appeared later, possibly introduced as eggs by predatory waterfowl (Lovas-Kiss et al. 2020). Experimental animals were handled and used per Concordia University Animal Research Ethics protocol #30000255. Following capture and sorting, the bycatch, including any

finescale or reproductively active redbelly dace, was returned to the pond. Healthy adults were used for conspecific skin extract (alarm cue) production or retained as experimental subjects and held under semi-natural conditions.

The large semi-natural enclosures used in this experiment were meant to avoid the imposition of barren conditions used in previous experiments (Joyce and Brown 2020a; 2020b), which had limited space and restricted sightlines which may constrain plasticity (Fong et al. 2019; Mes et al. 2020). The enclosures consisted of mesh bags, approximately (0.6 m × 0.8 m) attached to weighted baskets (Figure 1A). The holes in the mesh (3 mm) were small enough to prevent escape but large enough to permit the entrance of drift fodder. Airline tubing was strung to each of the enclosures for the delivery of cues and supplementary food and the tops were covered with netting to prevent jump-outs. To ensure adequate nutrition, each occupied enclosure received 2–3 g of brine shrimp flakes once a day. Enclosures were suspended in the water column off the sides of square rafts and had a volume of approximately 110 L. The rafts were tethered approximately 9 m from shore to ensure a constant water depth and they were kept a minimum of 6 m apart. Each raft received 20 mL per enclosure of either pond water (ambient risk) or alarm cue (heightened risk) twice a day, at haphazard times between 06:00 and 21:00. The heightened and ambient risk groups were kept approximately 24 m apart. Light exposure and water temperature were the same for both groups.

Alarm cue production

The conspecific skin extract used for the alarm cue was prepared from healthy redbelly dace euthanized by a sharp blow to the head. Skin fillets were removed, and their dimensions were recorded, before homogenization with distilled water. The solution was filtered through polyester floss and diluted to produce a stock solution of approximately 0.15 cm^2 mL^{-1} which was frozen in 20 mL aliquots until needed. The alarm cue for the behavioral assay was prepared as needed by serial dilution of the stock cue to concentrations of 10%, 1%, 0.01%, and 0.001%, with distilled water (0%) as the control (as in Dupuch et al. 2004).

Experimental setup

The dace were evaluated individually in 20-L tanks fitted with equal lengths of 3 mm tubing to deliver air and olfactory cues. The tanks were filled with well water and held around 20 °C. Behavior was recorded using high-definition digital

Table 1. Predictions and outcomes for (a) predation-induced morphological and behavioral changes and (b) correlations between brain morphology and behavior.

Prediction	Result	
a) Preexposed to heightened risk		
(1) Increased investment in optic tecta/ olfactory bulbs	True	
(2) Decreased hypothalamic investment	True	
(3) Reduced antipredator response (more proactive individuals)	True	
b) Overall correlations		
(4) Latency to emerge correlates positively with hypothalamus size	Mixed	(AR+/HR−)
(5) Antipredator response correlated with hypothalamus size	False	
(6) Antipredator response intensity correlates with olfactory investment	Mixed	(PR only)

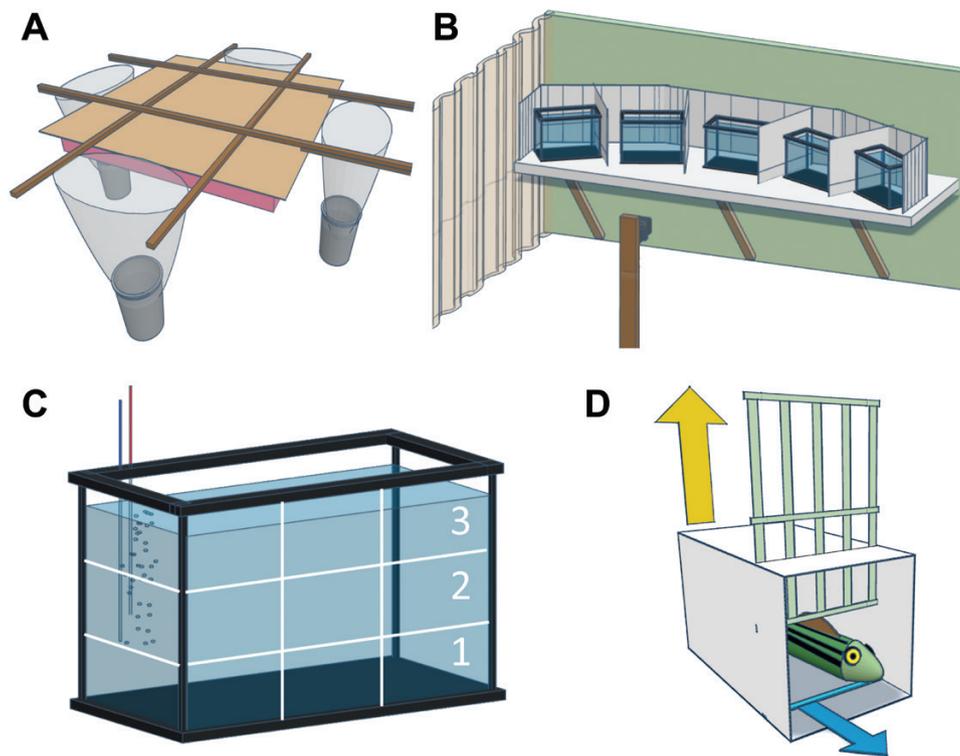HR, heightened risk; AR, ambient risk; PR, proactive individuals.

