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# The relative roles of genes and rearing environment on the spatial cognitive ability of two sympatric species of threespine stickleback

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## ABSTRACT

**Background:** Recently diverged populations provide a powerful model for studying trait evolution. Benthic sticklebacks primarily occupy vegetated areas of lakes, a spatially complex environment. Limnetic sticklebacks primarily occupy open water in lakes, a spatially simple environment. In a T-maze spatial learning assay, wild-caught benthic sticklebacks perform better than wild-caught limnetic sticklebacks. It is not known whether this difference has a genetic basis and is thus the result of evolution or is instead a plastic response to the contrasting environments.

**Question:** To what extent are differences in the spatial cognitive ability of benthic and limnetic sticklebacks influenced by genetic differences, rearing environment, or the interaction between the two?

**Methods:** Using wild-caught limnetic and benthic fish from Paxton and Priest Lakes, we made pure-species crosses in the lab. We reared the fertilized eggs in spatially simple or spatially complex lab environments. We used a previously validated T-maze spatial learning assay to quantify the ability of adult fish from each rearing environment to learn an association between a visual landmark and a reward location.

**Results:** Lab-reared benthic fish learned the spatial task faster and made fewer errors than lab-reared limnetic fish, which supports a genetic basis underlying species differences in spatial learning ability. However, we found no significant differences between fish raised in different artificial environments.

*Keywords*: cognitive evolution, common garden, genetic basis, phenotypic plasticity, spatial learning, threespine stickleback.

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## INTRODUCTION

A substantial body of research demonstrates that animals evolve foraging, social, and mating behaviours in response to selection from distinct environments. Less well understood is how cognitive abilities evolve in response to distinct ecological and social environments (Bond *et al.*, 2003; Lyon, 2003; Dukas, 2004; Shettleworth, 2010; Croston *et al.*, 2015b). Despite decades of work on 'cognitive ecology,' animal behaviourists are still seeking answers to many fundamental questions. For example, heritable variation is critical for evolutionary change, yet little is known about the heritability of cognitive abilities and associated brain structures in natural populations (Croston *et al.*, 2015a). A related issue that is even less well studied is whether genetic differences contribute to observed differences in cognitive abilities between populations. In addition, it is now recognized that behavioural plasticity can have important effects on evolutionary processes (West-Eberhard, 2003; Snell-Rood, 2013). Therefore, it is important to try and quantify the relative role of genes and environment in determining cognitive performance (Buchanan *et al.*, 2013).

Enhanced cognition likely has large costs due to the expensive neurological tissue required (Aiello and Wheeler, 1995; Isler and van Schaik, 2006), and so is likely to be selected for only when there are large associated benefits. Recently diverged populations provide powerful models for studying trait evolution, including cognitive abilities. For example, populations of black-capped chickadees (*Poecile atricapillus*) from harsh environments outperform those from more benign environments on spatial learning tasks and have neurological differences in the hippocampus, the brain region implicated in spatial learning (Croston *et al.*, 2015b). However, population-level studies often lack true evolutionary replication, because of the opportunity for gene flow between populations living in similar, geographically nearby habitats. On the other hand, species that are highly divergent and no longer exchanging genes may differ in ways unrelated to the divergent selection factor of interest, creating additional noise in the data or leading to spurious associations. Threespine sticklebacks (*Gasterosteus aculeatus*) allow us to capitalize on the strength of the comparative approach and mitigate the above concerns because there are multiple independent replicates of the same recent evolutionary divergence.

Approximately 12,000 years ago, following retreat of the glaciers from British Columbia, populations of anadromous threespine sticklebacks became isolated in multiple lakes near the Strait of Georgia (Schluter and McPhail, 1992; McPhail, 1994; McKinnon and Rundle, 2002). A few lakes, including Paxton and Priest, had independent concurrent speciation events that gave rise to sympatric 'benthic' and 'limnetic' populations (Taylor and McPhail, 1999, 2000) that are reproductively isolated (Rundle *et al.*, 2000; Boughman *et al.*, 2005; Gow *et al.*, 2006) and thus considered good biological species under the Biological Species Concept. These repeated independent speciation events provide an excellent model for exploring the evolutionary processes that cause new species to diverge from each other (Schluter and McPhail, 1992) and provide statistically and genetically independent 'evolutionary experiments'. Benthic fish spend the majority of their lives foraging in the densely vegetated littoral zone, whereas limnetic fish spend the majority of their lives feeding in the open-water pelagic zone. Besides differences in habitat, benthic and limnetic fish differ in morphology and behaviour. For example, benthic fish are larger with downturned snouts and large facial musculature that allow for optimal foraging for insects in the substrate and attached to vegetation, whereas limnetic fish are smaller with slender snouts and many more gill rakers that aid in capturing plankton (Bentzen and McPhail, 1984; Schluter and McPhail, 1992; McGee *et al.*, 2013). In

this study, we leverage this model system to study questions related to the evolution of spatial cognition.

