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RESEARCH ARTICLE

Simulated predator stimuli reduce brain cell proliferation in two electric fish species, *Brachyhypopomus gauderio* and *Apteronotus leptorhynchus*

Kent D. Dunlap[1,*], Geoffrey Keane[1], Michael Ragazzi[1,2], Elise Lasky[1] and Vielka L. Salazar[3]

ABSTRACT

The brain structure of many animals is influenced by their predators, but the cellular processes underlying this brain plasticity are not well understood. Previous studies showed that electric fish (*Brachyhypopomus occidentalis*) naturally exposed to high predator (*Rhamdia quelen*) density and tail injury had reduced brain cell proliferation compared with individuals facing few predators and those with intact tails. However, these field studies described only correlations between predator exposure and cell proliferation. Here, we used a congener *Brachyhypopomus gauderio* and another electric fish *Apteronotus leptorhynchus* to experimentally test the hypothesis that exposure to a predator stimulus and tail injury causes alterations in brain cell proliferation. To simulate predator exposure, we either amputated the tail followed by short-term (1 day) or long-term (17–18 days) recovery or repeatedly chased intact fish with a plastic rod over a 7 day period. We measured cell proliferation (PCNA + cell density) in the telencephalon and diencephalon, and plasma cortisol, which commonly mediates stress-induced changes in brain cell proliferation. In both species, either tail amputation or simulated predator chase decreased cell proliferation in the telencephalon in a manner resembling the effect of predators in the field. In *A. leptorhynchus*, cell proliferation decreased drastically in the short term after tail amputation and partially rebounded after long-term recovery. In *B. gauderio*, tail amputation elevated cortisol levels, but repeated chasing had no effect. In *A. leptorhynchus*, tail amputation elevated cortisol levels in the short term but not in the long term. Thus, predator stimuli can cause reductions in brain cell proliferation, but the role of cortisol is not clear.

KEY WORDS: Brain plasticity, Predation, Tail injury, Cortisol, Proliferating cell nuclear antigen

INTRODUCTION

Predators exert a potent influence on the brain of their prey (Gonda et al., 2013; van der Bijl and Kolm, 2016; Stankowich and Romero, 2017). For example, animals living among abundant predators commonly have brains that differ in size and shape from those living with few or no predators (Gonda et al., 2012; Walsh et al., 2016). However, in most cases, it is not clear how predators alter cellular processes that shape brain structures. The size and proportions of the brain depend on the relative abundance and distribution of brain cell proliferation and cell death. In field studies, we previously documented that brain cell proliferation in a free-living electric fish (*Brachyhypopomus occidentalis*) is inversely related to their predator exposure: fish living among a high density of predators (*Rhamdia quelen*) and experiencing high rates of tail injury show reduced levels of cell proliferation in telencephalic brain regions that participate in the behavioural response to predators (Dunlap et al., 2016). These field studies demonstrated a correlation between predator exposure, tail injury and brain cell proliferation, but it was uncertain whether interactions with predators indeed caused the inhibition of cell proliferation. Here, we tested these causal relationships through laboratory experiments in which we compared brain cell proliferation in intact, undisturbed fish with that in fish exposed experimentally to simulated predator exposure and tail amputation.

In many vertebrates, experimental exposure to predators, like other psychological stressors, causes an increase in glucocorticoids (e.g. cortisol or corticosterone) (Barcellos et al., 2007; Falconer and Galea, 2003; Tanapat et al., 2001). However, in field-active electric fish, we found no correlation between plasma cortisol levels and predator exposure or brain cell proliferation (Dunlap et al., 2016). These results suggested that either electric fish do not have a glucocorticoid response to predator stimuli or the time course of the response was not detected in our sampling scheme. To assess the glucocorticoid response to stress in electric fish and to better understand its possible role as a mediator of predator-induced changes in brain cell proliferation, we also measured plasma cortisol levels in fish experimentally exposed to simulated predator stimuli and tail amputation.

We investigated the effect of simulated predation on two species of gymnotiform electric fish, *Brachyhypopomus gauderio* and *Apteronotus leptorhynchus*. We chose to examine fish in the genus *Brachyhypopomus* to experimentally test hypotheses arising from our field studies. These field studies were conducted on *B. occidentalis*; however, this species is not readily available for laboratory studies, and so we instead examined the congener *B. gauderio*, which is available from captive laboratory populations. *Brachyhypopomus gauderio* is native to Uruguay, Paraguay and southern Brazil. Previous field studies have documented the effects of seasonality and social interactions on brain cell proliferation in this species (Dunlap et al., 2011), but little is known about predation pressures on this species in the wild.