Figure 1. Diagrams of (A) the rafts used to suspend the mesh enclosures, (B) the single camera setup for recording multiple tanks for behavioral testing, (C) an experimental test tank with 3 × 3 grid, and (D) a refuge used for emergence time testing; (see text for dimensions).

cameras, each facing an array of 5 tanks that occupied equal portions of the field of view; opaque plastic dividers between the tanks prevented fish from seeing one another (Figure 1B). The tanks were marked with lines dividing the front of the tank space into a 3 × 3 grid (Figure 1C). A floor-length curtain hung behind the camera which screened the experimenters from view during testing. To control for the impact of water temperature on expressed behavior (Biro et al. 2010), water from the test tanks was gradually introduced to the transfer buckets until they were within 2 °C of the experimental tanks. After 30 min of acclimation, subjects were individually transferred to refuge boxes for individual emergence time testing. The refuges (Figure 1D) were square PVC tubes 10 cm on a side and 15 cm long, with plastic mesh fixed across one end and as a sliding mesh gate at the other.

Emergence time assay

Individual dace were tested for latency to emerge from their shelter and explore a novel environment (emergence time) in the face of unknown risk, a proxy for boldness (Burns 2008). Emergence time into a novel arena correlates with measures of exploration, and when associated with a perceived threat, also with measures of fear and escape (Toms et al. 2010). In terms of stress, the process of being captured by dip-net and later released into the refuge was analogous to escaping a predator (Donaldson et al. 2010). After a further 10-min acclimation period in the refuges, the gates were lifted and the time until more than half of the subject's body crossed the threshold (emergence time) was recorded. This relatively short time ensured that the free choice to emerge was first presented before the dissipation of the secondary stress response (Barton 2002; Wong et al. 2019) and may increase repeatability (O'Neill et al. 2018). Any trials where an obvious external

stimulus that may have influenced the results, for example, a door slammed shut, were excluded. After 18 min, the refuges were removed and subjects who never emerged were coded as taking the maximum time of 1,080 s.

Antipredator response assay

Following an additional post-emergence acclimation period of at least 60 min, 10 min of pre-stimulus behavior was recorded. After the initial observation, a syringe was used to withdraw and discard two 60 mL aliquots of water, which cleared the injection line and primed it with clean water. Subsequently, 5 mL of the stimulus was introduced, followed by 60 mL of well water. The moment of injection for each tank was recorded and the tanks were filmed for 10 min post-stimulus. Between tests, the tanks were thoroughly drained, repeatedly rinsed, and then refilled with oxygenated well water held at room temperature (20–22 °C). Following the behavioral assays, subjects were individually euthanized with an overdose of clove oil (0.03% v/v) and transferred to 15 mL centrifuge tubes containing 4% paraformaldehyde in phosphate-buffered saline and refrigerated until dissection.

Video coding

Videos were coded by recording the position of the subject within the tank as well as its swimming speed at regular intervals. The videos were coded twice, and the 10-min pre/post video was coded at 20-s intervals. The horizontal and vertical positions were expressed as their 2D grid coordinates as seen from the camera's position (X: 1–3, Y: 0–3, Activity 0–3). The vertical position of zero refers to an additional area comprising the very bottom of the tank, from 0 to 2.5 cm. Activity level was defined by swimming speed. Subject unmoving/hovering in position within the water column (0), directed

movement 0.5–1 body lengths per second (1), 1.5–2.5 per second (2), and dashing (3) at 2.5+. The combination of being at the very bottom and not moving (Y:0, A:0) was considered a special condition that may reflect a strong APR.

