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Article

Brain differences in ecologically differentiated sticklebacks

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Abstract

Populations that have recently diverged offer a powerful model for studying evolution. Ecological differences are expected to generate divergent selection on multiple traits, including neurobiological ones. Animals must detect, process, and act on information from their surroundings and the form of this information can be highly dependent on the environment. We might expect different environments to generate divergent selection not only on the sensory organs, but also on the brain regions responsible for processing sensory information. Here, we test this hypothesis using recently evolved reproductively isolated species pairs of threespine stickleback fish *Gasterosteus aculeatus* that have well-described differences in many morphological and behavioral traits correlating with ecological differences. We use a state-of-the-art method, magnetic resonance imaging, to get accurate volumetric data for 2 sensory processing regions, the olfactory bulbs and optic tecta. We found a tight correlation between ecology and the size of these brain regions relative to total brain size in 2 lakes with intact species pairs. Limnetic fish, which rely heavily on vision, had relatively larger optic tecta and smaller olfactory bulbs compared with benthic fish, which utilize olfaction to a greater extent. Benthic fish also had larger total brain volumes relative to their body size compared with limnetic fish. These differences were erased in a collapsed species pair in Enos Lake where anthropogenic disturbance has led to intense hybridization. Together these data indicate that evolution of sensory processing regions can occur rapidly and independently.

Key words: brain evolution, divergent selection, magnetic resonance imaging, olfactory bulb, optic tectum, threespine stickleback.

Understanding how the brain evolves in response to selection remains a central goal of biological science. In the past, studies addressing this issue tended to focus on comparisons of diverse taxonomic groups (Finlay and Darlington 1995; Barton and Harvey 2000; Sol et al. 2005; Yopak et al. 2010; MacLean et al. 2014). Although the comparative method is powerful, such groups have lengthy and complicated evolutionary histories that may obscure signals of selection and it can be difficult to disentangle the influence of history from selection. An alternative approach is to compare recently diverged species or populations and determine how these differ along an ecological gradient (Healy et al. 1994, 1996; Pollen et al. 2007; Shumway 2008; Gonda et al. 2009; Roth and Pravosudov 2009; Schulz-Mirbach et al. 2016). The power of this approach comes from testing whether neurobiological traits vary in conjunction with environmental differences along a gradient. Selection is the likely cause especially when such a correlation occurs repeatedly in independently evolved populations, although this pattern can also arise from a convergent response to selection on correlated traits (Losos 2011; Riesch et al. 2013).

We use this approach here to test whether brain regions dedicated to processing visual and olfactory information have evolved in conjunction with the environment in threespine stickleback fish *Gasterosteus aculeatus*. Marine threespine sticklebacks colonized multiple lakes in British Columbia, and subsequently adapted to 2 distinct ecological niches, the open water pelagic (limnetic) and the in-shore littoral (benthic) that differ ecologically in many ways (Schluter and McPhail 1992; McPhail 1994). Similarity between same-type niches and parallel divergence of many traits (e.g., number of gill rakers, body size, nuptial coloration, and behavior) across these lakes suggests that divergent selection acts in a similar manner within each lake (Boughman et al. 2005). In the process of divergent adaptation, sticklebacks evolved into 2 species that are strongly reproductively isolated and exhibit numerous ecological, behavioral, and genetic differences, making them good species according to the Biological Species Concept (McPhail 1984; Schluter and McPhail 1992; Rundle et al. 2000; Boughman 2001; Gow et al. 2007). This divergence and speciation has occurred repeatedly and independently in several lakes within the last 12,000–15,000 years (McPhail 1994; Rundle et al. 2000; McKinnon and Rundle 2002).

The limnetic and benthic niches differ conspicuously in ways that suggest divergent selection on sensory systems. For example, the amount and quality of light, the amount of physical structure provided by vegetation and the substrate, and the prey and predators present all differ between the niches (Bentzen and McPhail 1984; Bentzen et al. 1984; McPhail 1984, 1994; Schluter and McPhail 1992; Boughman 2001; Vamosi 2002; Rafferty and Boughman 2006). The open water (limnetic) niche is brightly lit with relatively full spectrum light and little physical structure, and odors are likely to diffuse rapidly. Major prey are zooplankton and major predators are cutthroat trout and diving birds, which the sticklebacks detect by relying on vision. In contrast, the inshore littoral (benthic) niche is more dimly lit with spectrally shifted red light and has complex physical structure formed by logs, rocks, and vegetation of many types. Visibility is more limited than in the open water both by the quality of light and obstruction by structural elements, and odors are likely to diffuse more slowly and be easier to localize than in the open water. Major prey are macroinvertebrates found on or under the substrate or attached to plants and major predators include invertebrates, cutthroat trout, and birds; benthic fish likely use vision and olfaction to detect these prey and predators. Therefore, the limnetic niche should favor good vision and supporting this, eyes of limnetic fish are larger (McPhail 1984) and are more sensitive to red light than eyes of benthic fish (Boughman 2001). On the other hand, the benthic niche should favor good olfaction and supporting this, benthic fish are more sensitive to olfactory information, showing stronger discrimination of mates based on odor (Rafferty and Boughman 2006; Mobley et al. 2016) and stronger effects of imprinting due to odor exposure (Kozak et al. 2011).

We use a powerful method to quantify variation in brain regions that underpin these senses. To date, the most common method for measuring brain region size variation is estimating volume from photographs of the brain's exterior (e.g., Pollen et al. 2007; Gonda et al. 2009; Gonzalez-Voyer et al. 2009; Eifert et al. 2014; Schulz-Mirbach et al. 2016; Ahmed et al. 2017), which gives relatively coarse information and requires an assumption that the structure is ellipsoid, but it can be done easily and cheaply in large numbers. Histology is less frequently used (e.g., Kotrschal and Palzenberger 1992; Day et al. 2005), and although it can provide quite detailed information, it requires careful slicing, staining, and then imaging of the brain structures, and can be prone to distortions as the slices are mounted onto slides, making measurements of volume inaccurate. We used magnetic resonance imaging (MRI), an alternative technique that can visualize detailed neuroanatomical structure (including fiber tracts) of intact brains in 3 dimensions. MRI has been little used in studies of fish (Ullmann et al. 2010a, 2010b; DePasquale et al. 2016), perhaps because of its expense and the time required for segmentation and 3-D reconstruction. However, MRI produces comparable contrast-based results to traditional histological studies (Oelschläger et al. 2008; Ullmann et al. 2010a, 2010b) and has several additional advantages. For example, post-processing allows the size, shape, and stereotaxic location of specific brain structures to be reconstructed and measured precisely. Rehydration and staining methods are also hard to reproduce accurately from section to section in histological studies, whereas with MRI, the brain is intact (Simões et al. 2012). In addition, since the brain is already fixed prior to imaging, MRI still allows for future cytoarchitectural analysis through sectioning and staining specific regions for closer inspection.

