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Exposure to predators does not lead to the evolution of larger brains in experimental populations of threespine stickleback

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Natural selection is often invoked to explain differences in brain size among vertebrates. However, the particular agents of selection that shape brain size variation remain obscure. Recent studies suggest that predators may select for larger brains because increased cognitive and sensory abilities allow prey to better elude predators. Yet, there is little direct evidence that exposure to predators causes the evolution of larger brains in prey species. We experimentally tested this prediction by exposing families of 1000–2000 F2 hybrid benthic-limnetic threespine stickleback to predators under naturalistic conditions, along with matched controls. After two generations of selection, we found that fish from the predator addition treatment had significantly smaller brains (specifically smaller telencephalons and optic lobes) than fish from the control treatment. After an additional generation of selection, we reared experimental fish in a common environment and found that this difference in brain size was maintained in the offspring of fish from the predator addition treatment. Our results provide direct experimental evidence that (a) predators can indeed drive the evolution of brain size—but not in the fashion commonly expected and (b) that the tools of experimental evolution can be used to the study the evolution of the vertebrate brain.

KEY WORDS: **Brain, experimental evolution, predation, stickleback.**

Brain size and structure varies greatly within and between vertebrate species (Kotrschal et al. 1998; Ullmann et al. 2010). While a great deal of brain size variation is due to differences in body size (with larger individuals having larger brains), vertebrate species also display impressive levels of diversity in the relative (body size-corrected) size of their brains (Kotrschal et al. 1998). Relative brain size is strongly associated with a large variety of fitness-related cognitive and sensory abilities (Krebs et al. 1989; Garamszegi and Eens 2004; Roth and Pravosudov 2009). However, being highly metabolically active, brain tissue is also involved in strong energetic trade-offs with other tissues (Raichle and Gusnard 2002). As such, the evolution of relative brain size is thought to be mediated by a balance between energetic constraints and natural selection for enhanced cognitive and sensory abilities (Isler and van Schaik 2009; Navarrete et al. 2011; Moran et al. 2015). This trade-off framework forms the basis of the modern evolutionary view of the vertebrate brain.

That said, we are only beginning to understand the specific agents of selection that shape brain size variation. For example, observational and correlative studies have shown that both relative brain size and structure are statistically associated with a wide variety of ecological variables. These include spatial complexity of the environment, water depth, predation, light environment, and the complexity of the social environment (Kotrschal et al. 1998; Irschick and Losos 1999; Safi and Dechmann 2005; Edelaar et al. 2008; Moran et al. 2015). These associations are compelling, but difficult to interpret because they only hint at the role of individual agents of selection. For example, a difference in aquatic light environment (e.g., cave vs surface) is typically correlated with many other ecological differences including primary production,

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prey species availability, predation regime etc. (Kotrschal et al. 1998; Gonda et al. 2013; Sylvester et al. 2013). Any of these variables may change the strength and direction of selection on particular cognitive functions and, in turn, the brain. Thus, if we wish to understand how the brain is directly or indirectly shaped by individual agents of selection, we must use experiments to isolate the effects of individual agents of selection. Of the ecological factors known to affect brain morphology, predation has the strongest body of evidence, and is highly amenable to experimental manipulation (Gonda et al. 2009; Kotrschal et al. 2015; Noreikiene et al. 2015).

PREDATORS AND BRAIN SIZE

Predators have been long thought to exert strong selection on brain size in prey species. This stems from the idea that the increased cognitive and sensory abilities afforded by a larger brain allow individuals to better avoid predators, increasing fitness (Møller and Erritzøe 2013). This hypothesis has both observational and experimental support in the literature, largely from studies of fish. For example, an analyses of 623 pairs of predator and prey species of fish found that on average, prey species tend to have larger brains than the species that predate them, perhaps suggesting a "cognitive arms race" (Kondoh 2010). Further, a number of natural history studies have shown that fish from high predation environments tend to have larger brains than fish from low predation environments (Kotrschal et al. 1998; Gonda et al. 2011). The best direct experimental evidence of the connection between increased relative brain size and predation comes from the work of Kotrschal et al. (Kotrschal et al. 2013, 2015). In their first study, these authors artificially selected lines of Trinidadian guppies (*Poecilia reticulata*) for either large or small brains (Kotrschal et al. 2013). The authors found that larger brained guppies performed better on basic cognitive tasks such as solving a simple maze (Kotrschal et al. 2013). In a later study, Kotrschal et al. exposed mixed groups of large-brained and small-brained guppies to predation in artificial tanks (Kotrschal et al. 2015). The authors reported that large brained individuals had 13.5% higher survival than their smaller brained counter parts, although the effect was limited to females. While not a study of intergenerational evolution per se, these results suggest that predation may exert a direct selective pressure on brain size via its contribution to cognitive performance.

That said there are reasons to believe that predation may actually favor the evolution of smaller brains. As previously mentioned, brain tissue has a high metabolic cost. Many anti-predatory strategies employed by prey species are either highly energetic (e.g., escape behaviors) or cause an overall reduction in foraging rate (e.g., vigilance or crypsis (Lima 1992). Thus, depending on the particular way in which a prey species deals with predation, predation may favor the evolution of reduced energy allocation to the brain and increased allocation to other tissues (e.g., swimming muscles, defensive structures, etc.). The idea that predation can favor the evolution of smaller brains is supported by a recent observational study on natural populations of Trinidadian killifish, *Rivulus hartii* (Walsh et al. 2016). These authors found that killifish from sites with high levels of predation had smaller brains than killifish from low-predation sites. Importantly, this pattern held when individuals from wild populations were reared in a common environment, suggesting a genetic basis for the brain size difference (Walsh et al. 2016).

While the majority of the literature has focused on the direct effects of consumptive predation on prey brain size, predators can also generate a plethora of indirect forms of selection. For example, prey often adjust how they use their habitats in response to the presence of predators–perhaps favoring covered areas over more open ones (Lima and Dill 1990). Prey may also alter their frequency of foraging, mating behaviors, and competitive interactions in response to predator presence (Velema et al. 2012). Finally, a reduction in prey size populations due to consumption can also have wide-ranging indirect effects on the ecology of prey habitats (e.g., increasing resource availability, Rudman et al. 2016).

However, while there have been many studies of the connection between brain size and predators, the use of artificially selected lines (Kotrschal et al. 2013, 2015), and an absence of multigenerational selection experiments makes it unclear how predators influence the evolution of brain size in natural systems. While studies to date have been suggestive, we will ultimately require detailed experimental evolutionary studies which, to our knowledge, have not yet been undertaken.