We also examined *A. leptorhynchus*, which is distributed widely across tropical South America in Colombia, Venezuela, Peru and northern Brazil. This species is easily accessible commercially and is one of the most thoroughly studied teleost fish in terms of environmental influences on cell proliferation and its hormonal

[1]Department of Biology, Trinity College, Hartford, CT 06106, USA. [2]Department of Molecular Biology and Biophysics, University of Connecticut Health Center, Farmington, CT 06030, USA. [3]Department of Biology, Cape Breton University, Sydney, NS, Canada B1P 6L2.

*Author for correspondence (kent.dunlap@trincoll.edu)

K.D.D., 0000-0002-1153-7001

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control (Dunlap, 2016; Dunlap et al., 2013). However, nothing was known about predator or injury effects on brain cell proliferation. Because *A. leptorhynchus* is easily available commercially, our present study serves as a basis for future studies to address how predator stimuli interact with other environmental factors (e.g. social interactions) in an accessible experimental model.

MATERIALS AND METHODS

Animals and housing conditions

Adult (mean body length: 16.6±1.9 cm; range: 14.0–19.5 cm) and juvenile (mean body length: 9.04±0.78 cm; range: 7.1–10.6 cm) *Brachyhypopomus gauderio* Giora & Malabarba 2009 were obtained from laboratory breeding colonies (M. Kawasaki, University of Virginia; P. Stoddard, Florida International University) and adult *Apteronotus leptorhynchus* (Ellis 1912) (mean body length: 12.3 ±1.8 cm; range: 10.5–14.5 cm) were obtained commercially. All fish were housed individually in 38 l aquaria that were part of 1230 l circulating aquatics facility. Water conditions (27–28°C, pH 6.6–7.0) and lighting conditions (12 h:12 h light:dark) were held constant. Fish were fed commercial blackworms and brine shrimp. All fish were acclimated for at least 14 days under these conditions before experimentation. All housing conditions and procedures involving animals were approved by the Trinity College Institutional Animal Use and Care Committee.

Experimental tail amputation

To simulate predator-induced injury, we experimentally amputated the tails of both *B. gauderio* and *A. leptorhynchus*. Fish were placed under anaesthesia (0.5% 2-phenoxyphenol in aquarium water) and their body lengths were measured. The tails of experimental fish were then cut with a scalpel to remove the caudal 20% of their total body length. This degree of tail loss replicates the mean degree of tail loss in wild captured *Brachyhypopomus occidentalis* (Tran, 2014). (No data are available on tail injuries in wild *Apteronotus*.) Control fish were anaesthetized and handled similarly, but their tails were left intact. Fish were then returned to their home aquaria for recovery and tail regeneration. For *B. gauderio*, all fish, both juveniles (N=8 experimental fish and N=7 controls) and adults (N=6 experimental fish and N=6 controls), were killed 17 days post-amputation, when experimental fish had regenerated approximately 8% of their body length. This replicates the mean degree of regeneration found in wild captured *B. occidentalis* (Tran, 2014). For *A. leptorhynchus*, fish were killed 1 day post-amputation (28–29 h; short-term recovery, N=6) or 18 days post-amputation (long-term recovery, N=5), and control fish were left intact (N=6). To control for the effect of capture and anaesthesia, all fish were anaesthetized 24 h prior to being killed.

Simulated predator exposure

To simulate non-injurious predator stimuli, we experimentally exposed adult *B. gauderio* and *A. leptorhynchus* to repeated light taps on the tail. Control fish were left undisturbed. In each simulated predator encounter, the shelter tube in which the fish retreat during the day was removed, and the fish was lightly touched on the tail with a clear Plexiglas rod (39 cm long×1 cm diameter) once every 15 s over the course of 1 min. Thus, our simulated predator includes removal from shelter as well as chasing. Following the series of four taps on the tail, the shelter tube was returned to the tank. Such simulated predator exposures were repeated three times per day (separated by 2–3 h intervals) for 7 days. Thus, all together, fish were chased 21 times. To measure the effect of this simulated predator exposure on brain cell proliferation and plasma cortisol, we killed fish and collected the brain and blood 30 min after the last chase trial.