Given the ecological differences between the limnetic and benthic niches, we predict that the neuroanatomical structures responsible for processing visual and olfactory information have responded to divergent selection in benthic and limnetic fish. Our first step in testing this hypothesis is to compare the relative size of the brain regions responsible for processing olfactory information (olfactory bulbs) and visual information (optic tecta) in the 2 species. Animals that rely more on vision are predicted to have larger optic tecta, whereas those that rely more on odor are predicted to have larger olfactory bulbs. This is in part because brain tissue is expensive and thus likely to show strong tradeoffs (Aiello and Wheeler 1995; Isler and van Schaik 2006). In addition to comparing relative size of brain regions, we also compare overall brain size between the species, which have been shown to differ in learning ability (Odling-Smee et al. 2008; Martinez et al. 2016; Keagy J, Boughman JW, unpublished data), and overall brain size has previously been shown to be positively correlated with habitat complexity (cichlids: Pollen et al. 2007; Shumway 2008, 2010; zebrafish, *Danio rerio*: DePasquale et al. 2016). Both Paxton and Priest Lakes have reproductively isolated limnetic and benthic species (McPhail 1994), and by including both we gain power to test the importance of the environment for brain evolution. We also include fish from Enos Lake, which historically had a pair of reproductively isolated species that showed substantial differences in behavior and ecological traits (McPhail 1984). However, recently they have hybridized extensively after anthropogenic disturbance (introduction of crayfish), resulting in the loss of morphological and genetic differentiation (Gow et al. 2006; Taylor et al. 2006; Cooper et al. 2011; Taylor and Piercey 2017), as well as loss of reproductive isolation (Lackey and Boughman 2013). We were interested to see whether this hybridization could have led to a decoupling between ecologically divergent characters such as body shape and brain morphology.

Materials and Methods

Specimen preparation

Each fish was euthanized using an overdose of anesthetic (MS-222, Tricaine Methane Sulfonate). We took photos to measure standard length using Image J v1.34i (National Institutes of Health, Washington, DC, USA). The fish were then rinsed with water, and fixed in a 10% neutral buffered formalin solution for at least 72 h. Prior to imaging, fish were immersed in a 2% Magnevist (Bayer HealthCare, Berlin, Germany) phosphate-buffered solution for 1 week and stored at 4 °C. Magnevist is a contrast agent allowing for faster imaging (DePasquale et al. 2016).

Imaging

After being immersed in Magnevist for 1 week, fish heads were cut off with a razor blade. Each head was put in a separate sample vial and packed in cotton wool soaked in Fluorinert Electronic Liquid FC-43 (3 M, Maplewood, MN, USA). To prevent the fish heads from drying out and to minimize magnetic susceptibility artifacts during scanning, the entire vial was filled with Fluorinert. In order to reduce artifacts in the images, air bubbles were carefully dislodged from the surface of the specimen by gently tapping the outside of the vial or by using tweezers. The vial top was wrapped with Parafilm and a small amount of Fluorinert was injected just prior to the vial being completely sealed to remove the rest of the air. All specimens were scanned at the High-Field MRI Facility in the Huck Institutes of the Life Sciences at Pennsylvania State University, in a vertical 14.1 tesla Agilent imaging system using a home built saddle coil. We used a standard 3-dimensional spin echo sequence with TE (echo time) = 9.05 ms and TR (repetition time) = 70 ms. Parameters such as the field of view, acquisition matrix, and scan time (1.01–4.91 h) were varied slightly to end up with an isotropic resolution of 40 µm for all samples.

Image post-processing

Matlab (The Math Works Inc., Natick, MA, USA) was used for post-processing. Data were zero-filled by a factor of 2 in each direction prior to application of a Fast Fourier Transform algorithm. This zero-filling has the effect of smoothing the image, resulting in an apparent 20 µm isotropic pixel resolution for each sample. The volume of olfactory bulbs, optic tecta, and whole brains was measured using 3-dimensional data visualization and reconstruction software (Avizo 8.1.1, FEI Visualization Sciences Group, Hillsboro, OR, USA). We first realigned brains, and then used the segmentation editor in which brain regions were manually outlined. We verified our segmentation using a variety of neuroanatomical references (primarily Wullimann et al. 1996). The olfactory bulbs and optic tecta were segmented every third slice (60 µm) in the sagittal perspective (out of 400–854 slices for the entire width of the head) and confirmed by careful inspection in the 2 other orthogonal views (transverse and horizontal). The posterior end of the olfactory bulb is very distinct and easily distinguished from the telencephalon. The anterior end of the olfactory bulb could be distinguished from the olfactory nerve because the nerve tended to be a different color and was narrow and approximately the same diameter along its entire length. For the optic tectum, we included the tectum opticum and periventricular gray zone (PGZ) of the optic tectum but not the torus longitudinalis (TL). In the middle of each lobe, a small number of nuclei in the diencephalon form a fingerlike border between the optic tectum and the diencephalon. Elsewhere, the PGZ is distinct and unmistakable making clear delineation simple. The whole brain was segmented every sixth slice (120 µm) in the transverse perspective (out of 1,000–1,230 slices for the entire length of the head) and again confirmed using the 2 other orthogonal views. The whole brain was defined as extending from the most rostral area of the olfactory bulb to the terminus of the rhombencephalic ventricle caudally. These boundaries were primarily used because they provided clear borders that were easily distinguishable and repeatable in each specimen. The whole brain boundaries were used successfully in a previous MRI study of zebrafish brains (DePasquale et al. 2016). Finally, we used Avizo to interpolate the volume between the measured slices and then calculate total volumes (in μm^3).