PLASTICITY, GENETIC VARIATION, AND CORRELATED CHARACTERS

Experimentally testing the role of predators in driving the evolution of relative brain size presents a number of challenges. First, brain size exhibits moderate levels of plasticity in most taxa, and thus experimental changes in brain phenotype need to be confirmed by rearing experimental animals in a common environment (Gonda et al. 2012b). Secondly, to observe an evolutionary response in a practical experimental timeline, there must be sufficient additive genetic variation in brain size within and among experimental families. Recent studies in various taxa have found that relative brain size has an additive, quantitative genetic basis akin to other morphological characters of similar complexity (Noreikiene et al. 2015). One way to maximize additive variation available to natural selection in the context of an experiment is to create F_2 hybrid families derived from F_1 crosses between individuals from phenotypically divergent (but interfertile) populations. This type of "variation inflation" approach has been widely applied in many experimental evolution and breeding studies (Kato and Wada 1999; Hawthorne and Via 2001; Wright and Stanton

2007; Arnegard et al. 2014). The F_2-hybrid (or more advanced generation hybrid) approach has the added benefit of breaking down linkage disequilibrium between co-occurring alleles, weakening linkage-mediated genetic correlations among traits and decreasing problems association with correlated selection (Arnold 1992; Sinervo and Svensson 2002; McGlothlin et al. 2005).

THE EXPERIMENTAL EVOLUTIONARY APPROACH

With the above challenges in mind, we set out to conduct a multigenerational experiment in which we subjected F_2-hybrid families of threespine stickleback to predators in semi-natural ponds and compared them to F_2-hybrids in control ponds without predation. These ponds (described in Arnegard et al. 2014) mimic the natural lake habitat of benthic and limnetic species pairs of threespine stickleback in British Columbia, Canada (Schluter and McPhail 1992). These species pairs are particularly useful for our purposes because they (1) have been adapted to differential levels of predation with benthics experiencing less predation by trout than limnetics (Vamosi 2005; Vamosi and Schluter 2007) and (2) vary in brain size–benthic-adapted stickleback generally having smaller telencephelons than limnetics (Park and Bell 2010). Further, benthics and limnetics are naturally interfertile (Peichel et al. 2001), allowing us to create hybrids with increased phenotypic variation and genetically decouple traits correlated with brain size (Hager et al. 2012; Noreikiene et al. 2015).

Using this experimental approach, we asked: does natural selection via exposure to a vertebrate predator result in the evolution of larger brains? To do this, we examined the size of the four major lobes of the stickleback brain: the olfactory and optic lobes, the telencephalon and the cerebellum. These regions have been shown to correlate with a variety of ecological variables in other systems (Kotrschal et al. 1998; Broglio et al. 2003; Hamdani and Døving 2007). Further, the size and shapes of these lobes have been linked to increased performance in predator avoidance and related cognitive tasks (Garamszegi and Eens 2004; Roth and Pravosudov 2009; van der Bijl et al. 2015).

We followed up our experimental evolution study with a "common garden" experiment to determine whether changes in brain size arising from exposure to a predator were the result of plasticity or heritable evolutionary change. This was done to account for the considerable plasticity in brain size among sticklebacks–particularly in response to predators (Gonda et al. 2012a; Walsh et al. 2016; Dunlap et al. 2016).

Methods

PONDS

The experiment was conducted in ten experimental ponds located at the University of British Columbia. These ponds harbor a naturally sourced assemblage of nonfish aquatic life (see Arnegard et al. 2014 for more details). Each pond has a shallow littoral zone with vegetation and an open water habitat (total size of 25 m × 15 m, with a maximum depth of 6 m). Prior to the introduction of experimental fish the ponds were paired based on macrophyte coverage, phytoplankton, zooplankton, and insect abundance.

COLLECTION

Benthic and limnetic threespine stickleback were collected from Paxton Lake on Texada Island, British Columbia, Canada and subsequently crossed in May 2011. Four wild-caught benthic females and four limnetic males were artificially crossed to produce four distinct F_1 families. These F_1 families were raised in 100 L tanks in the laboratory without predators from May 2011 to May 2012 (see Schluter 1993 for rearing protocol). A group of advanced generation benthic-limnetic hybrids (approximately 29 generations old) were also collected from First Lake on Texada Island in May 2012. These fish are the descendants of artificially generated multiple families of F_1 benthic-limnetic hybrids from Paxton Lake that were introduced to First Lake on Texada Island in 1981 (McPhail 1993) and thus represent approximately F_{30} hybrid intercrosses between Paxton benthics and limnetics. Pedigrees obtained from genotyping indicate that these fish generated multiple hybrid lineages (11–18 per pond). We had no a priori reason to treat these fish differently than fish derived from a cross, and thus considered them an additional replicate cross in all analyses presented herein.

TROUT EXPOSURE EXPERIMENT

In May 2012, each of the five benthic-limnetic hybrid families (four F_1 families and the First Lake hybrids) was split into a pair of ponds at the University of British Columbia's experimental pond facility (Fig. 1). Between 21 and 31 adult fish (depending on the size of the F_1 family) were introduced into each pair of ponds, with the same number of individuals used for each family. These fish bred over the spring and summer of 2012, which produced F_2 hybrid progeny (or F_{31} for the First Lake ponds). Censuses carried out before the experiment estimated these F_2 founding populations to be composed of 1746 fish ± 235 individuals per pond (Rudman et al. 2016). After the first round of breeding, one pond from each pair was randomly assigned to the predator treatment and the other was assigned the control treatment. In September 2012, two cutthroat trout (*Oncorhynchus clarkii*, 10–12 inches in length) from Placid Lake in the Malcolm Knapp Research Forest, BC, Canada were introduced to each predator treatment pond. After six months of predation pond populations with trout had an average population decline of 65% whereas control pond without trout predation had population declines of only 25% (Rudman et al. 2016).