Dissection and photography

The subjects' weights and standard lengths were recorded. Their brains were extracted and photographed, dorsally and ventrally, in the same manner, described in Joyce and Brown (2020a). In brief, the skull was split open, the olfactory nerves were severed at the nares, and the spinal cord was cut where it exits the base of the skull. Finally, the optic nerves were severed as the brain was lifted out. The brains were kept moist until photographed at a fixed 10X magnification and full illumination using a Leica EZ4W dissection scope. The focal plane of the scope was used as a gauge for positioning. Correct positioning required that all the regions to be examined were visible within the focal plane.

The apparent cross-sectional area of each region served as a proxy for volume (Joyce and Brown 2022). It was found by tracing its outline in ImageJ (Figure 2) (Schneider et al. 2012) using a series of discrete anatomical points (landmarks) and points delineating the perimeter between them (semi-landmarks). They were placed using tpsDig2 (Rohlf 2018); see Park and Bell (2010) and Figure 2A. Brain areas were log-transformed and expressed as a proportion of the whole brain; regional symmetry ratios are all left/right.

Statistical analysis

All linear models, correlations, regressions, cluster analysis, and standard analysis of variance (ANOVA) were computed using SPSS v.27 (SPSS Statistics for Windows, 2020). Overall, brain shape was analyzed using MorphoJ v1.07a (Klingenberg 2011). A multivariate general linear model (GLM) was used to assess the relationship between the regional area values and symmetry rations by treatment and body size; significant

results were reexamined with a univariate GLM. We compared regional investment and symmetries between heightened and ambient risk subjects using one-way ANOVA. Linear models were performed factorially using standardized values, with replicate number as a factor, and with all interaction terms. In subsequent runs, terms were excluded from the model in order of nonsignificance (Bolker et al. 2009).

The 2-step cluster analyses for grouping behavioral phenotypes into early/late emergers and strong/weak antipredation responders used log-likelihood as the distance measure and Schwarz's Bayesian criterion for clustering; it was performed on the standardized residuals for emergence time and on changes in vertical area use, activity level, and time spent stationary on the substrate post-stimulus. In both cases, the number of clusters (2) was determined automatically. Chi-square analysis was used to determine if the proportion of subjects in each category differed by treatment. APR intensity in the dace was quantified using their activity level, proximity to the substrate, and time spent stationary at the bottom of the tank (Geffroy et al. 2020; Wisenden et al. 2009). Later, a single principal component, APR Intensity, was extracted which encompassed all 3 variables.

Results

Behavior

The latency to emerge was shorter in heightened-risk subjects, 165 ± 173 versus 308 ± 338 s ($F_{1,116} = 4.053$, $P = 0.046$, $\eta p^2 = 0.03$). Additionally, 6 ambient risk subjects failed to emerge in the allotted time. Emergence time in both groups was uncorrelated with weight and only correlated with body length in ambient risk fish (Pearson $r = -0.312$, $N = 65$, $P = 0.011$). Multivariate GLM of the APR variables found a significant effect of treatment (Pillai's trace = 0.065, $F_{3,126} = 3.172$, $P = 0.038$) and treatment by concentration level (Pillai's trace = 0.228, $F_{12,384} = 3.172$, $P = 0.002$). Post hoc LSD analysis found

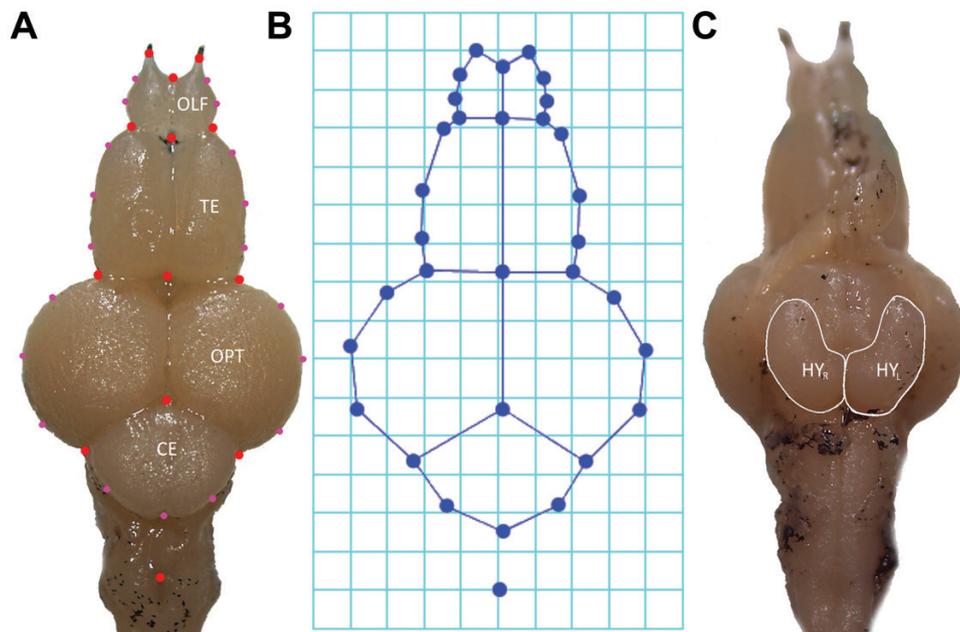**Figure 2.** (A) The dorsal surface of a dace brain, showing the positions of the landmarks (red) and semi-landmarks (orange) used for morphometrics; (B) a wireframe showing the mean configuration of landmark points for all specimens examined, and (C) the ventral surface of a dace brain showing the outline of the left and right inferior lobes of the hypothalamus. Grid: approximately 0.25 mm; scale bar: 4 mm.