Spatial cognition is the means by which animals process information about their environment in order to navigate (Hughes and Blight, 1999). A previous study demonstrated that wild-caught benthic fish from Paxton and Priest Lakes outperform wild-caught limnetic fish from the same lakes in spatial learning trials (Odling-Smee *et al.*, 2008). Park (2013) surveyed a number of single-population lakes in Alaska and found that wild-caught benthic-like fish tended to learn faster than wild-caught limnetic-like fish. Another study compared the spatial learning abilities of wild-caught Scottish populations of sticklebacks from either rivers or ponds. The authors found that fish from ponds (where there is little water flow and likely long-term stability in visual landmarks) outperformed fish from rivers on visual cue association tasks (Girvan and Braithwaite, 1998). Thus, there is strong evidence that stickleback populations differ in spatial cognitive abilities and that these cognitive differences are associated with environmental differences. However, in all of these cases, we do not know the extent to which population differences in spatial cognitive abilities are the result of plastic responses to the different environments, or are genetically based and the result of evolution in response to divergent selection.

Previous studies on the importance of experience on spatial cognitive abilities in sticklebacks have been inconclusive. For example, lab-reared benthic-like fish from Corcoran Lake, Alaska learned more slowly than field-caught benthic-like fish from the same lake (Park, 2011), suggesting that experience can affect the spatial cognitive ability of sticklebacks. However, Park (2011) pointed out the possibility that wild fish with poor spatial learning abilities die at a very young age in that lake and thus the wild-caught fish were a non-random sample. Park (2013) also raised anadromous sticklebacks from Rabbit Slough, Alaska, in contrasting lab environments: spatially complex or spatially simple. In that study, rearing environment did not influence spatial learning ability (Park, 2013).

The present study addresses whether there is a genetic basis to the previously documented spatial learning differences in wild-caught benthic and limnetic fish from Paxton and Priest Lakes, British Columbia. We bred pure-species fish in the lab from wild-caught parents and then raised them in a common garden experiment. We also addressed whether environmental complexity has effects on spatial learning ability by raising these fish in two environments that differed in spatial complexity. We then assessed the spatial learning abilities of these fish using a T-maze previously validated with threespine sticklebacks (Odling-Smee and Braithwaite, 2003; Odling-Smee *et al.*, 2008; Park, 2011, 2013). We predicted that benthic fish would outperform limnetic fish in the T-maze spatial learning task. We further predicted that plasticity in spatial cognition would be manifested by better performance by the fish raised in the complex rearing environment compared with those raised in the simple environment.

## METHODS

### Subjects

Benthic and limnetic threespine sticklebacks were collected from two lakes, Paxton and Priest (Texada Island, British Columbia) in April 2014 and brought to Michigan State University. These wild sticklebacks were crossed in June to August 2014 to create an $F_1$ generation. A single male was crossed with a single female from the same lake and species, and each pair of fish contributed a single clutch to our experiment. We reared the offspring

of these crosses from the moment of hatching in either a spatially complex environment or a spatially simple environment (110-litre aquaria with a 76 × 31 cm footprint and water depth of 43 cm). The spatially complex rearing environment contained six nylon packs of crushed coral (to mediate water quality), two shelters (half of a ceramic pot), and 11 artificial plants. In contrast, the spatially simple environment had only one of each of these objects (Fig. 1).