Specimen sources

Benthic fish from Priest and Paxton Lakes (Texada Island, British Columbia, Canada) were collected, euthanized, and fixed at the point of capture in April 2012. They were imaged in November 2012. Limnetic fish from Paxton Lake were collected in April 2012, brought back to Michigan State University for experiments, and then euthanized, fixed, and imaged in November 2012. Limnetic fish from Priest Lake were collected, euthanized, and fixed at the point of capture in April 2013 and imaged in November 2013. Fish were caught from Enos Lake (Vancouver Island, British Columbia, Canada) in April 2011 and classified according to whether they resembled benthic fish, limnetic fish, or a hybrid based on overall body shape (Taylor et al. 2006; Malek et al. 2012; Lackey and Boughman 2013). Only fish with unambiguous limnetic or benthic body shapes were used in this study. These fish were used in unrelated experiments and then euthanized, fixed, and imaged in November 2013. We scanned 2 brains from benthic(-like) and limnetic(-like) populations in Priest and Enos Lakes. We scanned 5 benthic and 2 limnetic fish from Paxton Lake (Table 1). We mostly used females to make comparisons more straightforward, because sexual dimorphism in brain size has been described in some populations of threespine sticklebacks from British Columbia (Samuk et al. 2014, but see Ahmed et al. 2017). However, we were unable to do this for Enos Lake limnetic fish, as they are now particularly rare in nature and the females are especially hard to collect, limiting access. In one population (Paxton Lake benthic), we did have male and female fish that could be directly compared.

Statistical analysis

All data analyzed can be found in Table 1. All statistical analyses were done in R v3.3.2 (R Core Team 2016). We were primarily interested in what the investment in different sensory regions was relative to the total brain size. Therefore, we first used 2 separate ANOVAs in which the ratio of sensory region to total brain size was predicted by lake, species, and the interaction between lake and species. We investigated differences between species within each lake using preplanned contrasts with the "contrast" package (Kuhn et al. 2016). Because of strong linear relationships between brain region volume and body size, we also ran separate models using the ratio of brain region size to body size (standard length). We examined the ratio of total brain size to body size using the same predictors. Finally, we repeated these analyses with Paxton Lake benthic fish including sex as a covariate to ask whether there was strong sexual dimorphism in this population and reran analyses with Paxton and Priest lakes including only females.

Table 1. Data used in analysis

Lake	Species	Sex	OB volume (mm^3)	OT volume (mm^3)	Total brain volume (mm^3)	SL (mm)
Paxton	Benthic	F	0.36768	6.21894	26.46347	53.06
Paxton	Benthic	F	0.32898	5.67898	23.59506	49.03
Paxton	Benthic	F	0.34339	8.71007	36.43300	68.18
Paxton	Benthic	M	0.28044	5.31150	21.51460	45.74
Paxton	Benthic	M	0.22390	5.69972	22.56650	55.45
Paxton	Limnetic	F	0.15652	5.13639	17.91072	44.41
Paxton	Limnetic	F	0.16563	5.67007	19.55784	49.99
Priest	Benthic	F	0.34750	6.20454	26.27415	51.29
Priest	Benthic	F	0.41974	8.03585	30.00989	64.72
Priest	Limnetic	F	0.16226	4.52910	14.57407	42.93
Priest	Limnetic	F	0.09770	3.74352	13.91006	44.86
Enos	Benthic	F	0.28524	8.86926	36.79705	64.72
Enos	Benthic	F	0.26285	8.39383	35.55666	70.03
Enos	Limnetic	M	0.25374	8.73868	34.94348	58.81
Enos	Limnetic	M	0.41594	11.21361	42.94004	63.81

Notes: This is the complete dataset analyzed in this study. OB, olfactory bulb; OT, optic tectum; SL, standard length.

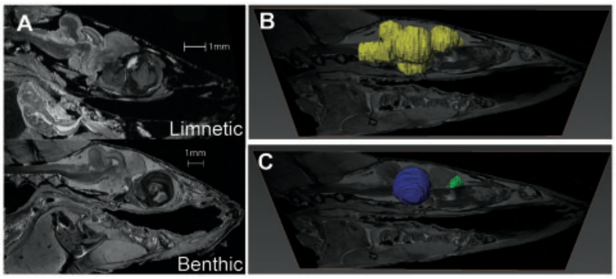

Figure 1. MRI of limnetic and benthic sticklebacks. (**A**) MRI images of a limnetic and benthic stickleback fish. (**B**) The whole brain volume is indicated in yellow against a slice along the midline. (**C**) Optic tectum (blue) and olfactory bulb (green) volumes are indicated against a slice long the midline.

Results

We show example MRI images in Figure 1 for limnetic and benthic sticklebacks. Olfactory bulbs made up a smaller proportion of brains for limnetic fish compared with benthic fish ($F_{1,9} = 6.15$, $P = 0.035$, Figure 2A). Pre-planned contrasts confirmed a significant difference between limnetic and benthic fish in Priest Lake ($t_9 = -2.43$, $P = 0.038$) and a nearly significant difference between limnetic and benthic fish in Paxton Lake ($t_9 = -2.21$, $P = 0.054$). However, this pattern was not maintained with limnetic-like and benthic-like fish from Enos Lake ($t_9 = 0.48$, $P = 0.64$). There was no significant interaction between lake and species ($F_{2,9} = 2.45$, $P = 0.14$). However, there was a suggestive trend toward an effect of lake ($F_{2,9} = 3.55$, $P = 0.073$) driven by the reduction in relative olfactory bulb size in benthic-like fish in Enos Lake compared with benthic fish in the other 2 lakes ($t_9 = -3.44$, $P = 0.007$).