Blood and brain collection

At the conclusion of the experimental treatment, fish were anaesthetized (0.075% 2-phenoxyethanol) and, within 2 min of capture, bled from the caudal vein with a heparinized 25 G needle and syringe. Blood was stored at 4°C for several hours until centrifugation, and plasma was stored at −80°C until hormone assays.

After blood collection, the brain was dissected from the cranium, placed immediately in 4% paraformaldehyde for 90 min, rinsed in PBS (3×30 min), cryoprotected overnight in 25% sucrose, frozen in isopentane, and sectioned (30 µm) on a freezing microtome. The sections were mounted on slides and stored at −20°C until immunolabelling.

Immunohistochemistry

To identify proliferating cells, we labelled cells expressing proliferating cell nuclear antigen (PCNA) using an immunohistochemical procedure identical to that reported previously (Dunlap et al., 2016). Sections were treated in 2 mol l^{-1} HCl (30 min) at 37°C. All subsequent steps were carried out at room temperature. Sections were placed in 0.1 mol l^{-1} borate buffer (pH 8.5, 2×10 min), blocking solution (5% donkey serum, 0.3% Triton X-100, in PBS, 1 h), primary antibody (rabbit anti-PCNA, 1:50, overnight; FL-261, Santa Cruz Biotechnology, Dallas, TX, USA), secondary antibody (donkey anti-rabbit, 1:300 for 2 h; Jackson ImmunoResearch, West Grove, PA, USA) and then coverslipped.

To quantify the rate of cell proliferation, we estimated the density of PCNA+ cells in a 100 µm band at the periphery of the telencephalon (sections 30–36 in the *Apteronotus* brain atlas; Maler et al., 1991) unilaterally in three regions (dorsolateral, dorsomedial and ventral telencephalon) and in a 100 µm band surrounding the ventricle, the periventricular zone (PVZ), of the diencephalon (sections 17–19 of the *Apteronotus* brain atlas). The areas of each sampled region were estimated using NIH ImageJ (v. 4.0). The density of proliferating cells was calculated by dividing the number of PCNA+ cells by the area of the region and section thickness (30 µm).

Hormone assays

Plasma cortisol levels were quantified using enzyme immunoassays (Cortisol EIA kit, Cayman Chemical Co., Ann Arbor, MI, USA) with a detection limit of 1.2×10^{-2} ng ml^{-1}. Plasma samples were diluted 1:200 for *B. gauderio* and 1:100 for *A. leptorhynchus* to ensure that cortisol concentrations were within the detection range of the assay. For each species, all plasma samples were measured in triplicate using one 96-well plate. The intra-assay coefficient of variation was 4.78 for the samples from *B. gauderio* and 6.87 for the samples from *A. leptorhynchus*. Plates were developed at room temperature in the dark for 60–120 min, and read at 410 nm with an Eon microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT, USA). Plasma cortisol levels were calculated against an 8-point standard curve using the Gen5 v2.04 microplate reader and data analysis software (Biotek Instruments, Inc.).

Statistics

To determine the effect of predator stimuli on brain cell proliferation, we used two-way repeated measures ANOVA with treatment (amputated versus intact or chased versus undisturbed) as

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the independent variable, brain region (dorsolateral, dorsomedial, ventral telencephalon) as the repeated measure, and density of PCNA+ cells as the dependent variable. To identify differences among brain regions, we used Šidák's multiple comparisons as a *post hoc* test. In all cases, all telencephalic regions responded similarly to treatment (i.e. no significant brain region×treatment interaction; Table 2). We then calculated an overall proliferating cell density across the telencephalon, and repeated the analysis using the telencephalon and diencephalon as brain regions. To analyse cortisol measurements, we used ANOVA with predator treatment (tail amputation or simulated chase) as the independent variable and plasma cortisol level as the dependent variable.

Each species was run in separate experiments, and within *B. gauderio*, juveniles and adults were run in separate experiments. Thus, data from each species and age class were analysed in separate statistical tests. All statistical analysis was conducted with Prism 7.0 software. All data are expressed as means±s.e.m. $P<0.05$ was considered statistically significant.