that the minimum concentration of AC which produced an overt response, with significant reductions in vertical position and activity levels relative to the controls, was 0.001% for heightened risk (MD = –0.189, SE = 0.086 (position), MD = –0.180, SE = 0.080, P = 0.028 [activity]), and 0.01% for ambient risk fish; heightened risk (MD = –0.219, SE = 0.069, P = 0.002 [position]), MD = –0.140, SE = 0.077, P = 0.072 [activity]). A GLMM found that the change in the number of time intervals spent stationary and at the bottom (Position: 0, Activity: 0) was lower in heightened-risk fish (M = 2.59, SD = 8.20 [heightened] versus M = 4.42, SD = 8.52 [ambient] GLMM $\chi^2_{3, N = 141}$ = 7.721, P = 0.005). Time stationary on the substrate decreased with body length and olfactory investment (Exp(β) =0.119, GLMM $\chi^2_{3, N = 141}$ = 6.754, P = 0.009 (length) and Exp(β) = 1.042, $\chi^2_{3, N = 141}$ = 4.794, P = 0.029 (investment). A total of 17 fish across the 8 replicates were excluded due to external interference while testing.

Risk-taking phenotypes

The 2-step cluster analysis for the bolder early emerging and shyer late emerging fish groupings was based on the latency to emerge from the refuge box. Individuals were classified as either early emerging (bolder) (35.5%, mean Z-score = –1.12) or late emerging (shyer) (64.5%, mean Z-score = 0.61). The proportions of shyer and bolder individuals were not significantly different within the heightened-risk subjects (32 vs. 27, $\chi^2_{1, N = 59}$ = 0.424, P = 0.515), while in ambient risk fish, shy fish were significantly more common (48 vs. 17, $\chi^2_{1, N = 65}$ = 14.785, P < 0.001). Individuals were identified as having a weak (more proactive) or strong (more reactive) APR based on the proportional change in their vertical position, activity level, and time spent stationary on the substrate; variable importance = 1 (position), 0.94 (activity), and 0.5 (time stationary). The strong APR (reactive) group represented 45.4% of individuals, while 55.6% were proactive (weak APR); (mean Z-scores = –0.71 vs. 0.59 [vertical position], –0.69 vs. 0.59 [activity], 0.5 vs. –0.42 [time stationary]); (mean Z-scores = 0.59 [vertical position], 0.57 [activity], –0.42 [time stationary]). Heightened-risk fish were marginally more likely to have a weaker APR (42 vs. 27, $\chi^2_{1, N = 69}$ = 3.261, P = 0.071), while for ambient risk fish, the proportions of weak APR and strong APR fish were comparable (35 vs. 37, $\chi^2_{1, N = 72}$ = 3.261, P = 0.814).

Within the ambient risk treatment, the proportion of individuals found in each of the 4 phenotypic combinations of early/late emergence time and strong/weak APR (Figure 3A), was significantly different ($\chi^2_{3, N = 65}$ = 2.627, P ≤ 0.001), while the distribution among the heightened-risk fish was roughly equal ($\chi^2_{3, N = 59}$ = 2.627, P = 0.453). Between treatments, the number of early emerging/weak APR individuals was significantly higher in the heightened-risk group (N = 17 vs. N = 5, $\chi^2_{1, N = 22}$ = 0.6545, P = 0.011) (EW, Figure 3A). The proportions of the remaining combinations were not significantly different (χ^2, P > 0.1, for all).