The results were generally the same when we examined olfactory bulb size relative to body size; olfactory bulbs were relatively smaller in limnetic fish compared with benthic fish ($F_{1,9} = 8.55$, $P = 0.017$). There was no difference between lakes in average olfactory bulb size ($F_{1,9} = 0.00$, $P = 1.00$), but the interaction between lake and species was significant ($F_{1,9} = 6.20$, $P = 0.020$). This was caused by limnetic fish having relatively smaller olfactory bulbs than benthic fish in Paxton ($t_9 = -2.68$, $P = 0.025$) and Priest ($t_9 = -3.48$, $P = 0.007$) Lakes, but this difference being nonsignificant (and in the opposite direction) in Enos Lake ($t_9 = 1.28$, $P = 0.23$).

In contrast, the optic tecta made up a larger proportion of brains for limnetic fish compared with benthic fish ($F_{1,9} = 23.11$, $P = 0.009$, Figure 2B). Pre-planned contrasts confirmed that this pattern was driven by significant differences between limnetic and benthic fish in both Paxton ($t_9 = 4.03$, $P = 0.003$) and Priest ($t_9 = 2.82$, $P = 0.020$) Lakes. However, this pattern was not maintained with limnetic-like and benthic-like fish from Enos Lake ($t_9 = 1.27$, $P = 0.24$). There was no significant interaction between lake and species ($F_{2,9} = 1.34$, $P = 0.31$). There was a nonsignificant trend for an effect of lake ($F_{2,9} = 3.32$, $P = 0.083$) driven by the reduction in relative optic tecta size in limnetic-like fish in Enos Lake compared with limnetic fish in the other 2 lakes ($t_9 = -2.87$, $P = 0.018$).

The pattern was different when we examined optic tectum size relative to body size. There was a lake effect ($F_{1,9} = 13.12$, $P = 0.002$) driven by Enos Lake fish having larger optic tecta given their body size compared with fish from the other lakes (Enos vs. Paxton: $t_9 = 4.21$, $P = 0.002$, Enos vs. Priest: $t_9 = 4.75$, $P = 0.001$). Species did not consistently differ ($F_{1,9} = 0.03$, $P = 0.86$). However, the interaction between lake and species was significant ($F_{1,9} = 8.02$, $P = 0.010$); in Enos Lake, limnetic-like fish had larger optic tecta given their body size than benthic-like fish ($t_9 = 3.08$, $P = 0.013$), there was no species difference in Paxton Lake ($t_9 = -0.15$, $P = 0.88$), and in Priest lake, benthic fish had larger optic tecta given their body size than limnetic fish ($t_9 = -2.56$, $P = 0.030$). These patterns mirror differences in total brain size relative to body size described below and are therefore likely driven by a very strong positive relationship between optic tectum size and total brain size ($F_{1,13} = 294.90$, $R^2 = 0.96$, $P \ll 0.001$). Whereas olfactory bulb size has a weaker but still positive correlation with total brain size ($F_{1,13} = 12.98$, $R^2 = 0.46$, $P = 0.003$) and thus may evolve more independently.

Finally, we found that overall brain size relative to body size was greater in benthic fish compared with limnetic fish ($F_{1,9} = 5.76$, $P = 0.040$, Figure 2C). However, there was a significant interaction between lake and species ($F_{2,9} = 10.35$, $P = 0.004$). Pre-planned contrasts showed that this was due to benthic fish having relatively larger brains than limnetic fish in Paxton ($t_9 = -2.35$, $P = 0.044$) and Priest ($t_9 = -3.95$, $P = 0.003$) Lakes, but this pattern was reversed in Enos Lake ($t_9 = 2.31$, $P = 0.046$). There was also a

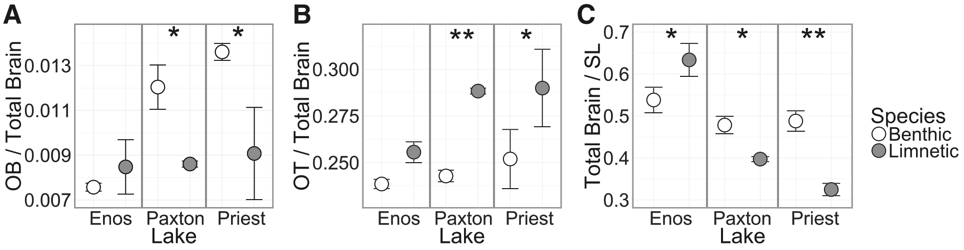

Figure 2. Differences in brain morphology between limnetic and benthic sticklebacks. (**A**) Ratio of olfactory bulb to total brain volume. (**B**) Ratio of optic tectum to total brain volume. (**C**) Ratio of total brain volume to standard length (mm^3/mm). Circles indicate means and error bars correspond to 1 standard error. Asterisks indicate significant differences between species within each lake (*≤ 0.05, **≤ 0.01).

significant effect of lake ($F_{2,9} = 21.75$, $P < 0.001$) driven by the Enos Lake limnetic-like fish having relatively larger brains than limnetic fish from Paxton and Priest Lakes ($t_9 = 7.62$, $P \ll 0.001$).

We primarily measured the brains of female fish. We had male and female samples for Paxton Lake benthic fish, but found no strong evidence of sexual dimorphism (Welch 2 sample *t*-tests; olfactory bulb/total brain: $t_{2.60} = 0.44$, $P = 0.70$; optic tectum/total brain: $t_{1.72} = -3.46$, $P = 0.09$; total brain/standard length: $t_{1.50} = 1.87$, $P = 0.24$). If we focus our analysis on female fish from Paxton and Priest lakes, we get qualitatively the same results as we do including the male Paxton Lake benthic fish; olfactory bulbs are relatively larger in benthic fish ($F_{1,5} = 8.48$, $P = 0.033$), optic tecta are relatively larger in limnetic fish ($F_{1,5} = 15.74$, $P = 0.011$), and brains are relatively larger in benthic fish ($F_{1,5} = 61.60$, $P = 0.001$).

Discussion

Limnetic and benthic fish from 2 lakes (Paxton and Priest Lakes) differ in their relative brain investment for olfaction and vision, consistent with specialization for different senses predicted from their ecology. It remains unclear what underlies the relative size differences (e.g., it could be changes in the amount of glia, the number of neurons, or the relative size of the neurons). However, our discovery of parallel patterns of evolutionary change lends credence to the hypothesis that stickleback brains have adapted to an environmental gradient and argues against drift as the evolutionary mechanism. Therefore, the rapid and repeated divergent local adaptation in ecological and behavioral traits that characterize threespine stickleback fish (Schluter and McPhail 1992; McKinnon and Rundle 2002; Boughman 2006) can be seen as extending to neuroanatomy.