RESULTS

Distribution of proliferating cells in two species of electric fish

Brachyhypopomus gauderio and *A. leptorhynchus* showed patterns of proliferating cells similar to each other and similar to those reported previously (Dunlap et al., 2011; Zupanc and Horschke, 1995). In the telencephalon, PCNA+ cells were concentrated at the lateral margins of each lobe, with the great majority (>95%) within the 100 µm band that we quantified. In the diencephalon, the majority of proliferating cells were in the lateral wall of the ventricle, the PVZ, but, compared with the telencephalon, a greater fraction of proliferating cells was distributed throughout the section. In both species, the three subdivisions of the telencephalon differed significantly: the ventral telencephalon had a greater density of proliferating cells than the dorsomedial and dorsolateral telencephalon, and the dorsomedial telencephalon had a greater density than the dorsolateral telencephalon (Tables 1 and 2). It is difficult to make direct comparisons between species and, within *B. gauderio*, between age classes as both species and age classes were run as separate experiments and analyses. Nevertheless, it appears that the density of PCNA+ cells in the ventral telencephalon is greater in *A. leptorhynchus* than in *B. gauderio*, and within *B. gauderio*, juvenile fish had proliferating cell densities 2–3 times greater than in adult fish in all telencephalic brain regions (Table 1).

Tail amputation and brain cell proliferation

In both species, tail amputation followed by long-term (17–18 days) recovery decreased the density of proliferating cells by about half in the telencephalon (Tables 1 and 2, Fig. 1A). The three telencephalic regions responded similarly to amputation, as indicated by the non-significant treatment×region interaction (Table 2). However, while the telencephalon responded significantly as a whole, the diencephalon showed no significant response to tail amputation (Table 1). That is, when including the telencephalon and diencephalon as repeated measures, there was a significant treatment×region interaction (*B. gauderio*: $F_{1,6}=13.2$, $P<0.005$); *A. leptorhynchus*: $F_{1,6}=10.6$, $P<0.01$), and *post hoc* tests showed that, in both species, there were significant effects of treatment on the telencephalon ($P<0.01$), but not on the diencephalon ($P>0.05$).

In *B. gauderio*, for which fish of different ages were available, juvenile and adult fish showed a similar percentage decrease in the density of proliferating cells in response to tail amputation; just as in adults, tail amputation in juveniles decreased cell proliferation by about half in the telencephalon (Table 1; no data were available for the diencephalon).

In *A. leptorhynchus*, brain cell proliferation was drastically lower after tail amputation followed by short-term (1 day) recovery than in the control, intact condition (Table 1, Fig. 1A). The effect occurred globally across all telencephalic and diencephalic regions examined, and there was no significant treatment×region interaction ($F_{2,17}=1.4$, $P>0.05$). In fish with long-term recovery (18 day), cell proliferation was also lower than in intact fish, but higher than in fish with short-term recovery. In addition, there was a significant treatment×region interaction ($F_{2,17}=5.8$, $P<0.05$), and *post hoc* tests showed that suppression of cell proliferation was found only in the telencephalon ($P<0.005$) and not in the diencephalon ($P>0.05$) (Tables 1 and 2).

Simulated predator exposure and brain cell proliferation

In both species, simulated exposure to a predator reduced telencephalic cell proliferation (Table 1, Fig. 2A). Quantitatively, the effect was similar to that of tail amputation, with chased fish showing proliferating cell densities that were about half those of undisturbed fish. In *B. gauderio*, the effect was region specific, with treatment reducing cell proliferation across the telencephalon but having no effect on the diencephalon (Tables 1 and 2). When including the diencephalon in the analysis, there was a significant treatment×region interaction ($F_{1,6}=12.3$, $P<0.005$), and *post hoc*

Table 1. Effect of stimulated predator treatments on brain cell proliferation in two species of electric fish: *Brachyhypopomus gauderio* and *Apteronotus leptorhynchus*

			Density of proliferating cells (PCNA+ cells mm^{-3})			
			Telencephalon			
Treatment	Species	Treatment group (*N*)	Dorso-lateral	Dorso-medial	Ventral	Diencephalon PVZ
Tail amputation	*B. gauderio* (adult)	Amputated[a] (5)	6523±1086*	7104±1977*	9063±1427*	9018±1062
		Intact (6)	11,727±1969	15,033±1147	17,405±2162	9233±751
	B. gauderio (juvenile)	Amputated[a] (8)	14,049±1675*	20,284±1160*	22,897±2887*	NA
		Intact (7)	28,445±2036	34,507±3390	38,373±5451	
	A. leptorhynchus	Amputated: ST[b] (6)	1548±270*	1444±385*	1532±334*	684±213*
		Amputated: LT[a] (5)	5941±558*	6709±1384*	7919±2315*	6073±1145
		Intact (6)	11,278±575	14,274±2695	25,325±2430	8636±1365
Chase	*B. gauderio*	Chased (6)	3847±929*	6277±1721*	8010±1938*	7220±641
		Undisturbed (7)	8430±641	10,340±1265	15,932±2108	7334±785
	A. leptorhynchus	Chased (7)	7344±1426*	10,387±853*	12,981±2954*	NA
		Undisturbed (6)	14,387±1642	18,834±2259	23,983±4388	