Brain shape and regional areas

Between treatments, ambient risk fish had a greater overall left-side bias versus heightened-risk fish; 5.95% ± 0.06 versus 3.43% ± 0.06 ($F_{1,139}$ = 5.849, P = 0.017, ηp^2 = 0.04) for their telencephalons and 8.89% ± 0.05 versus 6.59% ± 0.06 ($F_{1,139}$ = 5.744, P = 0.018, ηp^2 = 0.04) for their optic tecta. We found that individuals from the heightened-risk treatment had proportionally larger optic tecta (0.51 ± 0.02 vs. 0.50 ± 0.02,
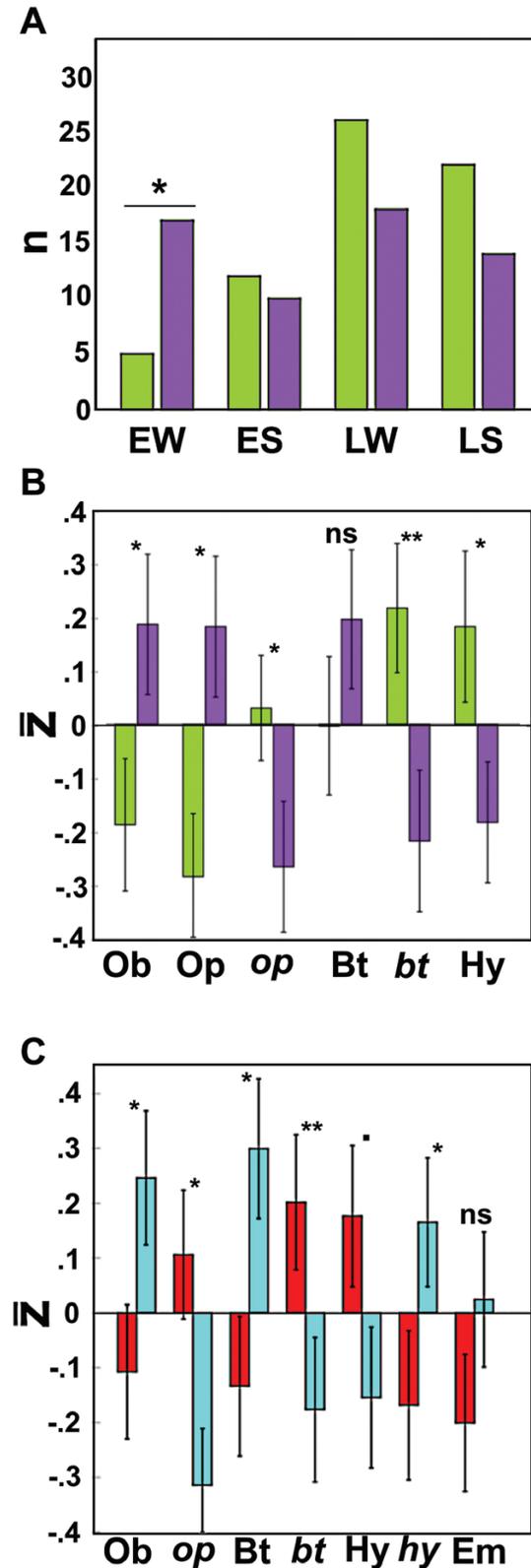

Figure 3. (A) Bar chart showing the number of individuals (n) in each combination of early (E) and late (L) emergers and weak (W) and strong (S) antipredator responders for ambient risk (light fill) and heightened risk (dark fill); notable differences in mean brain morphology and symmetry ($\bar{z}$, standardized variables) by (B) background risk—ambient risk (light fill) and heightened risk (dark fill), and (C) by antipredator response—strong response (reactive, dark fill) group (dark fill) and weak (proactive) responders (light fill); Ob – olfactory bulb size, Op—optic tecta size, op—optic tecta ratio, Bt—total brain area, bt—brain laterization ratio, Hy – hypothalamus, hy—hypothalamic ratio, Em—emergence time; ns (P > 0.05), *P < 0.05, **P < 0.01.

$F_{(1,139)} = 6.385$, $P = 0.013$, $\eta p^2 = 0.04$), smaller hypothalami (0.14 ± 0.009 vs. 0.15 ± 0.01, $F_{1,139} = 4.413$, $P = 0.037$, $\eta p^2 = 0.03$) and, controlling for standard length, larger olfactory bulbs ($F_{1,139} = 4.293$, $P = 0.04$, $\eta p^2 = 0.03$) (Figure 3B).