The divergence we found between benthic and limnetic fish suggests local adaptation in the neuroarchitecture underpinning sensory systems. Specifically, larger olfactory bulbs in benthic fish likely enable a heightened sense of smell, facilitating detection of prey buried in the substrate, olfactory detection of hidden predators, and odor-mediated social behavior (Rafferty and Boughman 2006; Mobley et al. 2016). Larger optic tecta and larger eyes in limnetic fish likely facilitate zooplanktivory, visual detection of predators, and visually mediated social behavior (Bentzen and McPhail 1984; Boughman 2001). The optic tectum is also relatively large in cichlids that live in shallow water (Gonzalez-Voyer and Kolm 2010). Evolutionary change in the brain along a visual/olfactory axis might be mediated by patterns of early neural development (Sylvester et al. 2010, 2011). Our finding that benthic fish, which live in a spatially complex habitat, have relatively large olfactory bulbs contrasts with some studies in cichlids that have found a negative relationship between olfactory bulb size and habitat complexity (Pollen et al. 2007; Gonzalez-Voyer and Kolm 2010), although a similar positive relationship with habitat complexity has been found in ninespine sticklebacks *Pungitius pungitius* (Gonda et al. 2009). These discrepancies could arise for several reasons, including that they are very different fish families with different ecologies.

The total brain size differences between benthic and limnetic fish from Paxton and Priest Lakes are interesting because we know that benthic fish from these same lakes are better spatial learners (Odling-Smee et al. 2008; Martinez et al. 2016) and better at a delayed local enhancement task (Keagy J, Boughman JW, unpublished data) compared with limnetic fish. Habitat complexity, which is thought to select for differences in spatial learning, has been found to also correlate with brain size in cichlids (Pollen et al. 2007; Shumway 2008, 2010). While this raises the possibility that the difference in brain size is the result of selection on learning ability, this is speculative without a stronger test. In addition, attributing brain volume differences to complex behaviors or cognition is controversial and probably overly simplistic as it remains unclear what generates the increased size (Healy and Rowe 2007). Moreover, others have argued that connectivity and processing ability may be equally or more important (Chittka and Niven 2009). We agree it is wise to heed these cautions. Nonetheless, numerous studies show consistent differences in various cognitive tasks for large compared with small brained species or populations (Sol et al. 2005; Finarelli and Flynn 2009; MacLean et al. 2014; Kotrschal et al. 2015; Benson-Amram et al. 2016), suggesting that large brains may provide the substrate for heightened cognitive ability, even if this relationship is not straightforward. Another possible explanation for our data is that the much more narrow skull shape of limnetic fish constrains the total volume that is possible for their brains. A trade-off between skull morphology and brain size or morphology has previously been suggested for hominids (Stedman et al. 2004), carnivores (Wroe et al. 2005; Wroe and Milne 2007), and fish (Striedter and Northcutt 2006). Interestingly, our MRI images suggested that benthic brains often did not fill the entire skull cavity, suggesting that skull size is not always an important or primary constraint.

The patterns we found in Paxton and Priest Lakes were not maintained in Enos Lake, in which formerly reproductively isolated benthic and limnetic fish hybridized extensively shortly after the introduction of crayfish to that lake (Taylor et al. 2006; Taylor and Piercey 2017). Species differences were reduced for the optic tectum and olfactory bulb, and were reversed for total brain size. Smaller differences are unsurprising, given the mixing of genomes resulting from hybridization and that similar homogenization of phenotypes has been found for morphological traits including body shape and nuptial color (Taylor et al. 2006; Behm et al. 2010; Cooper et al. 2011; Malek et al. 2012). More surprising is the reversal for total brain size, where Enos Lake limnetic-like fish have larger brains than benthic-like fish. In addition, total brain size of Enos Lake fish appears to be slightly larger than either Priest or Paxton Lake fish. It is possible that these patterns with total brain size are due to the Enos Lake limnetic-like fish being male and most of the other fish tested being female. For example, sexual dimorphism in total brain size has been detected in other populations of threespine stickleback fish from British Columbia (Samuk et al. 2014, but see Ahmed et al. 2017), although we did not detect it in our Paxton Lake benthic fish.

Our study did have several limitations which warrant further study. Use of MRI is expensive and segmentation of images is time-intensive, limiting the number of populations we sampled and the number of individuals we sampled from each population. However, our sample sizes are similar to those using histological methods (e.g., Day et al. 2005). Indeed, it would be an interesting future direction to add either the one other remaining intact species pair (Little Quarry Lake) or benthic and limnetic populations from single-species lakes to further study adaptive specialization of sensory processing regions. Aiding this comparison would be the addition of marine fish which resemble the ancestors of benthic and limnetic fish. Another issue is that by sampling fish across multiple years with varying time in the laboratory, we may have introduced some noise into our data. For example, phenotypic plasticity could generate differences between individuals that were sampled differently. Indeed, plasticity in total brain size and in brain region size or shape has been demonstrated in fishes, including stickleback (Gonda et al. 2011; Park et al. 2012; Eifert et al. 2014; Herczeg et al. 2014). However, these sampling differences cannot explain the consistent differences seen in benthic and limnetic fish across 2 lakes with intact species pairs. Since plasticity may be an important contributor to variation within and between species in neuroanatomical features (Ebbesson and Braithwaite 2012; Park et al. 2012), we suggest that a critical future study will be to determine the degree of phenotypic plasticity in these brain regions for each of these populations as well as the marine sticklebacks that represent the ancestors to all freshwater populations.