*Significantly different from intact or undisturbed controls. [a]17–18 day recovery period following amputation (LT, long term); [b]1 day recovery period following amputation (ST, short term). PVZ, periventricular zone; NA, not available.

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Table 2. ANOVA results for the effect of brain region, simulated predator treatment and their interaction on telencephalic cell proliferation

Treatment	Species	Effect	d.f (nominator, denominator)	*F*	*P*
Tail amputation	*B. gauderio* (adult)	Treatment	1, 9	12.0	**0.001**
		Brain region	2, 18	6.8	**0.006**
		Treatment×brain region	2, 18	0.6	0.527
	B. gauderio (juvenile)	Treatment	1, 13	56.4	**0.001**
		Brain region	2, 26	4.3	**0.02**
		Treatment×brain region	2, 26	<0.1	0.97
	A. leptorhynchus	Treatment	1	4.7	**0.001**
		Brain region	2	2.1	**0.032**
		Treatment×brain region	2	1.5	0.41
Chase	*B. gauderio*	Treatment	1, 11	11.4	**0.006**
		Brain region	2, 22	20.4	**0.001**
		Treatment×brain region	2, 22	2.8	0.078
	A. leptorhynchus	Treatment	1, 11	9.1	**0.001**
		Brain region	2, 22	9.8	**0.009**
		Treatment×brain region	2, 22	0.6	0.534

tests showed that there was a significant effect of treatment on the telencephalon ($P<0.01$), but no significant effect in the diencephalon ($P>0.05$).

Predator stimuli and cortisol levels

In *B. gauderio*, tail amputation followed by long-term recovery caused a significant increase in plasma cortisol (Fig. 1B). In *A. leptorhynchus*, tail amputation and short-term recovery also caused an elevation in plasma cortisol; however, plasma cortisol levels were not different from those of intact controls after long-term recovery. The decrease in cortisol levels observed between short-term and long-term recovery matches the time line of cell proliferation increase (Fig. 1). In *B. gauderio*, repeated chases by a simulated predator had no effect on plasma cortisol concentration (Fig. 2B).

Cortisol levels in intact, undisturbed laboratory *B. gauderio* (35–45 ng ml^{-1}) were similar or slightly higher than in intact field-captured *B. occidentalis* (~21–37 ng ml^{-1}) (Dunlap et al., 2016). Cortisol levels in control *A. leptorhynchus* in this study were also somewhat higher (8–9 ng ml^{-1}) than those in previous laboratory studies (1–5 ng ml^{-1}) (Dunlap et al., 2013).

DISCUSSION

Brachyhypopomus gauderio

We found that *B. gauderio* exposed in the laboratory to simulated predator stimuli (tail amputation and repeated chasing) showed reductions in telencephalic cell proliferation that were quantitatively and qualitatively similar to those of free-living *B. occidentalis* exposed to naturally occurring tail injuries and high predator densities (Tables 1 and 2, Figs 1A and 2A). These experimental results are consistent with the hypothesis that predators are significant causal agents in influencing brain cell dynamics of prey in the wild.