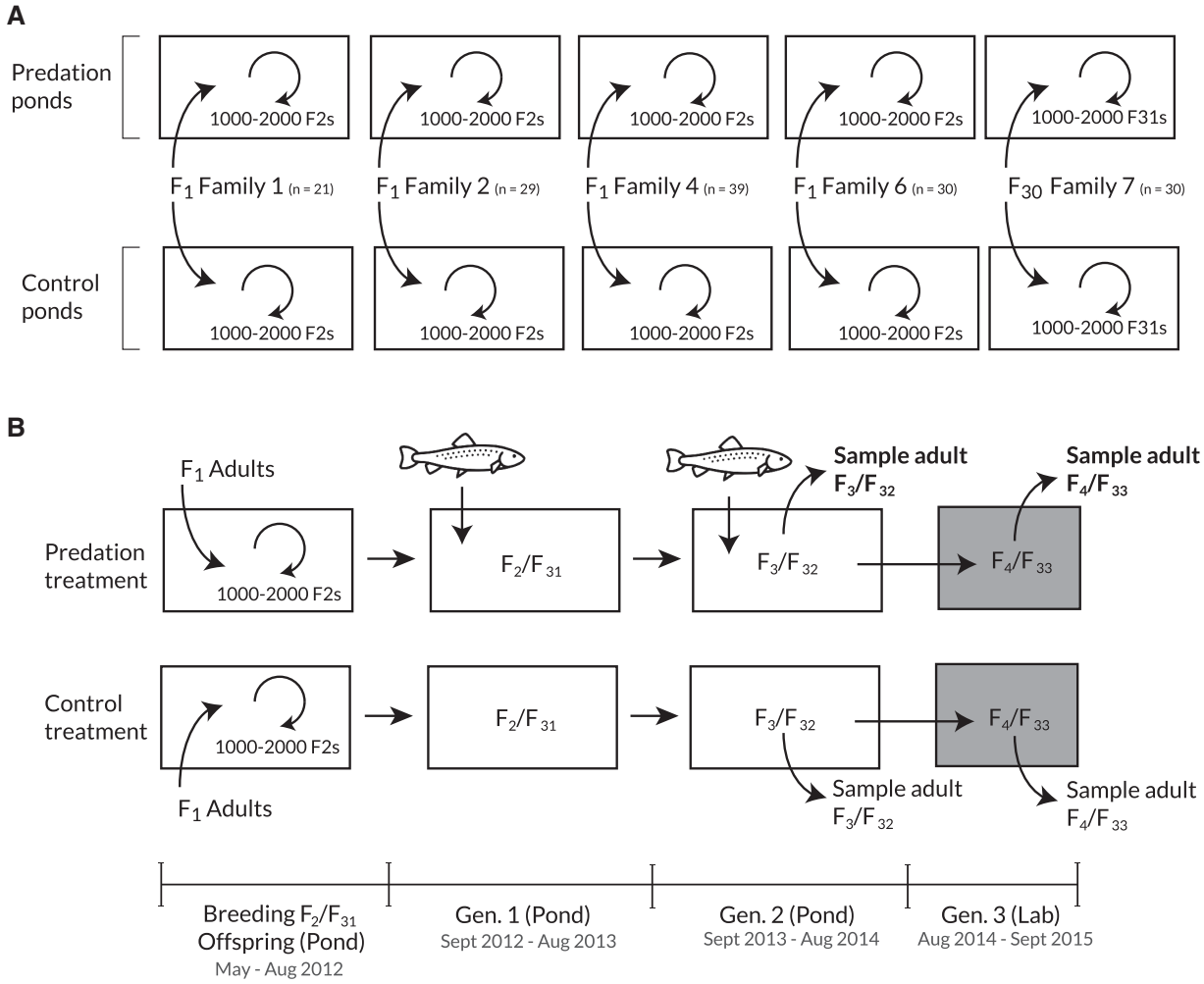

Figure 1. **The experimental design of the predation evolution experiment. (A) Five families of F_1 fish were each split into two ponds, with each pond receiving the indicated number of fish (e.g., $n = 21$ per pond for Family 1). These F1s were allowed to freely breed, resulting in a starting population of between 1000–2000 F_2 individuals (or F_{31}s in the case of the First Lake fish, see text for details). (B) Each pond in a pair received one of two treatments, predation or control. White rectangles represent ponds, and arrows between ponds represent the passage of a single generation. Gray rectangles represent laboratory tanks. Addition of trout (fish icon) and sampling of individuals is annotated where applicable. The time line depicts the number of generations since the experiment began.**

In May 2013, the F_2 and F_{31} fish reproduced to create a new post selection generation of F_3/F_{32} hybrids. In September 2013, a small subset of these juvenile offspring (500) were captured from each pond using a combination of unbaited minnow traps, open water seining, and dip netting. Two-hundred of these fish (100 per treatment, 20 per pond) were used for the brain phenotyping described below.

By September 2013, all the experimental trout had died, and were replaced with new trout. After collecting the F_3/F_{32} sample described above, three new trout were introduced into each of the predator addition ponds. The hybrid stickleback bred again in May 2014 creating F_4/F_{33} generations respectively, these offspring were used in the common garden experiment described below.

COMMON GARDEN EXPERIMENT

Between May and June 2014, one or two day-old F_4 and F_{33} fry were collected by snorkelers from the experimental ponds (See Fig. 1 for timeline) and reared to adulthood under laboratory conditions. These collections were done in lieu of full lab rearing (i.e., breeding adults in the lab and rearing their offspring) because the majority of adult fish in the experimental ponds were of low body condition by the end of the experiment and would have generated very small clutches or fail to reproduce at all. Thus, collecting 1–2 day-old fry maximized the number of individual fish we could rear in the laboratory. We employed a standard stickleback rearing regime (described in detail in (Schluter 1993). Briefly, fish were reared in groups of 10–15 individuals (2–3 tanks of 15 individuals per pond) in 100 L freshwater tanks kept at 16C on a 14:10