One-way ANOVA found that individuals with a stronger APR invested proportionally less in their olfactory bulbs, 5.28% of total brain area versus 5.49% ($F_{1,139} = 4.629$, $P = 0.033$, $\eta p^2 = 0.03$) and their brains on average were 3.61% smaller than those of the weak APR (Figure 3C). The size of other regions did not differ significantly between treatments, nor did body length, weight, or condition. The brains of weak APR individuals were more symmetric than those of strong APR fish (5.6% vs. 7.4%, $F_{1,139} = 5.530$, $P = 0.020$, $\eta p^2 = 0.04$). Strong APR fish had more left-side lateralization of their optic tecta, $6.34\% \pm 4.72$ versus $9.47\% \pm 6.53$ ($F_{(1,139)} = 10.837$, $P = 0.001$, $\eta p^2 = 0.07$). Their hypothalamic lobes had significantly more right-side bias, $4.89\% \pm 6.69$ versus $2.44\% \pm 6.43$ ($F_{1,139} = 4.880$, $P = 0.029$, $\eta p^2 = 0.03$) and were marginally larger relative to the total brain area (14.7 vs. 14.4%, $P = 0.086$). Regional sizes within the brain were highly correlated in the shy group; suggesting concerted growth (Table 2).

Brain morphology and behavioral correlates

After controlling for body size, the latency to emerge from shelter correlated positively with hypothalamus size for ambient and negatively for heightened-risk fish (Figure 4A, Pearson $r = 0.274$, $F_{1,63} = 5.131$, $N = 65$, $P = 0.027$ [ambient risk] versus -0.407, $F_{1,57} = 11.317$, $N = 59$, $P = 0.001$ [heightened risk]). Linear regression found a significant effect for hypothalamic ratio in heightened risk only (Pearson $R = 0.487$, $F_{2,56} = 8.692$, $P = 0.001$, $\beta_{hypothalamus} = -0.447$, $P = 0.001$, $\beta_{hypothalamic\ ratio} = -0.270$, $p_{hypothalamic\ ratio} = 0.026$). When the water-only controls relative were excluded, olfactory bulb size was found to correlate positively with vertical displacement; especially in the heightened-risk fish (Pearson $r = 0.203$, $F_{1,112} = 4.810$, $P = 0.030$, Pearson $r = 0.273$, $F_{1,52} = 4.202$, $P = 0.045$). A GLMM ($\chi^2_{19,\ N = 141} = 31.114$, $P = 0.039$), looking at APR intensity versus olfactory investment found significant [alarm cue] by [risk level] ($\chi^2_{4,\ N = 141} = 17.218$, $P = 0.002$) and [alarm cue] by [risk] by [olfactory investment] ($\chi^2_{9,N = 141} = 38.624$, $P < 0.001$) differences between heightened and ambient risk for the 0.001% and 0.1% levels. Spearman correlations were used to detect any monotonic relationships between olfactory and hypothalamic measures with APR intensity by treatment and cue concentration (Table 3).

A follow-up univariate GLM revealed that the relationship between olfactory investment and response intensity varied significantly by APR phenotype ($P < 0.001$); with olfactory investment only correlating with APR intensity in the weak response (proactive) grouping. The number of intervals spent stationary on the substrate (an overt APR) and olfactory investment were significantly correlated for only the weak APR fish (weak APR: Pearson $r = 0.319$, $N = 77$, $P = 0.005$, versus strong APR: Pearson $r = 0.089$, $P = 0.483$).

Discussion

We found that in redbelly dace, exposure to perceived predation led to increased olfactory investment and led to a response reflective of proportional response intensities from the heightened-risk fish at alarm cue concentrations above and below the response thresholds of the ambient risk group. This may imply that relatively larger olfactory bulbs may

Table 2. Spearman's Rho correlations between the $\log_{10}$ transformed size of brain regions for dace in the ambient risk ($N = 72$) versus heightened risk ($N = 69$) treatments (Table 2A), classified as having a strong ($N = 77$) or weak ($N = 64$) antipredator response phenotypes (Table 2B), or classified as early emergers ($N = 44$) or late emergers ($N = 80$).

(A) Predation Pressure	TE	OP	CE	HY
Ambient risk OB	0.219	–0.256*	0.061	–0.18
TE		–0.448**	–0.075	–0.225
OP			–0.478**	–0.198
CE				–0.167
Heightened risk OB	–0.059	–0.072	–0.301*	–0.082
TE		–0.516**	–0.312**	0.152
OP			–0.088	–0.558**
CE				–0.209
(B) Antipredator Response	TE	OP	CE	HY
Strong (reactive) OB	0.184	–0.185	–0.157	–0.087
TE		–0.434**	–0.177	–0.208
OP			–0.312*	–0.220
CE				–0.307*
Weak (proactive) OB	–0.036	–0.135	–0.155	–0.117
TE		–0.486**	-0.200	0.084
OP			–0.302**	–0.458**
CE				-0.099
(C) Emergence Time	TE	OP	CE	HY
Early (bolder) OB	0.187	0.200	0.226	0.113
TE		0.512**	0.419**	0.513**
OP			0.351*	0.547**
CE				0.228
Late (shyer) OB	0.586**	0.565**	0.334**	0.384**
TE		0.710**	0.507**	0.623**
OP			0.575**	0.547**
CE				0.444**

OB, olfactory bulbs, TE, telencephalon, OP, optic tecta, CE, cerebellum, HY, hypothalami.
*$P < 0.05$, **$P < 0.01$.

facilitate behavioral decision-making when navigating alarm cue concentration gradients at the extremes of their detection range (Holmes and McCormick 2011). However, confidence in this conclusion is tempered by the observation that the relationship between regional size, symmetry, and behavioral outcomes may be dependent on recent experience with predation, as with the hypothalamus size and emergence time.