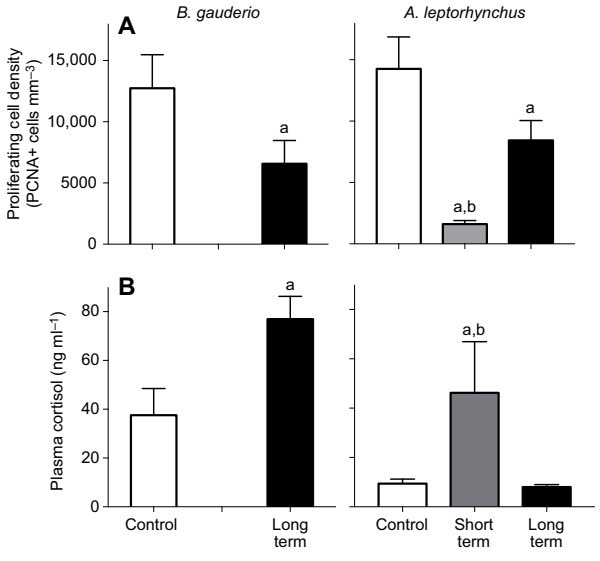

Fig. 1. Effect of tail amputation. *Brachyhypopomus gauderio* (left) and *Apteronotus leptorhynchus* (right) underwent tail amputation followed by short-term recovery (1 day, grey bar) or long-term recovery (17–18 days, black bar). Effects on (A) telencephalic cell proliferation and (B) plasma cortisol are shown. No short-term data were collected for *B. gauderio*. Sample sizes are presented in Table 1. [a]Significantly different from controls; [b]significantly different from long-term recovery.

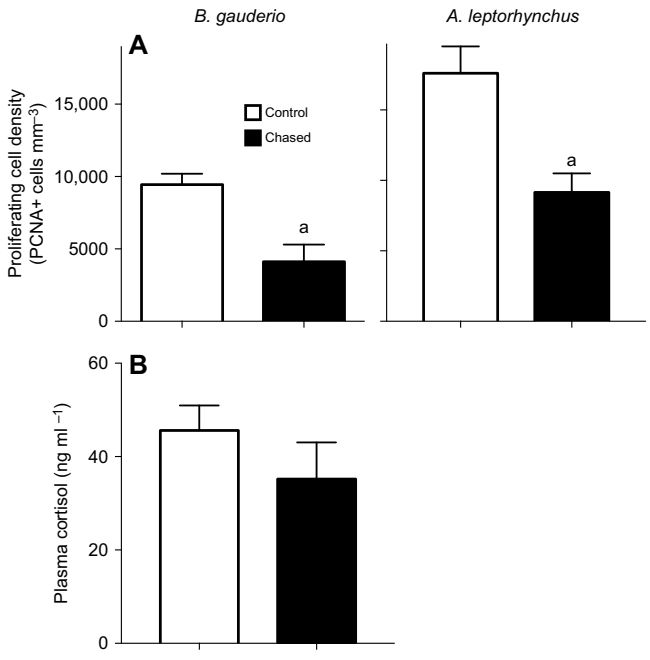

Fig. 2. Effect of simulated predator chase. *Brachyhypopomus gauderio* (left) and *A. leptorhynchus* (right column) were treated with repeated tail taps to simulate predator exposure. Effects on (A) telencephalic cell proliferation and (B) plasma cortisol are shown. Sample sizes are presented in Table 1. No cortisol data were collected for *A. leptorhynchus*. [a]Significantly different from controls.

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Comparison with field-collected *B. occidentalis*

In Panama, the incidence of tail injury in *B. occidentalis* varies considerably among populations, ranging from 12% to 46% of individuals showing injured tails (Dunlap et al., 2016). This injury rate correlates closely with the local density of a predatory catfish, *R. quelen*, a known predator of *B. occidentalis*. There is no evidence of intraspecific aggression causing tail injury in adults, and thus it appears that most naturally occurring tail injuries are attributable to predation attempts. On average, naturally injured fish had ∼20% of their original body length clipped from the tail and ∼8% of the body length had subsequently regenerated (Tran, 2014). In the field, injured fish, compared with intact fish, had lower proliferating cell densities in the telencephalon, with no difference in the diencephalon. When we experimentally mimicked the extent of tail injury and regeneration in *B. gauderio* by tail amputation followed by a 17 day recovery period, amputated fish had a similar region-specific decrease in cell proliferation rate in the telencephalon. This indicates that direct but sub-lethal contact with predators in the wild probably causes suppression of brain cell proliferation.

Interestingly, the effect of predators in the field appears to extend beyond direct injuries. Even among intact fish, those living in populations with high predator densities had lower rates of brain cell proliferation than those living among fewer predators (Dunlap et al., 2016). That is, cell proliferation rates also correlated with the abundance of predators, not just tail injury from predators. Thus, we hypothesized that chases and non-injurious exposure might also serve as stimuli that affect cell proliferation.