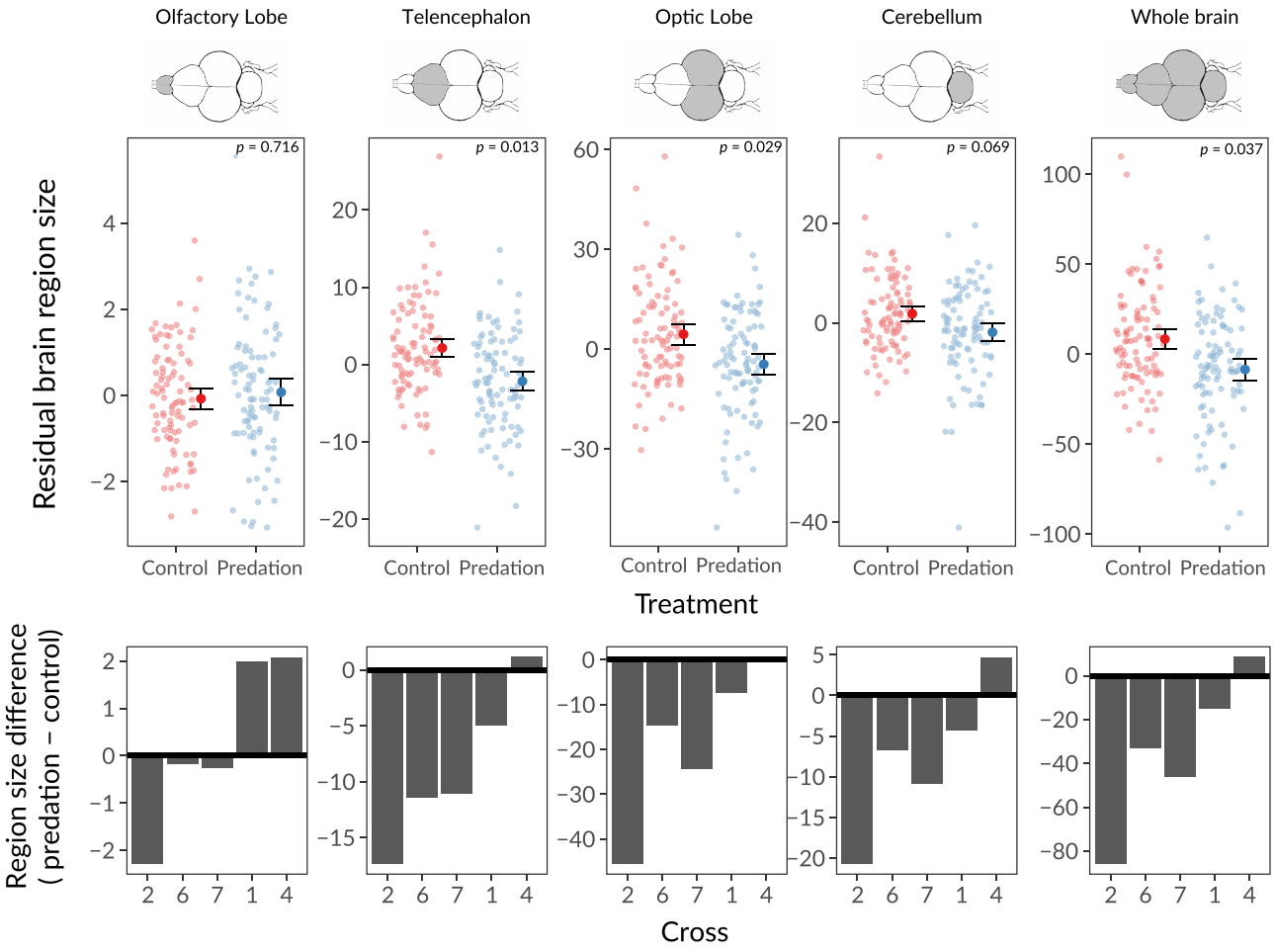

Figure 2. **Individuals exposed to predation in experimental ponds had smaller telencephalons, optic lobes, cerebellums, and overall brains compared to individuals from the control treatment. Panels depict the size of each brain region in the predation (red) and control (blue) treatments. Small, transparent points represent sex and size-corrected brain region sizes of individuals from all crosses. Large, solid points represent means; error bars represent 95% confidence intervals. Bar plots depict the difference in mean sex and size corrected brain size (predation minus control) within each individual cross, with negative values representing cases where the brain region was smaller in the predation treatment. The numbering of the crosses corresponds to Figure 1A.**

light cycle We initially fed fry chopped Hikari brand bloodworms (Hikari Corp. USA, thawed from frozen), eventually transitioning to full-sized bloodworms when fish had reached approximately 2 cm in length. A total of 300 F_4 fish were sacrificed when they had reached exactly 8 months of age (December or January 2015, depending on initial collection date) and phenotyped as described below.

DISSECTIONS AND MEASUREMENTS

We euthanized fish collected from the ponds in September 2013 (F_3) and the common garden experiment (F_4) with an overdose of buffered tricaine mesylate (MS-222) at a concentration of 0.5 mg/L. We then fixed each fish for 3–5 days in a 15 mL Falcon tube containing 10% phosphate buffered (pH 7.0) formalin, and then transferred them to 40% isopropyl alcohol.

We carried out measurements and dissections of the experimental pond fish during October and November 2015. Measurements and dissections for the common garden fish occurred in March 2016. We sexed the experimental pond fish using genotypic data (see Supporting Information for details), and the common garden fish by dissection of the gonads.

After sexing, we dissected brains from fixed specimens under a Lecia S8APO stereomicroscope using a scalpel, fine forceps (Roboz Super Fine #5 Dumonts), and precision scissors (Vannas Scissors, 8.5 cm, Curved, 7 mm Blades WPIInc). All dissections were performed blind with respect to treatment. To begin, we used a scalpel to score a medial incision along the dorsal surface of the skull of each fish, extending from the nose to the back of the head. We then scored two diagonal incisions extending from behind each eye to the back of the hyomandibular bone. We next used precision scissors to bisect the skull between the

Table 1. Results of significance tests (Wald chi-squared tests) for linear-mixed models fit to the experimental pond data.

Brain region	Model term	X^2_1	*P* value
Olfactory	**Standard length**	**11.59**	**6.6×10^{-4}**
	Sex	1.85	0.17
	Treatment	0.06	0.80
	Standard length: Treatment	0.03	0.86
	Sex: Treatment	0.08	0.78
Telencephalon	**Standard length**	**111.18**	**5.38×10^{-26}**
	Sex	**16.05**	**6.17×10^{-05}**
	Treatment	**12.36**	**4.4×10^{-4}**
	Standard length: Treatment	3.51	0.061
	Sex: Treatment	0.17	0.68
Optic Lobe	**Standard length**	**89.39**	**3.24×10^{-21}**
	Sex	3.09	0.079
	Treatment	**10.88**	**9.7×10^{-4}**
	Standard length: Treatment	**7.75**	**0.01**
	Sex: Treatment	0.03	0.87
Cerebellum	**Standard length**	**67.71**	**1.89×10^{-16}**
	Sex	1.39	0.24
	Treatment	3.78	0.051
	Standard length: Treatment	3.19	0.07
	Sex: Treatment	0.00	0.96
Total Size	**Standard length**	**94.99**	**1.91×10^{-22}**
	Sex	**4.35**	**0.037**
	Treatment	**8.51**	**0.0035**
	Standard length: Treatment	**5.49**	**0.02**
	Sex: Treatment	0.03	0.86

Each of the four brain region and total size were analyzed separately (see text). Bold values indicate a significant effect ($\alpha = 0.05$). Cross and pond were modeled as nested random effects (intercepts, not shown). In cases where the interaction term(s) were not significant, the main effects were reestimated by fitting a model without interaction terms.

eyes posterior to the olfactory lobe. Using this cut and the scored incisions as a baseline, we then made further incremental cuts along the skull, gradually exposing the brain. After the brain was exposed, we severed the optic nerves and caudal section of the brain stem to free the brain from the skull.