The proportionally larger olfactory and optic sensory regions seen in the heightened-risk dace suggest a generalized response to a perceived increase in predation risk; both sensory modalities may facilitate their detection of predators (Fischer et al. 2017). Correspondingly, the decreased investment in the hypothalamic lobes of the heightened-risk dace may suggest a reduction in the reactivity of their HPI axis; as seen in proactive fish, and those from high-predation
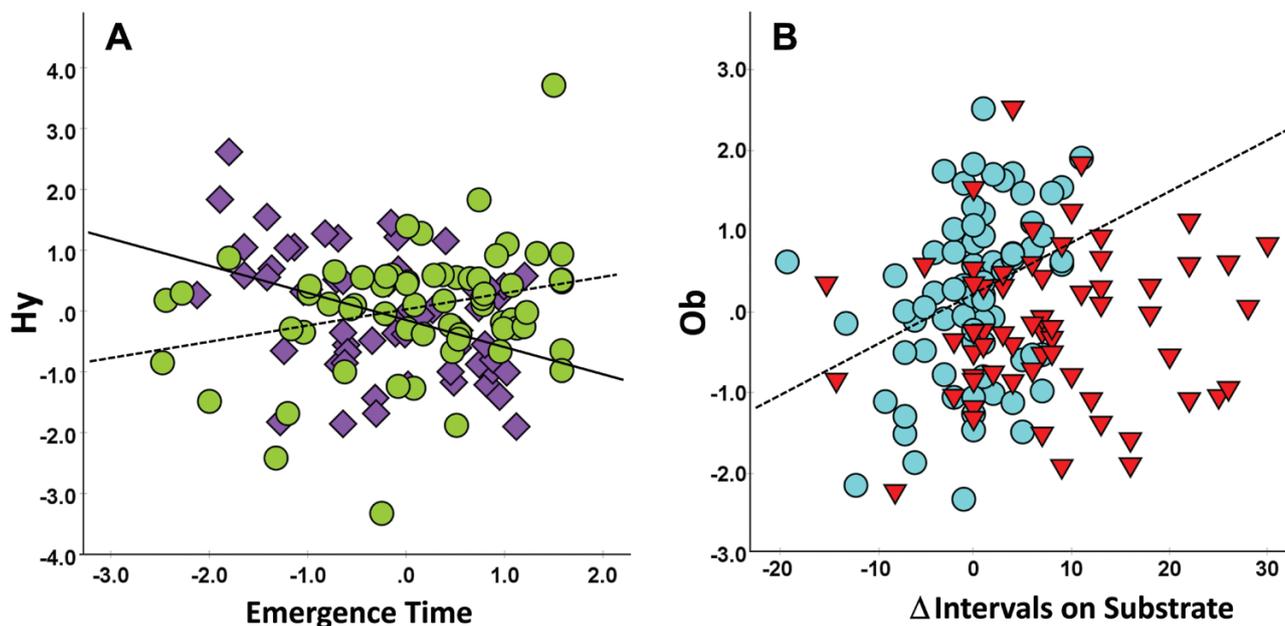
Figure 4. The standardized values for (A) relative hypothalamic investment controlling for body size (Hy) versus $\log_{10}$ transformed latency to emerge from shelter—HR (diamonds, solid line) and AR (circles, dashed line) subjects; and (B) relative olfactory investment (Ob) the change in the number of intervals spent stationary on the substrate, weakly responding (proactive) fish (circles, dashed line) and strongly responding (reactive) fish (triangles).

Table 3. Spearman Rho correlations between relative olfactory or hypothalamic investment versus the change in vertical area use (VAU), activity level (ACT), and time spent stationary (TS). Pearson r correlations with antipredator response intensity (ARI), in dace exposed to (A) ambient risk or (B) heightened-risk treatments.