In total, we produced 14 successful crosses, each housed in separate tanks (Table 1). Following hatching, fish were fed first-instar brine shrimp (*Artemia* spp.) nauplii twice daily. Once fish reached ~1.5 cm, they were fed a mixture of first-instar brine shrimp nauplii and finely chopped defrosted bloodworms (chironomid larvae). Once fish reached ~2.5 cm, they were fed whole defrosted bloodworms and brine shrimp. Tanks were checked once a week to determine whether fish were large enough to attempt 'upgrading' their diet. Whenever diets were switched, we ensured through observation that every individual fish successfully consumed the new diet; if not all fish consumed the new diet, we did not upgrade the diet of the tank that day, and tried again one week later. Therefore, by the time fish were used in experiments at 7 months of age, they were all very experienced with eating bloodworms and brine shrimp, both in the water column as the food drifted downwards, and also off of the

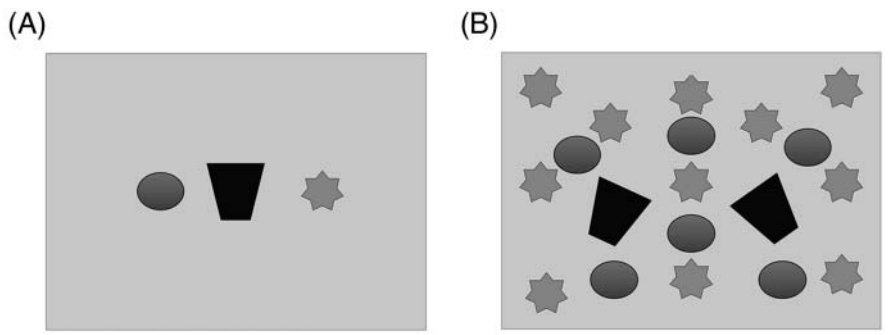


**Fig. 1.** Rearing environments. Diagrams of rearing environments as if looking down on the tank from above: trapezoids represent clay pots, stars represent artificial plants, and ovals represent nylon packs of crushed coral. (A) Spatially simple rearing environment. (B) Spatially complex rearing environment.

**Table 1.** Sample sizes

| Lake | Species | Rearing environment | Number of families | Number of fish tested |
|---|---|---|---|---|
| Paxton | Limnetic | Simple | 0 | 0 |
| | | Complex | 2 | 0 |
| | Benthic | Simple | 3 | 5 |
| | | Complex | 1 | 10 |
| Priest | Limnetic | Simple | 3 | 10 |
| | | Complex | 3 | 12 |
| | Benthic | Simple | 1 | 5 |
| | | Complex | 1 | 6 |

*Note*: The number of families refers to the number of families initially generated through crosses that successfully hatched. The number of fish tested refers to the number of fish used in our T-maze spatial learning assay.

bottom of the tank. We also determined through experimentation that fish preferred bloodworms to brine shrimp, and that satiation on bloodworms occurred only at very large numbers (75+), making it an excellent food reward.

Once the fish reached 7 months of age, groups of 2–5 fish of similar size (standard length = 42.43 ± 6.02 mm; mean ± S.D.) that were from the same lake, species, and treatment were haphazardly selected and removed from their home tanks. These fish were then marked with elastomer (Northwest Marine Technology, Shaw Island, WA) to allow them to be uniquely identified. Following marking, the fish groups were placed into holding tanks with the same spatial complexity (simple or complex) as their rearing tanks. These holding tanks were located in the room that held the T-maze. After 2 days of acclimation to the new holding tanks, the fish were subjected to a 5-day pre-training phase in which they familiarized themselves with the maze (see 'Pre-training' below). Following pre-training, training trials began in which a landmark indicated which direction a fish needed to turn to reach a double reward (see 'Training' below). Each fish was housed in a holding tank with their group until the entire group successfully reached criterion (six out of seven trials correct) or completed a total of 45 trials, whichever came first.

### T-maze apparatus

One four-arm maze was submerged in a shallow circular pool (Fig. 2) and was similar to those used in previous studies with threespine sticklebacks (Odling-Smee and Braithwaite, 2003; Odling-Smee *et al.*, 2008; Park, 2011, 2013). The maze was constructed from white corrugated plastic. Each