After dissections, we transferred the brains to an agar plate containing 40% isopropyl alcohol for imaging. To hold the brains in place, we placed them in a shallow, triangular divot in the agar (Kotrschal et al. 2012). We then moved the agar plate to the imaging stage of a Lecia S8APO stereomicroscope with an integrated digital camera and imaged the dorsal view of the brain (the two-dimensional dorsal areas of brain lobes are strongly correlated with their overall mass and volume, Naslund 2014). We then used Image-J to measure the length and width of the olfactory, telencephalon, optic, and cerebellum lobes from the photographs (Fig. S2). All measurements were standardized against a metal ruler present in each image. We estimated the two-dimensional surface area of each lobe assuming each lobe was approximately ellipsoidal (i.e., area = pi $\times$ width $\times$ height, a 2D procedure similar to Gonda et al. 2009). Total brain size was estimated as the sum of all the surface areas of the individual lobes. If a brain was damaged in the process of dissection, or was otherwise unusual (e.g., poorly preserved), we excluded it from the dataset. The photographs were also initially blinded with respect to pond and treatment. In total, we measured 196 brains for the predator experiment and 232 brains for the common garden experiment (see Table S1).

STATISTICAL ANALYSES

All analyses were carried out using R version 3.2.4 (R Core Team 2015). To test the hypotheses that exposure to a predator causes the evolution of larger brains, we fit mixed effects linear models via REML using the R package *lme4* (Bates et al. 2015). In each model, standard length was included as a covariate in these models because it is known to scale positively with brain size (Kotrschal et al. 1998). We also included sex as a model term, as brain size is known to differ among the sexes in sticklebacks (Kotrschal et al. 2012; Samuk et al. 2014). We modeled family and pond (i.e., experimental block) as random effects to account

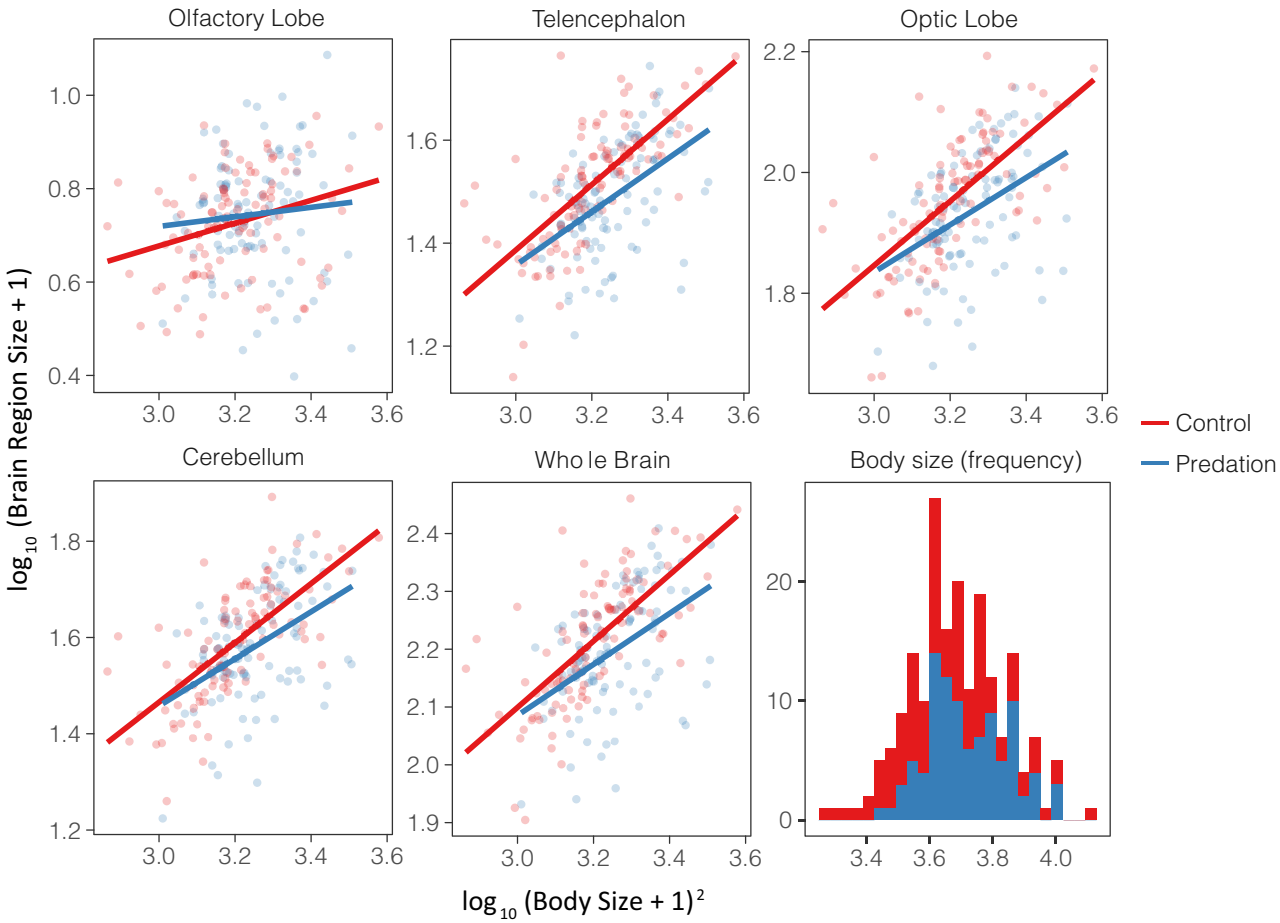

Figure 3. **Differences in body size (*x*-axis) and brain region size (*y*-axis) between the control and predation experimental treatments. Each panel depicts the relationship for a single brain region. The final panel (bottom right) depicts the distribution of body sizes for individuals drawn from each treatment (the two distributions are not significantly different, see text). Data are drawn from both males and females, as the treatment differ did not itself differ between sexes (see Figs. S3 and S4).**

for nonindependence due to shared environment and/or genetic relatedness. In the end, the models had the following form:

$$\begin{aligned}\text{Brain lobe area} = {} & \text{standard length} + \text{sex} + \text{treatment} + \text{sex} \\ & \times \text{treatment} + \text{standard length} \times \text{treatment} \\ & + \text{family (random effect)} + \text{pond (random effect)}\end{aligned}$$

We tested the significance of the model terms via Wald chi-squared tests implemented in the R package *car* (function "Anova," Fox and Weisberg 2011). In cases where the interaction term(s) were not significant, the main effects were reestimated by fitting a model without interaction terms (Engqvist 2005). We performed separate analyses for the experimental fish and the common garden fish. In the case of the common garden fish, only cross was included as a random effect (all fish were raised in the lab, thus "pond" is no longer a blocking factor). Note that the sizes of all the regions of the brain were correlated to some degree (Fig. S2), and thus the statistical results for each region are not entirely independent.