Risk treatment	Stimulus	N	ΔVAU	ΔACT	ΔTS	ARI
(A) Ambient risk *Olfactory bulbs*	DW	12	0.049	0.294	0.182	0.148
	0.001%	16	−0.224	0.041	0.08	−0.181
	0.01%	14	0.367	−0.134	0.262	−0.89
	1%	15	0.664**	0.656**	−0.056	0.500*
	10%	15	−0.175	−0.286	0.324	−0.290
Hypothalami	DW	12	−0.273	0.049	00.211	−0.063
	0.001%	16	0.379	0.065	−0.058	0.105
	0.01%	14	0.367	−0.134	0.262	−0.193
	1%	15	−0.374	−0.411	0.375	−0.511*
	10%	15	0.068	−0.339	0.159	−0.101
(B) High risk *Olfactory bulbs*	DW	15	−0.143	−0.079	0.023	−0.091
	0.001%	15	0.546*	0.543*	0.115	0.658**
	0.01%	13	-0.154	-0.429	0.469	-0.382
	1%	13	−0.311	−0.313	0.111	−0.342
	10%	13	0.615*	0.385	0.152	0.554*
Hypothalami	DW	15	−0.236	−0.129	0.05	0.126
	0.001%	15	0.225	0.068	0.116	0.150
	0.01%	13	0.066	−0.176	0.017	−0.189
	1%	13	0.028	−0.143	−0.108	0.025
	10%	13	−0.203	0.137	−0.380	0.148

*$P < 0.05$, **$P < 0.01$.

environments, which tend to have lower basal and post-stress cortisol levels (Fürtbauer et al. 2015; Vindas et al. 2017a).

Contrary to previous findings (Joyce and Brown 2020b), the heightened-risk subjects reduced their hypothalamic investment, and investment was negatively correlated with the latency to emerge from a shelter when controlling for body size. An analogous effect has been seen in fathead minnows, where elevating perceived predation not only promoted boldness (propensity for risk-taking) but disrupted the correlation between boldness and body size (Meuthen et al. 2019). This may suggest a reduced role of the hypothalamus in the decision to emerge from the shelter or a decrease in cortisol output among the heightened-risk fish, which were bolder overall (Magnhagen and Borcherding 2008; do Carmo Silva et al. 2018). Conversely, it may suggest a heightened stress response in the ambient risk fish to the handling associated with the emergence test; influencing their subsequent behavior (Fürtbauer et al. 2015).

APR intensity was used as a second behavioral axis a proxy for a proactive/reactive response to risk (Mesquita et al. 2015); defined by the relative change in vertical position and activity level in response to a stimulus. The heightened-risk fish measurably reacted at the lowest cue concentration (0.001%), and significant correlations between olfactory bulb size and response intensity in the heightened-risk fish were seen only at the highest (10%) and lowest (0.001%) concentrations. Whereas in the ambient risk group, olfactory investment only correlated with response intensity significantly at the 1% concentration. This contrasts with the observation that fish from higher predation populations tend to exhibit a more graded response to perceived threats, while individuals from lower predation populations tend to react in a more hypersensitive or "all or nothing" manner (Brown et al. 2006). An explanation for this increased responsiveness from the heightened-risk group may be that repeated exposure to perceived predation pressure broadened the sensitivity range for olfaction in the heightened-risk group.

A corresponding result, also contrary to expectation, was that the correlations between the change in their vertical position and activity level with olfactory bulb size were positive. The weak APR (proactive) responders were found to invest more in their olfactory bulbs and exhibited the expected

positive graded between response intensity and olfactory bulb size (Figure 4B); this graded response to olfactory cues is more consistent with a proactive stress-coping style and/or bolder personalities. These results suggest that, for the proactive individuals, an increased olfactory investment may provide more nuanced olfactory information upon which to base decisions (Kermen et al. 2013); possibly owing to larger or more diverse populations of olfactory receptors (Bazáes et al. 2013).

Looking at both behavioral axes together, the heightened-risk group had an even distribution of behavioral phenotypes, while the ambient risk group had remarkably few early emerging/ weak APR fish. The threat of predation has previously been found to strengthen the association (behavioral syndrome) between boldness and aggressiveness (Bell and Sih 2007). Similarly, here the association between boldness and proactivity appears to have increased in response to predation risk (Figure 3A).

Our findings illustrate the potential power and pitfalls of relating gross brain morphology to complex behavior. The interactions between stress-coping style, predation pressure, and neuroplastic responses within individual brains have the potential to impact outcomes behavioral in ways that are difficult to predict. Future investigations examining the relationship between the gross morphology and behavioral responses of the teleost brain should consider how concurrent changes in sensory capacity and stress-coping style may contextualize behavioral variation.

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Conflict of Interest

The authors have no competing interests to declare.

Author Contributions

BJ designed and executed the experiment; BJ and GB wrote the manuscript; both contributed critically to the drafts and gave final approval for publication.

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