Stickleback fish are increasingly being used to study brain and cognitive evolution, and several other studies have asked how different environments correlate with brain morphology. For example, comparing populations with varying habitat complexity has found small differences in telencephalon shape (Park and Bell 2010) and the size of other areas (Gonda et al. 2009, 2011; Ahmed et al. 2017), suggesting some degree of local adaptation, even though effect sizes were modest in most studies. Overall brain size differences have also been detected for populations of threespine (Samuk et al. 2014; Ahmed et al. 2017) and ninespine stickleback (Gonda et al. 2009, 2011). As with our study, the cause of these size differences requires further research. An exciting direction is to explore the genetic basis of these differences, using QTL mapping (Noreikiene et al. 2015) and transcriptomics (Keagy J, Hofmann H, Boughman JW, unpublished data).

Finally, there are 2 major competing hypotheses for evolution of differences in the size of brain regions. The "concerted" hypothesis proposes that developmental interdependencies constrain independent evolution of different brain regions; a brain region can only respond to selection if the entire brain changes (Finlay and Darlington 1995). Alternatively, the "mosaic" hypothesis suggests different brain regions can respond independently to selection (Barton and Harvey 2000). We do not have the data for a rigorous test of these hypotheses about brain evolution. However, several lines of our evidence argue against the "concerted" hypothesis. First, the "concerted" model cannot explain how limnetic fish, with smaller brains, could have larger optic tecta relative to their brain size than benthic fish. Second, the "concerted" hypothesis cannot explain how benthic fish would have relatively smaller optic tecta, but relatively larger olfactory bulbs than limnetic fish. There is independent evolution of brain regions in these divergent species, at least in part. Olfactory bulbs are evolutionary labile, show fast rates of evolution, and may evolve more independently than other regions (Gonzalez-Voyer et al. 2009; Yopak et al. 2010); thus, our findings on changes in olfactory bulb size are in line with this earlier work. Evolutionary changes in the optic tectum independent of other brain regions are less often found, and this may be because they are more interconnected with other regions (Whiting and Barton 2003; Gonzalez-Voyer et al. 2009). We also found that the optic tectum had a stronger scaling relationship with total brain size than olfactory bulbs. Numerous studies present data consistent with mosaic evolution of the brain by finding that brain size alone cannot explain variation among populations or species in size of brain regions and that ecological and/or social factors correlate with variation in size of particular brain regions (Barton and Harvey 2000; Iwaniuk et al. 2004; Healy and Rowe 2007; Gonzalez-Voyer et al. 2009). Moreover, quantitative genetics studies find low or non-existent phenotypic and genetic correlations between size of brain regions (Hager et al. 2012; Noreikiene et al. 2015). Thus, our data combined with these other studies suggest that the strong developmental constraints assumed by the concerted hypothesis do not severely limit independent evolution of brain regions.

In conclusion, we found evidence for parallel evolution of brain regions underlying different senses along an environmental gradient in 2 lakes that have reproductively isolated species pairs of stickleback fish (Paxton and Priest Lakes) and this evolutionary change has occurred fairly rapidly (since the last ice age). The repeated changes we see accord with predictions based on the species' differing ecology, providing evidence for adaptive change. These differences were erased in a lake where hybridization after anthropogenic disturbance has resulted in the loss of species distinctiveness. Our data also suggest that sensory regions of the brain can evolve in a mosaic fashion not entirely constrained to follow changes in other brain regions. To determine whether tradeoffs act in a constraining manner requires further work, however. Together these data indicate that evolution of differences in sensory processing regions can occur rapidly and opens the way for further work on sensory, cognitive, and brain evolution.

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Author Contributions

J.K., V.A.B., and J.W.B. conceived of the project idea and were involved in aspects of specimen collection and/or data generation. J.K. oversaw MRI segmentation and analyzed the data. J.K., V.A.B., and J.W.B. wrote and revised the manuscript.

References

Ahmed NI, Thompson C, Bolnick DI, Stuart YE, 2017. Brain morphology of the threespine stickleback *Gasterosteus aculeatus* varies inconsistently with respect to habitat complexity: a test of the clever foraging hypothesis. *Ecol Evol* 7:3372–3380.

Aiello LC, Wheeler P, 1995. The expensive-tissue hypothesis: the brain and the digestive system in human and primate evolution. *Curr Anthropol* **36**: 199–221.

Barton RA, Harvey PH, 2000. Mosaic evolution of brain structure in mammals. *Nature* **405**:1055–1058.

Behm JE, Ives AR, Boughman JW, 2010. Breakdown in postmating isolation and the collapse of a species pair through hybridization. *Am Nat* **175**: 11–26.

Benson-Amram S, Dantzer B, Stricker G, Swanson EM, Holekamp KE, 2016. Brain size predicts problem-solving ability in mammalian carnivores. *Proc Natl Acad Sci U S A* **113**:2532–2537.

Bentzen P, McPhail JD, 1984. Ecology and evolution of sympatric sticklebacks (*Gasterosteus*): specialization for alternative trophic niches in the Enos Lake species pair. *Can J Zool* **62**:2280–2286.

Bentzen P, Ridgway MS, McPhail JD, 1984. Ecology and evolution of sympatric sticklebacks (*Gasterosteus*): spatial segregation and seasonal habitat shifts in the Enos Lake species pair. *Can J Zool* **62**:2436–2439.

Boughman JW, 2001. Divergent sexual selection enhances reproductive isolation in sticklebacks. *Nature* **411**:944–948.

Boughman JW, Rundle HD, Schluter D, 2005. Parallel evolution of sexual isolation in sticklebacks. *Evolution* **59**:361–373.

Boughman JW, 2006. Divergence and speciation in sticklebacks. In: Ostlund-Nilsson S, Mayer I, Huntingford FA, editors. *The Biology of the Three-Spined Stickleback*. Boca Raton (FL): CRC Press, 83–126.

Chittka L, Niven J, 2009. Are bigger brains better? *Curr Biol* **19**: R995–R1008.

Cooper IA, Gilman RT, Boughman JW, 2011. Sexual dimorphism and speciation on two ecological coins: patterns from nature and theoretical predictions. *Evolution* **65**:2553–2571.

Day LB, Westcott DA, Olster DH, 2005. Evolution of bower complexity and cerebellum size in bowerbirds. *Brain Behav Evol* **66**:62–72.

DePasquale C, Neuberger T, Hirrlinger AM, Braithwaite VA, 2016. The influence of complex and threatening environments in early life on brain size and behaviour. *Proc R Soc Lond B* **283**:20152564–20152568.