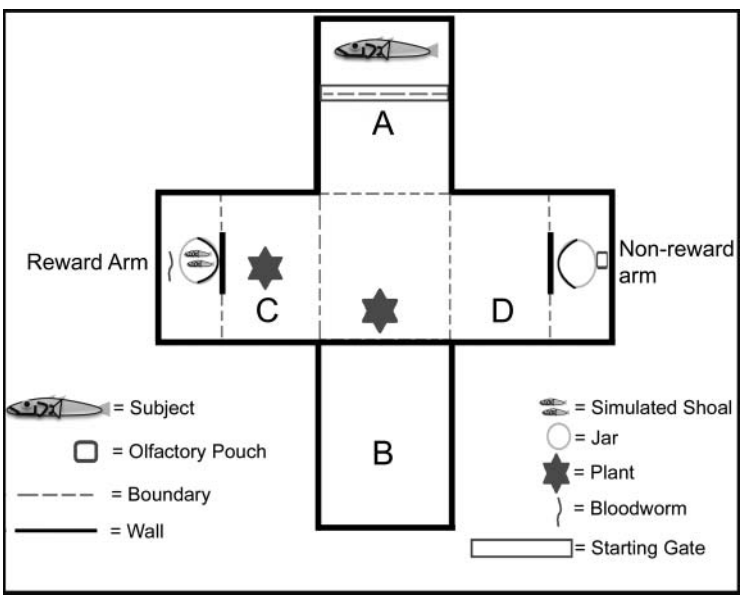


**Fig. 2.** Schematic representation of the T-maze spatial learning task. The starting arm (A or B) and the reward arm (C or D) were randomized using a random number generator. Both a simulated shoal (two fish) and a bloodworm served as rewards. An olfactory pouch (a bloodworm wrapped inside of window screen) was buried slightly under the sand in the non-reward arm to control for any differences in olfactory abilities between the species. The fish's choice of arm was determined when its pectoral fins crossed the visual barrier at the end of each arm in front of the corresponding jar.

arm was 30 cm long, 10 cm wide, and 20 cm deep. The water level was maintained to just below the top of the maze arms. The floor of the maze was lined with sand to minimize stress on the fish and also to maintain similar water chemistry to the rearing tanks. The four arms were set at 90° angles from each other and were referred to as A, B, C, and D. A fish began a trial in either arm A or B, which were directly opposite each other. During a trial, one of these arms was shut off, creating a T-maze. The opposite arm had a gate that could be opened by an experimenter pulling on transparent fishing line.

Subjects were given two rewards in either arm C or D for the successful completion of a trial (as in Odling-Smee and Braithwaite, 2003; Odling-Smee *et al.*, 2008). The first reward was a simulated shoal created by placing two non-experimental fish (haphazardly selected and similar in size and shape to the experimental subjects) into a mason jar filled with water. The jar was blackened out with black plastic on the side facing the centre of the maze, making the shoal viewable to a subject only after it committed to entry into the reward zone. The height of the water in the maze was lower than the height of the jar, preventing the simulated shoal from providing any olfactory information to the subject. The jar was placed 3 cm away from the far end of the reward arm. A second reward of a single bloodworm was placed between the clear side of the simulated shoal jar and the end of the reward arm.

The opposite 'non-reward' arm also contained a jar, identical in every way to the jar in the reward arm except that it did not contain a simulated shoal. A pouch made of window screen containing one bloodworm was shaken underwater and then buried underneath sand in the region between the clear side of the empty jar and the far end of the non-reward arm. This 'olfactory pouch' acted as a control ensuring that differences in olfactory ability (*sensu* Rafferty and Boughman, 2006) would not impact learning.

One piece of white corrugated plastic was placed in front of each of the jars as an additional measure preventing fish from observing which arm contained the rewards. Choice of arm was recorded only if the fish's pectoral fins passed one of these white visual barriers. The two rewards (bloodworm and shoal) acted as a double reward for the subject when it completed the task correctly.

A single plant landmark was placed in the centre of the maze. A second plant landmark was placed in the reward arm between the centre of the maze and the white visual barrier. This second landmark acted as the conditioned stimulus to the reward arm. An HD-camcorder (Canon VIXIA HF M41) was connected to a PVC pipe structure that suspended the camcorder above the maze, allowing recording of each session. The video feed was also transmitted in real time to an HD computer monitor connected with an HDMI cable that was on the other side of a black plastic curtain. The curtain acted as a 'blind' to shield the experimenter from the fish, and thus minimized any possible disturbance from the experimenter while the fish's behaviour was being observed.