If body size and brain size scaled allometrically (e.g., smaller fish have relatively larger brains), selection on body size per se could indirectly generate a difference in relative brain size. In addition to our main analyses, we tested for the presence of brain-body allometry in our dataset using the function "slope.test" in the R package *smart* (Warton and Ormerod 2007). This function performs a one-sample test, testing whether slope of a line fit to the size of each region versus the square of standard length is significantly different from a value of one. Because region sizes are ellipsoidal areas, an isometric relationship between the square body size value would be indicated by a slope value of one. We analyzed each lobe and sex combination separately, and log transformed both lobe area and squared standard length prior to regression (Table S2). Finally, we directly tested whether body size differed among the experimental treatments using the same mixed model approach described above. All analysis code and raw data are available at https://github.com/ksamuk/sticklebrains

Table 2. Results of significance tests (Wald chi-squared tests) for linear-mixed models fit to the common garden data.

Brain region	Model term	X^2_1	*P*-value
Olfactory	**Standard length**	**41.33**	**1.28×10^{-10}**
	Sex	1.54	0.21
	Treatment	5.52	0.019
	Standard length: Treatment	0.29	0.59
	Sex: Treatment	0.53	0.47
Telencephalon	**Standard length**	**29.13**	**6.77×10^{-8}**
	Sex	**35.05**	**3.21×10^{-9}**
	Treatment	0.15	0.70
	Standard length: Treatment	**7.33**	**0.0067**
	Sex: Treatment	**4.14**	**0.041**
Optic Lobe	Standard length	38.88	4.51 × 10-10
	Sex	2.62	0.10
	Treatment	**32.40**	**1.25×10^{-08}**
	Standard length: Treatment	2.00	0.16
	Sex: Treatment	3.66	0.06
Cerebellum	**Standard length**	**48.50**	**3.29×10^{-12}**
	Sex	3.42	0.064
	Treatment	1.63	0.20
	Standard length: Treatment	0.70	0.40
	Sex: Treatment	2.24	0.13
Total size	**Standard length**	**46.80**	**7.86×10^{-12}**
	Sex	**8.61**	**0.0033**
	Treatment	**11.01**	**9.0×10^{-4}**
	Standard length: Treatment	2.81	0.09
	Sex: Treatment	3.97	0.05

Each of the four brain region and total size were analyzed separately (see text). Bold values indicate a significant effect ($\alpha = 0.05$). Cross was modeled as a random effect (intercept, not shown). In cases where the interaction term(s) were not significant, the main effects were reestimated by fitting a model without interaction terms.

Results

PREDATOR TREATMENT DID NOT RESULT IN THE EVOLUTION OF LARGER BRAINS

We found that two generations of experimental exposure to predators resulted in significantly *smaller* relative brain sizes in the predation treatment populations compared to the control populations (Fig. 2; Table 1). This appears to have been driven by a significant difference in the size of the telencephalon and optic lobes in the experimental fish (Fig. 2, upper panels). This difference in size was largely consistent across experimental replicates: although the magnitude of the effect varied, four out of five of the replicates fish in the predation treatments had smaller brains in the predator treatment than in the paired control treatment (Fig. 2, lower panels). This difference was also reflected in a significantly shallower slope between brain region size and body size for the fish from the predation treatment (Fig. 3, Table 1, Standard Length: Treatment effects). Interestingly, the difference between the predator and control treatments was not sex-specific (Fig. S3, Table 1).

SMALLER BRAINS WERE NOT A RESULT OF ALLOMETRIC OR BODY SIZE DIFFERENCES

We found no evidence of positive body size versus brain size allometry in the experimental fish (Supporting Information Table 1, Fig. 3). Further, there was no significant difference in body size between the predator-exposed treatment and control fish (Likelihood ratio test, $X^2_1 = 2.351$, $P = 0.127$, Fig. 3). These two findings might be a consequence of our samples being composed largely of individuals of same age class (juveniles in the pond experiment, adults in the common garden), thus limiting the scope for allometry and body size differences between treatments.

SMALLER BRAINS WERE MAINTAINED AFTER ONE GENERATION IN THE LABORATORY

When reared under constant laboratory conditions, offspring of fish from the experimental predator addition treatment maintained a significantly smaller overall brain size relative to offspring of control fish (Fig. 4). The magnitude of this difference was similar

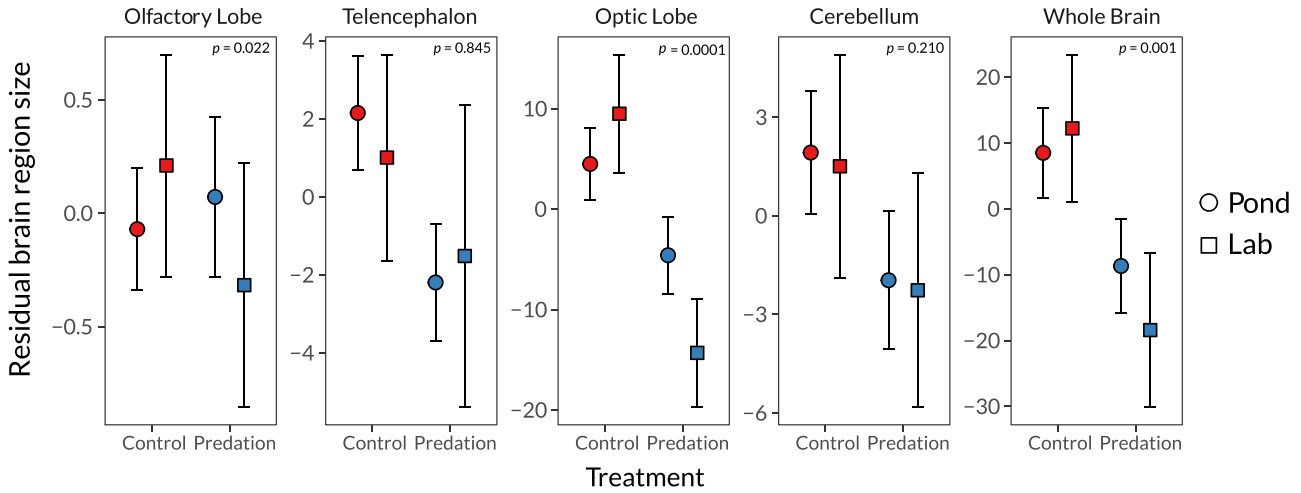

Figure 4. **Residual brain region size differences between the predation and control treatments shown for F_3/F_{32} pond-collected adults (circles) and lab-reared F_4/F_{33} adults (squares). To highlight the main effects of treatment, residuals were calculated by regressing raw region sizes on body size and sex, and extracting the resulting regression residuals (i.e., the models did not include any treatment effects or interactions). Colored points represent means for control (red) and predation (blue) groups. Error bars depict 95% confidence intervals. *P*-values correspond to the results of likelihood ratio tests comparing models with and without the treatment main effect and interactions (lab comparisons only).**

to that of the pond-reared fish. All the parts of the brain tended to be smaller in the lab-reared predator addition fish, with the optic lobe, telencephalon, and whole brain showing the strongest differences (Fig. 4, Table 2). There was no evidence that the effect of the predation treatment differed greatly between the sexes (Fig. S4, Table 2).