To simulate such non-injurious exposure, we removed captive *B. gauderio* from their shelter tube and repeatedly chased them with a plastic rod over a 7 day period. Such stimuli also caused a regionally specific decrease in telencephalic cell proliferation similar to that found in intact fish living in high predator streams (Table 1, Fig. 2A). We have no information on the frequency, duration or intensity of predator encounters in the wild and whether such encounters involve displacement from retreat sites, and so we cannot assess how well our manipulation mimics natural predation pressure. Nonetheless, our present laboratory experiment indicates that stressful pursuits without direct injury might plausibly inhibit brain plasticity of free-living fish.

The present experiments allow us to evaluate two alternative hypotheses explaining the negative correlation between brain cell proliferation and predation pressure in natural populations. First, it is possible that predators do not have a direct influence on cell proliferation, but rather have an indirect effect by reducing prey behaviours that otherwise promote cell proliferation. In many species, spatial learning and exploration behaviours tend to increase brain cell proliferation (LaDage et al., 2010; Barker et al., 2011; Opendak and Gould, 2015), and so predators may reduce brain cell proliferation in prey by altering these behaviours. However, in our experiments, fish exposed to predator stimuli had lower rates of cell proliferation than control fish even though they were housed in the same restricted, simple environment where they were limited to the same degree of spatial exploration. Thus, it appears that changes in behaviour are not required for predator-induced changes in brain cell proliferation.

Second, population differences could arise from genetic divergence among populations across evolutionary time rather than from plastic responses by individuals to predator exposure. However, here we found that predator-stimulated fish and control fish differed in cell proliferation rates even though these groups were formed randomly with respect to genetic background. Thus, phenotypic plasticity rather than genetic divergence is probably sufficient to explain population variation in the field.

Age class and brain cell proliferation

Experimental tail amputation reduced telencephalic cell proliferation by equivalent degrees (∼50%) in juvenile and adult fish (Table 1). However, given that juveniles are likely to have overall fewer brain cells (Zupanc and Horschke, 1995) and a greater baseline density of proliferating cells (Table 1), a far greater proportion of their brain cells are proliferating at any moment. Thus, an equivalent fractional decrease in proliferation rate in response to tail injury probably has a greater impact on the juvenile brain than the adult brain. In future studies, it will be important to examine the functional consequences of this apparent age-related effect and whether tail injury at the juvenile stage has lasting effects on adult brain structure or cell dynamics.

Apteronotus leptorhynchus

Species comparison

Similar to *B. gauderio*, *A. leptorhynchus* showed suppression of brain cell proliferation in response to tail amputation with long-term recovery and to simulated predator chase (Figs 1A and 2A). Moreover, for tail amputation, the effect occurred in all examined regions of the telencephalon, but not in the diencephalon (Table 1; the diencephalic response to chase was not examined in *A. leptorhynchus*). In most cases, the effect was quantitatively similar, with both tail amputation and chasing causing a reduction in the density of proliferating cells by about half. Thus, overall, future studies of predation and cell proliferation can reasonably examine *A. leptorhynchus* in the laboratory with the confidence that it mimics the response of free-living electric fish to natural predators.

Time course of response to tail amputation

Apteronotus was far more affected in the immediate aftermath of tail amputation than after a long recovery period. That is, fish that had recovered for only 1 day had an ∼85–95% lower proliferating cell density compared with intact fish while those that had recovered for 18 days had a ∼50% decrease (Fig. 2A). Moreover, in the short term, cell proliferation was reduced by amputation across both the telencephalon and diencephalon, whereas in the long term, the diencephalon of amputated fish had a similar level of cell proliferation to that of intact fish (Table 1). These results suggest that soon after tail amputation, cell proliferation is drastically reduced globally across the brain. Over the next 18 days, proliferation rates increased in a region-specific manner, with the diencephalon fully recovering to pre-injury levels and the telencephalon increasing to only half of pre-injury levels.

In the field, fish (*B. occidentalis*) with injured and partially regenerated tails have reduced brain cell proliferation, and we previously questioned whether this reduction was due to the injury itself or to the process of regeneration (Dunlap, 2016; Dunlap et al., 2016). Tail regeneration requires abundant cell proliferation, and if there is a trade-off between somatic and brain cell proliferation, tail regeneration may indirectly inhibit brain cell production. However, our present data on *A. leptorhynchus* indicate that injury quickly and drastically reduced brain cell proliferation and that proliferation rates increased in the brain during the period of tail regeneration. Thus, it appears that fish can elevate proliferation rates simultaneously in multiple body regions and are not required to differentially allocate proliferative 'energy' to different tissues. Moreover, the reduced cell proliferation in tail-injured fish in the wild probably results from the injury itself rather than the subsequent phase of regeneration.