Ebbesson LOE, Braithwaite VA, 2012. Environmental effects on fish neural plasticity and cognition. *J Fish Biol* **81**:2151–2174.

Eifert C, Farnworth M, Schulz-Mirbach T, Riesch R, Bierbach D et al., 2014. Brain size variation in extremophile fish: local adaptation versus phenotypic plasticity. *J Zool* **295**:143–153.

Finarelli JA, Flynn JJ, 2009. Brain-size evolution and sociality in Carnivora. *Proc Natl Acad Sci U S A* **106**:9345–9349.

Finlay BL, Darlington RB, 1995. Linked regularities in the development and evolution of mammalian brains. *Science* **268**:1578–1584.

Gonda A, Herczeg G, Merilä J, 2009. Adaptive brain size divergence in nine-spined sticklebacks *Pungitius pungitius*? *J Evol Biol* **22**:1721–1726.

Gonda A, Herczeg G, Merilä J, 2011. Population variation in brain size of nine-spined sticklebacks *Pungitius pungitius*: local adaptation or environmentally induced variation? *BMC Evol Biol* **11**:75.

Gonzalez-Voyer A, Kolm N, 2010. Sex, ecology and the brain: evolutionary correlates of brain structure volumes in Tanganyikan cichlids. *PLoS ONE* **5**:e14355.

Gonzalez-Voyer A, Winberg S, Kolm N, 2009. Brain structure evolution in a basal vertebrate clade: evidence from phylogenetic comparative analysis of cichlid fishes. *BMC Evol Biol* **9**:238.

Gow JL, Peichel CL, Taylor EB, 2006. Contrasting hybridization rates between sympatric three-spined sticklebacks highlight the fragility of reproductive barriers between evolutionarily young species. *Mol Ecol* **15**: 739–752.

Gow JL, Peichel CL, Taylor EB, 2007. Ecological selection against hybrids in natural populations of sympatric threespine sticklebacks. *J Evol Biol* **20**: 2173–2180.

Hager R, Lu L, Rosen GD, Williams RW, 2012. Genetic architecture supports mosaic brain evolution and independent brain-body size regulation. *Nat Commun* **3**:1079–1085.

Healy SD, Clayton NS, Krebs JR, 1994. Development of hippocampal specialisation in two species of tit (*Parus* spp.). *Behav Brain Res* **61**:23–28.

Healy SD, Gwinner E, Krebs JR, 1996. Hippocampal volume in migratory and non-migratory warblers: effects of age and experience. *Behav Brain Res* **81**: 61–68.

Healy SD, Rowe C, 2007. A critique of comparative studies of brain size. *Proc R Soc Lond B* **274**:453–464.

Herczeg G, Välimäki K, Gonda A, Merilä J, 2014. Evidence for sex-specific selection in brain: a case study of the nine-spined stickleback. *J Evol Biol* **27**: 1604–1612.

Isler K, van Schaik CP, 2006. Metabolic costs of brain size evolution. *Biol Lett* **2**:557–560.

Iwaniuk AN, Dean KM, Nelson JE, 2004. A mosaic pattern characterizes the evolution of the avian brain. *Proc R Soc Lond B* **271**:S148–S151. doi:10.1098/rsbl.2003.0127.

Kotrschal K, Palzenberger M, 1992. Neuroecology of cyprinids: comparative, quantitative histology reveals diverse brain patterns. *Environ Biol Fish* **33**: 135–152.

Kotrschal A, Corral-Lopez A, Amcoff M, Kolm N, 2015. A larger brain confers a benefit in a spatial mate search learning task in male guppies. *Behav Ecol* **26**:527–532.

Kozak GM, Head ML, Boughman JW, 2011. Sexual imprinting on ecologically divergent traits leads to sexual isolation in sticklebacks. *Proc R Soc Lond B* **278**:2604–2610.

Kuhn M, Weston S, Wing J, Forester J, Thaler T, 2016. *contrast: A Collection of Contrast Methods*. R package version 0.21 [cited 2017 December 7]. Available from: https://CRAN.R-project.org/package=contrast.

Lackey ACR, Boughman JW, 2013. Loss of sexual isolation in a hybridizing stickleback species pair. *Curr Zool* **59**:591–603.

Losos JB, 2011. Convergence, adaptation, and constraint. *Evolution* **65**: 1827–1840.

MacLean EL, Hare B, Nunn CL, Addessi E, Amici F et al., 2014. The evolution of self-control. *Proc Natl Acad Sci USA* **111**:E2140–E2148.

Malek TB, Boughman JW, Dworkin I, Peichel CL, 2012. Admixture mapping of male nuptial colour and body shape in a recently formed hybrid population of threespine stickleback. *Mol Ecol* **21**:5265–5279.

Martinez J, Keagy J, Wurst B, Fetzner W, Boughman JW, 2016. The relative roles of genes and rearing environment on the spatial cognitive ability of two sympatric species of threespine stickleback. *Evol Ecol Res* **17**:565–581.

McKinnon JS, Rundle HD, 2002. Speciation in nature: the threespine stickleback model systems. *Trends Ecol Evol* **17**:480–488.

McPhail JD, 1984. Ecology and evolution of sympatric sticklebacks (*Gasterosteus*): morphological and genetic evidence for a species pair in Enos Lake, British Columbia. *Can J Zool* **62**:1402–1408.

McPhail JD, 1994. Speciation and the evolution of reproductive isolation in the sticklebacks (*Gasterosteus*) of south-western British Columbia. In: Bell MA, Foster SA, editors. *The Evolutionary Biology of the Threespine Stickleback*. New York (NY): Oxford University Press, 399–437.

Mobley RB, Tillotson ML, Boughman JW, 2016. Olfactory perception of mates in ecologically divergent stickleback: population parallels and differences. *Evol Ecol Res* **17**:551–564.

Noreikiene K, Herczeg G, Gonda A, Balázs G, Husby A et al., 2015. Quantitative genetic analysis of brain size variation in sticklebacks: support for the mosaic model of brain evolution. *Proc R Soc Lond B* **282**:20151008.