## Pre-training

Pre-training commenced on the third day after subjects were transferred from the rearing tanks to holding tanks. The main purpose of pre-training was to allow the fish to familiarize themselves with the maze before trials began. No gates, plant landmarks, or jars were placed in the maze, allowing all fish to freely explore all four arms. At the end of each arm, 5–10 bloodworms were placed to reinforce exploration of all maze arms. For each experimental group, pre-training consisted of all subjects ($n = 2–5$) being placed together into the central region of the maze some time between 10.00 and 14.00 hours. Fish were allowed to explore

the apparatus for 60 minutes on each pre-training day. Three pre-training days were conducted on alternating days, each at the same time of day, over the course of 5 days before the training procedure commenced. The video feed of each pre-training trial was observed on the other side of a black plastic curtain to ensure that the subjects were successfully travelling around the maze. Feeding only took place in the maze (rather than the holding tanks) during pre-training to incentivize the fish to explore.

## Training

Groups were given three training trials every other day with feeding only taking place in the maze (the bloodworm reward) during a trial. This ensured a roughly 48-hour period without food before a trial session to maintain high motivation to successfully complete trials. The order in which fish received their first trial of the day was randomized using a random number generator and then this order was maintained for the two subsequent trials. A given trial (e.g. trial 1) had to be completed by each subject in a group before the next trial (e.g. trial 2) began; this resulted in a variable period of time between consecutive trials that was approximately one hour long. Trials were begun between 09.00 and 11.00 hours or between 13.00 and 15.00 hours. A given group began trials at the same time each trial session day.

The starting arm and reward arm were chosen at random for each trial using a random number generator. In these trials, fish learnt to associate the plant landmark placed in the reward arm (conditioned stimulus) with the dual rewards of the bloodworm and simulated shoal. After the reward and non-reward arms were set up (see 'T-maze apparatus' above), the arm opposite the starting arm was closed off, creating the T-maze. Next, the subject was placed into the starting arm. A gate located at the end of the starting arm prevented the subject from exploring the maze until the experimenter was ready to conduct the trial. Once the fish was placed into the starting arm behind the starting gate, the black plastic curtain was closed and the subject was allowed to acclimate for at least one minute before the trial was begun. The experimenter then began the trial by pulling up the starting gate using fishing line strung through a pulley. Each trial ended with the subject making a choice of one of the arms, or when 10 minutes had elapsed, whichever came first. A fish was determined to have made a decision when its pectoral fins had passed the white visual barrier hiding either jar. A correct choice was defined as the fish choosing the side with the bloodworm and simulated shoal rewards. If a fish chose correctly, it was allowed to feed on the bloodworm reward and was removed from the maze immediately following feeding. However, if a fish did not choose correctly, it was allowed to roam the maze until it reached the reward or until 10 minutes after the start of the trial, whichever came first. This was done so that the fish had an additional opportunity to find the reward in the other arm. In cases where a fish did not consume a bloodworm during any of the three trials in a day, that fish was fed 2–3 bloodworms at the end of the three trials to ensure fish were fed evenly and that poor learners did not starve.

We used the criterion measure for learning validated by Park (2013): a fish was determined to have successfully learned to associate the plant landmark stimulus with the double reward when it reached the criterion of completing six out of seven trials correctly. Training on this procedure ended after the fish reached this criterion or completed 45 trials, whichever came first. Training was terminated after 45 trials because it was previously shown that it is highly unlikely for learning to occur after this number of trials (Odling-Smee and

Braithwaite, 2003; Odling-Smee *et al.*, 2008). After fish reached criterion, they were kept in their holding tanks until the entire group had finished. Fish that had previously reached criterion were fed once a day; this feeding took place before the fish in their group that had not yet reached criterion were returned to their holding tanks.

## Statistical analysis

All statistical analyses were done in R v.3.2.0 (R Development Core Team, 2015). For each fish, we recorded the proportion of trials in which an incorrect choice was made ('error rate') and the number of trials required to reach criterion ('trials to criterion'; fish not reaching criterion were assigned 45). Five fish died before reaching criterion or 45 trials; for these fish we had error rate data, but not trials to criterion data.

Error rate was normally distributed (assessed by visual inspection of the histogram and normal Q-Q plot of the data). We were not able to test any Paxton Lake limnetic fish (Table 1), which meant that out of eight factor combinations (two lakes × two species × two rearing environments), two were missing (Paxton limnetic simple and Paxton limnetic complex). Therefore, we analysed the data as a one-way ANOVA with error rate as the response variable and a lake/species/rearing environment variable (with six levels) as the explanatory variable. We then performed pre-planned contrasts as described by Quinn and Keough (2002) to examine how error rate was influenced by lake, species, rearing environment, and the interaction between species and rearing environment.