Discussion

In this study, we tested the hypothesis that natural selection generated by predators can drive the evolution of brain size in hybrid threespine sticklebacks. To do this, we conducted a selection experiment over two generations in which we exposed families of hybrid stickleback to a predatory cutthroat trout or a predator-free control. Contrary to the "predation-brain" hypothesis, we found that exposure to predators resulted in smaller relative brain sizes in the predator-exposed treatment compared to the control treatment. This difference in brain size was reflected across the major regions of the brain, with the telencephalon and optic lobe showing the strongest differences in fish exposed to predators. The difference in brain size we observed was consistent across all but one of the five experimental families and persisted when fish were reared in a common environment. All differences in brain size were independent of sex. Our results show that predator-mediated selection does not necessarily result in the evolution of larger brains, suggesting that "cognitive arms races" between predators and prey may not be a broadly applicable model for brain size evolution in fish.

THE UTILITY OF NATURALISTIC MULTIGENERATIONAL SELECTION EXPERIMENTS

To our knowledge, ours is the first study to perform a multigenerational selection experiment addressing the effects of predators on brain size under naturalistic conditions. The strength of this method is that it combines the ability to isolate the effects of a particular agent of selection (e.g., Kotrschal et al. 2015) and with an approach for assessing evolved, heritable difference in brain size (e.g., Gonda et al. 2012a). Further, by performing our experiment in naturalistic experimental ponds, predator-prey interactions were allowed to play out in a much more realistic setting compared to previous experiments. We believe this is of key importance, because the way in which predator-mediated selection shapes the evolution of the brain will likely be highly dependent on how particular predator and prey species interact (Lima 1992). Thus, we believe naturalistic multigenerational selection experiments provide a key way forward for the study of brain evolution.

LIMITS AND CONSIDERATIONS OF THE EXPERIMENTAL APPROACH

While our experimental approach allowed us to test the hypothesis that predator exposure results in the evolution of larger brains, it does not allow us to directly assess whether the difference in brain size we observed was due to a reduction in brain size in the predator treatment, an increase in brain size in the control treatment, or both. Resolving this would require brain measurements of the F_2 parents of the experimental fish–which could not be measured because they were required to breed the F_3 generation in the experimental ponds. Note that, even without this information,

our results still demonstrably show that experimental exposure to predators does not result in the evolution of larger brains as suggested by some previous studies. We also note that this problem is common to nearly every other study of predators and brain size, with the exception of Kotrschal et al. (2012). Future studies could aim to more directly resolve this via repeated temporal sampling, or by measuring a base-line generation (e.g., the F_1s) before initiating the experiment in earnest.

At first, it seems surprising that we observed an evolutionary change in brain size after two generations of selection. However, note that we designed our experiment specifically to maximize our ability to observe adaptive evolution: the use of F_2 and advanced generation hybrids greatly increased the genotypic (and likely phenotypic) variation within and between families, and the relatively small size of the ponds likely resulted in fairly strong predator-mediated selection. The experiment was thus primed for detecting adaptive evolution (Kawecki et al. 2012). Indeed, previous experiments using this approach were able to readily detect natural selection on a similar (or shorter) time scale (Barrett et al. 2008; Arnegard et al. 2014).

Even though this study provides experimental evidence that in threespine sticklebacks exposure to predators does not result in the evolution of larger brains, a key question is whether the change in brain size we observed was generated by direct selection on brain size or was the result of selection on correlated traits. Indeed, this represents a classic challenge to all studies aiming to identify the true agents of selection (Arnold 1992; Sinervo and Svensson 2002). Our analyses ruled out the most obvious of these correlations, namely that between brain and body size, as a factor (Fig. 3). Moreover, our use of F_2 hybrids is an improvement over prior studies, as it allows linkage-mediated trait correlations to be broken down to some degree prior to selection (although pleiotropy-mediated trait correlations would be unaffected (Sinervo and Svensson 2002). Thus, while we cannot completely rule out correlated selection, our study strongly suggests that predators can drive the evolution of smaller brains in threespine sticklebacks.

One important consideration for our study is that our common garden experiment was initiated with 1–3 day old fry. This was done to maximize sample size and ensure detection of subtle differences in brain size (see Methods). However, as a result, we cannot completely rule out that early-acting plasticity may have contributed to the differences in brain size between treatments. That said, the fish in our common garden experiment experienced a common, predator-free environment for the vast majority (237–239 out of 240 days) of their lives. Previous work has shown that the brains of wild adult sticklebacks brought into a lab environment rapidly converge on the size of life-long lab reared fish within one month (Park et al. 2012). Thus, if we assume plasticity was strong enough to generate the difference in brain size we observed, why was it also not strong enough to homogenize the treatment and control fish by the time of dissection? Overall, it seems rather unlikely that exposure to predator cues in the first two days could generate the brain size differences we observed in our experiment. That said, very little is known about "sensitive periods" in the ontogeny of the fish nervous system, and early-acting plasticity remains a possible alternative explanation.

PREDATORS AS DIRECT AND INDIRECT AGENTS OF SELECTION

Another interesting question is whether predators were the direct or indirect agents of natural selection in our experiment. For one, did any consumptive predation actually occur during the experiment? While we were not able to directly quantify predation, two observations suggest that consumptive predation indeed occurred in the trout-addition ponds. First, during upkeep of the experiment, we repeatedly observed trout hunting and eating stickleback. Secondly, the density of stickleback was consistently lower in the predation ponds and declined much more rapidly (Rudman et al. 2016). Thus, consumptive predation was likely occurring at substantial levels in the ponds as planned.