Odling-Smee LC, Boughman JW, Braithwaite VA, 2008. Sympatric species of threespine stickleback differ in their performance in a spatial learning task. *Behav Ecol Sociobiol* **62**:1935–1945.

Oelschläger HHA, Haas-Rioth M, Fung C, Ridgway SH, Knauth M, 2008. Morphology and evolutionary biology of the dolphin (*Delphinus* sp.) brain: MR imaging and conventional histology. *Brain Behav Evol* **71**:68–86.

Park PJ, Bell MA, 2010. Variation of telencephalon morphology of the threespine stickleback *Gasterosteus aculeatus* in relation to inferred ecology. *J Evol Biol* **23**:1261–1277.

Park PJ, Chase I, Bell MA, 2012. Phenotypic plasticity of the threespine stickleback *Gasterosteus aculeatus* telencephalon in response to experience in captivity. *Curr Zool* **58**:189–210.

Pollen AA, Dobberfuhl AP, Scace J, Igulu MM, Renn SCP et al., 2007. Environmental complexity and social organization sculpt the brain in Lake Tanganyikan cichlid fish. *Brain Behav Evol* **70**:21–39.

Rafferty NE, Boughman JW, 2006. Olfactory mate recognition in a sympatric species pair of three-spined sticklebacks. *Behav Ecol* **17**:965–970.

R Core Team, 2016. *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing [cited 2017 December 7]. Available from: http://www.R-project.org/.

Riesch R, Plath M, Schlupp I, Tobler M, Langerhans BR, 2013. Colonisation of toxic environments drives predictable life-history evolution in livebearing fishes (*Poeciliidae*). *Ecol Lett* **17**:65–71.

Roth TC, Pravosudov VV, 2009. Hippocampal volumes and neuron numbers increase along a gradient of environmental harshness: a large-scale comparison. *Proc R Soc Lond B* **276**:401–405.

Rundle HD, Nagel L, Boughman JW, Schluter D, 2000. Natural selection and parallel speciation in sympatric sticklebacks. *Science* **287**:306–308.

Samuk K, Iritani D, Schluter D, 2014. Reversed brain size sexual dimorphism accompanies loss of parental care in white sticklebacks. *Ecol Evol* **4**: 3236–3243.

Schluter D, McPhail JD, 1992. Ecological character displacement and speciation in sticklebacks. *Am Nat* **140**:85–108.

Schulz-Mirbach T, Eifert C, Riesch R, Farnworth MS, Zimmer C et al., 2016. Toxic hydrogen sulphide shapes brain anatomy: a comparative study of sulphide-adapted ecotypes in the *Poecilia mexicana* complex. *J Zool* **300**: 163–176.

Shumway CA, 2008. Habitat complexity, brain, and behavior. *Brain Behav Evol* **72**:123–134.

Shumway CA, 2010. The evolution of complex brains and behaviors in African cichlid fishes. *Curr Zool* **56**:144–156.

Simões JM, Teles MC, Oliveira RF, Van der Linden A, Verhoye M, 2012. A three-dimensional stereotaxic MRI brain atlas of the cichlid fish *Oreochromis mossambicus*. *PLoS ONE* **7**:e44086.

Sol D, Duncan RP, Blackburn TM, Cassey P, Lefebvre L, 2005. Big brains, enhanced cognition, and response of birds to novel environments. *Proc Natl Acad Sci U S A* **102**:5460–5465.

Stedman HH, Kozyak BW, Nelson A, Thesier DM, Su LT et al., 2004. Myosin gene mutation correlates with anatomical changes in the human lineage. *Nature* **428**:415–418.

Striedter GF, Northcutt RG, 2006. Head size constrains forebrain development and evolution in ray-finned fishes. *Evol Dev* **8**:215–222.

Sylvester JB, Pottin K, Streelman JT, 2011. Integrated brain diversification along the early neuraxes. *Brain Behav Evol* **78**:237–247.

Sylvester JB, Rich CA, Loh Y-HE, van Staaden MJ, Fraser GJ et al., 2010. Brain diversity evolves via differences in patterning. *Proc Natl Acad Sci USA* **107**:9718–9723.

Taylor EB, Boughman JW, Groenenboom M, Sniatynski M, Schluter D et al., 2006. Speciation in reverse: morphological and genetic evidence of the collapse of a three-spined stickleback *Gasterosteus aculeatus* species pair. *Mol Ecol* **15**:343–355.

Taylor EB, Piercey RS, 2017. Going, going, gone: evidence for loss of an endemic species pair of threespine sticklebacks *Gasterosteus aculeatus* with implications for protection under species-at-risk legislation. *Conserv Genet*. doi:10.1007/s10592-017-1000-4.

Ullmann JFP, Cowin G, Collin SP, 2010a. Magnetic resonance microscopy of the barramundi *Lates calcarifer* brain. *J Morphol* **271**:1446–1456.

Ullmann JFP, Cowin G, Kurniawan ND, Collin SP, 2010b. A three-dimensional digital atlas of the zebrafish brain. *NeuroImage* **51**: 76–82.

Vamosi SM, 2002. Predation sharpens the adaptive peaks: survival trade-offs in sympatric sticklebacks. *Ann Zool Fennici* **39**:237–248.

Whiting BA, Barton RA, 2003. The evolution of the cortico-cerebellar complex in primates: anatomical connections predict patterns of correlated evolution. *J Hum Evol* **44**:3–10.

Wroe S, Milne N, 2007. Convergence and remarkably consistent constraint in the evolution of carnivore skull shape. *Evolution* **61**:1251–1260.

Wroe S, McHenry C, Thomason J, 2005. Bite club: comparative bite force in big biting mammals and the prediction of predatory behaviour in fossil taxa. *Proc R Soc B* **272**:619–625.

Wullimann MF, Rupp B, Reichert H, 1996. *Neuroanatomy of the Zebrafish Brain*. Basel: Birkhäuser Basel.

Yopak KE, Lisney TJ, Darlington RB, Collin SP, Montgomery JC et al., 2010. A conserved pattern of brain scaling from sharks to primates. *Proc Natl Acad Sci USA* **107**:12946–12951.