The trials to criterion distribution was rather bimodal (assessed by visual inspection of the histogram and normal Q-Q plot of the data); many fish did not learn after 45 trials (18 of 43 fish) and the trials to criterion distribution for fish that did learn was positively skewed. We analysed these data several different ways:

1. We conducted a one-way ANOVA with (log-transformed) trials to criterion as the response variable and a lake/species/rearing environment variable (with six levels; all possible combinations except those with Paxton limnetic fish) as the explanatory variable (as above with error rate). The residuals of this analysis were reasonably normal, as indicated by a Q-Q plot.
2. We analysed the trials-to-criterion data with two separate models: an ANOVA of the (log-transformed) trials to criterion, only focusing on successful learners, and a logistic regression predicting whether or not fish learned the task.
3. We analysed the trials-to-criterion data set with a Cox proportional hazards model (survival analysis), using the *coxph* function in the R package survival v.2.83-3 (Therneau and Grambsch, 2000; Therneau, 2015).

Models for (2) and (3) included as predictors: species, rearing environment, and the interaction between species and rearing environment. However, we did not include lake in these models because of the lack of Paxton Lake limnetic fish and because the analysis in (1) indicated no effect of lake (see 'Results'); this is equivalent to pooling benthic fish from Paxton and Priest Lakes and greatly simplified data analysis.

## RESULTS

### Error rate

There was a significant effect of species, with Priest Lake benthic fish performing fewer errors than Priest Lake limnetic fish, supporting a genetic basis for species differences in spatial learning ability ($F_{1,42} = 10.98$, $P = 0.0019$; Table 2, Fig. 3a). There was no difference in error rate between benthic fish from Paxton and Priest Lakes ($F_{1,42} = 1.69$, $P = 0.20$). The effect of rearing environment was not significant ($F_{3,42} = 0.94$, $P = 0.57$). In Priest Lake, the interaction between species and rearing environment was also not significant ($F_{1,42} = 1.95$, $P = 0.17$).

### Trials to criterion

There was a significant effect of species, with Priest Lake benthic fish reaching the learning criterion faster than Priest Lake limnetic fish, providing further support for a genetic basis for species differences in spatial learning ability ($F_{1,37} = 4.61$, $P = 0.039$; Table 3, Fig. 3b). There was no difference in error rate between benthic fish from Paxton and Priest Lakes ($F_{1,37} = 1.64$, $P = 0.21$). The effect of rearing environment was not significant ($F_{3,37} = 0.74$, $P = 0.46$). In Priest Lake, the interaction between species and rearing environment was also not significant ($F_{1,37} = 1.15$, $P = 0.29$).

When we focused only on the fish that reached the learning criterion, there were no significant main or interaction effects, but there was a very large reduction in sample size and hence power (species: $F_{1,21} = 1.26$, $P = 0.28$; rearing environment: $F_{1,21} = 0.01$, $P = 0.93$; species × rearing environment: $F_{1,21} = 0.63$, $P = 0.44$). We also found no significant

**Table 2.** The effect of species and rearing environment on error rate

| Source | SS | d.f. | MS | *F* | *P* | $\eta^2$ |
|---|---|---|---|---|---|---|
| **Cells** | **0.676** | **5** | **0.135** | **2.89** | **0.0249** | **0.26** |
| Lake | | | | | | |
| PrB vs. PaB | 0.079 | 1 | 0.079 | 1.69 | 0.20 | 0.03 |
| Species | | | | | | |
| **PrB vs. PrL** | **0.514** | **1** | **0.514** | **10.98** | **0.0019** | **0.19** |
| Rearing environment | 0.133 | 3 | 0.044 | 0.94 | 0.57 | 0.05 |
| PrBS vs. PrBC | 0.105 | 1 | 0.105 | 2.25 | 0.14 | 0.04 |
| PrLS vs. PrLC | 0.004 | 1 | 0.004 | 0.09 | 0.77 | 0.00 |
| PaBS vs. PaBC | 0.024 | 1 | 0.024 | 0.52 | 0.48 | 0.01 |
| Species × rearing environment | | | | | | |
| PrBS – PrBC vs. PrLS – PrLC | 0.091 | 1 | 0.091 | 1.95 | 0.17 | 0.03 |
| Residual | 1.965 | 42 | 0.047 | | | |

*Note*: Because we were unable to test any Paxton Lake limnetic fish, we conducted a one-way ANOVA with error rate as the response variable and a combined lake/species/rearing environment variable (with six levels) as the explanatory variable. 'Cells' refers to the between-groups source of variation and 'Residual' refers to the within-group source of variation from this analysis. Pre-planned contrasts were then used to test hypotheses regarding the influence of lake, species, rearing environment, and the interaction between species and rearing environment on error rate, using methods described in Quinn and Keough (2002). Pr = Priest, Pa = Paxton, L = limnetic, B = benthic, S = simple rearing environment, C = complex rearing environment. Significant results in **bold**.