That said, in addition to causing direct predation, the addition of predators likely had a number of other indirect effects. For example, in the experimental ponds, predators indirectly caused a variety of ecological changes such as changes in zooplankton and phytoplankton biomass and these effects were shown to be independent of consumption (Rudman et al. 2016). It is true, however, that these indirect effects are typical of predators in natural populations (Schmitz 1998; Walsh and Reznick 2008; Duffy et al. 2011), and thus it would be reasonable to consider them as part of the total selective effect of predators in wild populations. Indeed, these indirect effects may be a large component of the total selection imposed by predators in many natural populations (Walsh and Reznick 2008). Disentangling predation's indirect effects from the effects of consumptive predation per se, would be a fruitful area for future work–ideally with a focus on to specific controls for each indirect effect.

PREDATOR-EXPOSURE AND COGNITIVE PERFORMANCE

Brain size in fish is closely connected to cognitive abilities, such as ability to solve a maze or learn a simple task (Kotrschal et al. 2013). Assuming that this was one of the targets of selection in our study, our results suggest that predator exposure may favor a decrease in cognitive abilities in stickleback. Interestingly, there is some support in the literature for this idea (but see DePasquale et al. 2014 and Dingemanse et al. 2007 for counter-examples). For example, threespine stickleback from high-predation populations learn spatial memory tasks much more slowly than their low predation counterparts (Brydges et al. 2008). This connection has

also been reported in a variety of others species – for example, populations of the freshwater fish *Brachyraphis episcopi* from high predation areas take longer to learn the location of food in a multi-patch environment compared to their low predation counterparts (Brown and Braithwaite 2004), and Trinidadian guppies from high predation populations are more likely to make quick and inaccurate decisions (Burns and Rodd 2008). These studies support the idea predation can drive a decreased ability to perform complex learning tasks. Intriguingly, there is some suggestion that the threat of predation, perhaps via increased stress, can actually affect the development of the brain itself. For example, a recent study (Dunlap et al. 2016) found that electric fish (*Brachyhypopomus occidentalis*) from high-predation areas have decreased rates of cellular growth in their forebrains during development.

PREDATION AND BRAIN SIZE: CONFLICTING RESULTS

As discussed, studies of predator-mediated brain size differences vary considerably in the magnitude, direction, heritability, and sex-bias of their reported effects. For example, Kotrschal et al. (2015) found that large-brained individuals had increased survival in the presence of predators, but this effect was limited to females. In contrast, Walsh et al. (2016) and Gonda et al. (2011) found that individuals from populations with high levels of predation had smaller brains, with the difference being largest for males. Brain-size plasticity seems to be universal, with many published studies reporting some degree of plastic response in brain size as a result of predator exposure (Gonda et al. 2012b). Interestingly, other than the present study, only Walsh et al. (2016) have reported heritable natural variation in brain size directly linked to the presence of predators.

Why is there so little agreement among existing studies? Differences in methodology are one likely candidate: studies have varied widely their use natural versus artificially selected lines, experimental design, focal species, and realism of their experimental setting (mesocosms, naturalistic ponds, natural streams, etc.). As such, harmonizing methodology among studies will likely decrease interstudy variation.

However, this variation–particularly with respect to focal species–may actually be hinting at a more important point about the effect of predator-exposure on brain size. The role of predators in driving brain size evolution is likely intimately connected to the nature of species-specific predator-prey interactions. For example, the core classes of anti-predatory responses in fish are (1) a change in habitat, (2) increased vigilance, (3) decreased overall activity and (4) temporal shifts in the time of activity (Lima and Dill 1990; Jakobsen et al. 1994; Wooster and Sih 1995; Goldenberg et al. 2014). A fish that changes habitat (e.g., from open to sheltered) in response to predation will naturally experience a completely different suite of cognitive and sensory challenges than a fish that responds to predation via increased vigilance. The scope of these responses will also be shaped by ecological conditions. For instance, a prey species in an open environment with little cover will be forced to employ different anti-predatory strategies than one in a cover-rich environment (Lima 1992).

In the case of our experiment, behavioral observations of fish from predator addition ponds showed that they spend less time shoaling with other fish, and are generally less bold and active (Miller et al. 2016). There is also some indication that fish in the predator addition ponds spent the majority of their time in cover–generally in patches of vegetation (Rennison, pers. obs.). Together, these behavioral observations suggest that fish in the predator addition ponds were possibly experiencing a much less enriched environment than fish in the control ponds. Sitting motionless in cover likely provides much less scope for cognitive challenges than swimming in the open water and interacting with conspecifics (e.g. Shultz and Dunbar 2006). It may have been the case that fish in our experiment that reallocated energy away from the brain and toward reproduction or feeding performed better in the "simplified" environment imposed upon them by predators. This behavioral response illustrates the importance of taking ecological and behavioral data into close account when forming hypotheses about how predators (or any agent of selection) shape the evolution of the brain.

Conclusion

Contrary to previous research, we found that experimental exposure to predators did not result in the evolution of larger brains. Our results suggest that the connection between brain size and the presence of predators may not be as simple as previously thought. Instead, the ecological and behavioral context in which predators and prey interact may ultimately dictate how the brains of prey species evolve in response to predators, if at all. Moving forward, we advocate that studies of brain size evolution integrate naturalistic experimental studies with detailed behavioral, ecological, and morphological data.

AUTHOR CONTRIBUTIONS

K.S. and D.R. conceived of the study. D.R. designed and carried out the experiment and collected the samples. K.S. and D.R. reared the common garden fish. J.X. carried out the brain dissections and brain imaging. K.S. performed the brain measurements. K.S. and J.X. performed the statistical analyses and prepared the figures. K.S. and J.X. wrote the article, with input from D.R.

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DATA ARCHIVING

Brain size raw data are available at https://datadryad.org//resource/doi:10.5061/dryad.dh3h417.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Dorsal view of a threespine stickleback brain magnified under a stereomicroscope.
Figure S2. Correlations among brain regions measured in the experimental fish.
Figure S3. Individuals exposed to predation in experimental ponds evolved smaller telencephala, optic lobes, and cerebellums compared to individuals from the control treatment (n = 196).
Figure S4. When reared in a common environment, offspring of experimental fish showed a reduction in brain size, but it was limited to the optic lobe (n = 183).
Figure S5. Individuals exposed to the predation pressure treatment show a decrease in telencephalon, optic lobe, and cerebellum size and no decrease in olfactory lobe size compared to individuals from the control treatment (n = 196).
Figure S6. Common garden individuals show a decrease in optic lobe size only and no decrease in olfactory, optic lobe, or cerebellum size when compared to individuals from the control treatment with no predation exposure (n = 183).
Table S1. Summary of total number of individual sticklebacks sampled in the pond and common garden experiments.
Table S2. Tests of allometric scaling between brain lobe size and square standard length for sticklebacks sampled from experimental ponds.