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Cortisol and brain cell proliferation

In many vertebrates, including fish, the relationship between cortisol levels and brain cell proliferation or neurogenesis is complex (Koutmani and Karalis, 2015; Dunlap et al., 2016). Depending on the species and experimental treatment, cell proliferation can be positively correlated, negatively correlated or non-correlated with cortisol levels (Ebbesson and Braithwaite, 2012; Revest et al., 2009; Sørensen et al., 2013; Thomas et al., 2006). In electric fish (*A. leptorhynchus*), elevated endogenous cortisol levels associated with social interaction and exogenous cortisol treatment increase diencephalic cell proliferation (Dunlap et al., 2006, 2013), suggesting a positive relationship.

In laboratory *B. gauderio*, we found here that 1 week of daily experimental shelter removal and chasing had no effect on plasma cortisol levels despite its adverse effect on telencephalic cell proliferation (Fig. 2B). This is consistent with field studies of *B. occidentalis*, in which fish living in streams with high or low density of catfish predators had equivalent levels of cortisol (Dunlap et al., 2016). These combined laboratory and field studies suggest that basal glucocorticoid secretion in *Brachyhypopomus* is insensitive to the psychological stress of non-injurious exposure to predators. Alternatively, as shown in other studies, *B. gauderio* could have habituated to this repeated and predictable stressor by day 7 (Scott et al., 2008; Wong et al., 2008). By sampling 30 min after the last chase, we may have missed the onset of the cortisol response to chasing.

However, we found that *B. gauderio* with experimentally amputated tails and long-term recovery showed higher plasma cortisol levels than intact controls (Fig. 1B). This contrasts with field studies of *B. occidentalis*, in which fish with naturally occurring tail injuries and intact fish had similar cortisol levels (Dunlap et al., 2016). This discrepancy may reflect a species difference in the adrenocortical response to injury, an imperfect equivalence between natural injuries and experimental tail amputation, or a greater adrenocortical sensitivity to injury of fish in the laboratory compared with those in the field.

In *A. leptorhynchus*, we found elevated levels of plasma cortisol after short-term recovery from amputation during a period of greatly suppressed brain cell proliferation (Fig. 1B). Over the next 18 days, cortisol levels decreased corresponding to the period when brain cell proliferation is increasing. However, at day 18, when cell proliferation rate was still about half that of intact fish, cortisol levels were equivalent to those of intact fish. Thus, it appears that elevated cortisol in response to amputation is more transient in *A. leptorhynchus* than in *B. gauderio*. This species difference in cortisol response may arise from species differences in sociality. Our protocol, in which we both isolated fish and amputated their tails, may have had a more prolonged effect on *B. gauderio* because it is a more social species and thereby more stressed by isolation.

Conclusions

Our field studies and experimental results indicate that predators have a large influence on cell proliferation, one key determinant of brain morphology. Consistent with correlations described from natural populations, our laboratory experiments demonstrated that predator stimuli in the form of tail amputation and simulated predator chase decrease brain cell proliferation in two electric fish species. Future studies should expand on these findings to determine the effect of predators on overall brain size and relative proportions of brain components. However, these investigations will need to carefully examine the net balance of cell proliferation and cell death to clarify whether predators reduce the overall growth of the brain or only decrease the rate of cell turnover.

Our results on cortisol responses to predators are more ambiguous. Exposure to predators in the field and non-injurious simulated predator chase has little effect on cortisol levels, but tail amputation in the laboratory that mimics naturally occurring injury appears to increase cortisol levels. However, even the cortisol response to tail amputation differs between species and recovery phase.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.D.D., V.L.S.; Methodology: K.D.D., G.K., M.R., E.L., V.L.S.; Formal analysis: K.D.D.; Investigation: G.K., M.R., E.L., V.L.S.; Data curation: K.D.D.; Writing - original draft: K.D.D.; Writing - review & editing: K.D.D., G.K., M.R., E.L., V.L.S.; Supervision: K.D.D.; Project administration: K.D.D.; Funding acquisition: V.L.S.

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