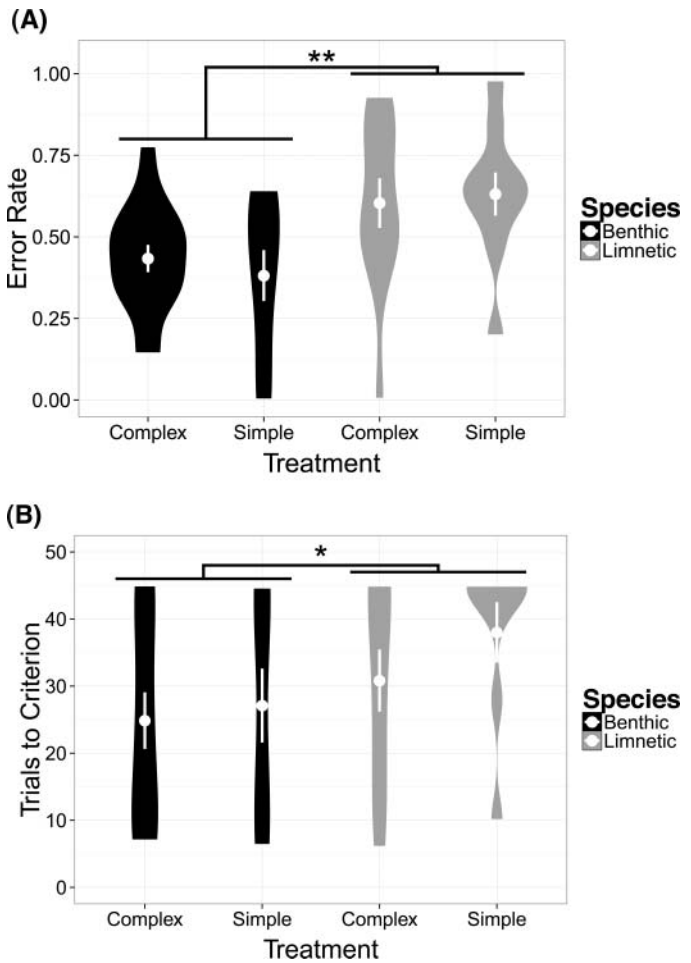


**Fig. 3.** Differences in spatial learning ability based on species and rearing environment. These violin plots visualize the distribution of the data in each group. The width corresponds to the probability of a sample being found at that value. The white dots indicate means and the white lines on either side indicate one standard error. (A) Error rate. (B) Trials to criterion. * $P < 0.05$, ** $P < 0.01$.

predictors for determining whether or not fish reached the learning criterion (species: $\chi^2 = 1.65$, $P = 0.20$; rearing environment: $\chi^2 = 0.86$, $P = 0.35$; species × rearing environment: $\chi^2 = 0.02$, $P = 0.89$).

The Cox proportional hazards model found a non-significant trend for benthic fish to learn faster than limnetic fish ($\chi^2 = 2.31$, $P = 0.13$; Fig. 4). This analysis did not indicate a significant main effect of rearing environment or a significant interaction between species and rearing environment on learning curves (rearing environment: $\chi^2 = 0.75$, $P = 0.39$; species × rearing environment: $\chi^2 = 0.14$, $P = 0.70$).

**Table 3.** The effect of species and rearing environment on trials to criterion

| Source | SS | d.f. | MS | $F$ | $P$ | $\eta^2$ |
|---|---|---|---|---|---|---|
| Cells | 3.287 | 5 | 0.657 | 1.27 | 0.30 | 0.15 |
| Lake | | | | | | |
| PrB vs. PaB | 0.853 | 1 | 0.853 | 1.64 | 0.21 | 0.04 |
| Species | | | | | | |
| **PrB vs. PrL** | **2.388** | **1** | **2.388** | **4.61** | **0.0385** | **0.11** |
| Rearing environment | 1.149 | 3 | 0.383 | 0.74 | 0.46 | 0.05 |
| PrBS vs. PrBC | 0.241 | 1 | 0.241 | 0.46 | 0.50 | 0.01 |
| PrLS vs. PrLC | 0.398 | 1 | 0.398 | 0.77 | 0.39 | 0.02 |
| PaBS vs. PaBC | 0.511 | 1 | 0.511 | 0.99 | 0.33 | 0.02 |
| Species × rearing environment | | | | | | |
| PrBS – PrBC vs. PrLS – PrLC | 0.598 | 1 | 0.598 | 1.15 | 0.29 | 0.03 |
| Residual | 19.184 | 37 | 0.519 | | | |

*Note*: Because we were unable to test any Paxton Lake limnetic fish, we conducted a one-way ANOVA with trials to criterion as the response variable and a combined lake/species/rearing environment variable (with six levels) as the explanatory variable. 'Cells' refers to the between-groups source of variation and 'Residual' refers to the within-group source of variation from this analysis. Pre-planned contrasts were then used to test hypotheses regarding the influence of lake, species, rearing environment, and the interaction between species and rearing environment on trials to criterion, using methods described in Quinn and Keough (2002). Pr = Priest, Pa = Paxton, L = limnetic, B = benthic, S = simple rearing environment, C = complex rearing environment. Significant results in **bold**.

### Relationship between error rate and trials to criterion

Error rate and trials to criterion were highly correlated ($R = 0.81$, $t_{41} = 9.00$, $P \ll 0.00001$, Fig. 5). Lower residuals from the regression describing this relationship would indicate fish that learned with fewer errors than expected given their learning speed. Benthic fish had lower residuals than limnetic fish, implying they learned with fewer errors ($F_{1,39} = 5.25$, $P = 0.027$). This difference was most pronounced when examining those fish that did not reach criterion after 45 trials ($t_{16} = -3.80$, $P = 0.0016$; Fig. 5). Rearing environment and the interaction between species and rearing environment did not explain variance in these residuals (rearing environment: $F_{1,39} = 2.05$, $P = 0.16$; species × rearing environment: $F_{1,39} = 0.38$, $P = 0.54$).

## DISCUSSION

Using wild-caught limnetic and benthic fish from Paxton and Priest Lakes, we made pure-species crosses in the lab. We then lab-reared the resulting benthic and limnetic offspring in two different environments that varied in spatial complexity. Finally, we tested their ability to learn to use visual landmarks to find the arm of a T-maze that had a double reward of food and a simulated shoal. Unfortunately, we were not able to assess the spatial learning ability of lab-reared Paxton Lake limnetic fish. We found that Priest Lake benthic fish learned significantly faster and with fewer errors than Priest Lake limnetic fish, confirming previous findings with wild-caught fish from Paxton and Priest Lakes (Odling-Smee *et al.*, 2008). In addition, benthic fish (from both lakes) that did not reach criterion nevertheless made significantly fewer errors than limnetic fish (from Priest Lake) that did not reach criterion. It is possible that these benthic fish were on their way to successfully learning the association

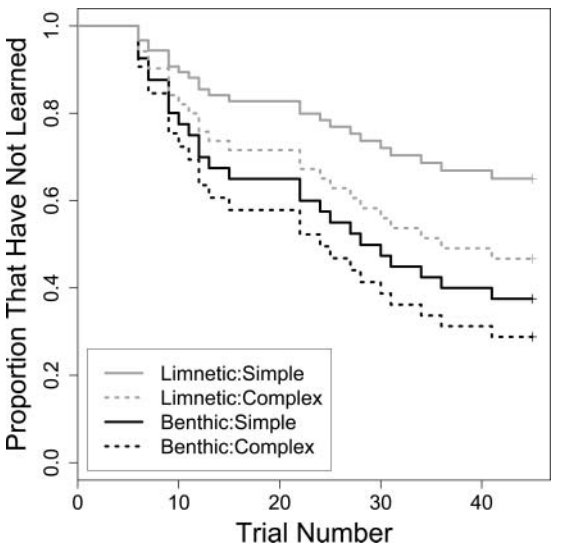


**Fig. 4.** Survival plots indicating learning progression. These results are from a Cox proportional hazards model that included species, rearing environment, and the interaction between species and rearing environment as predictors.

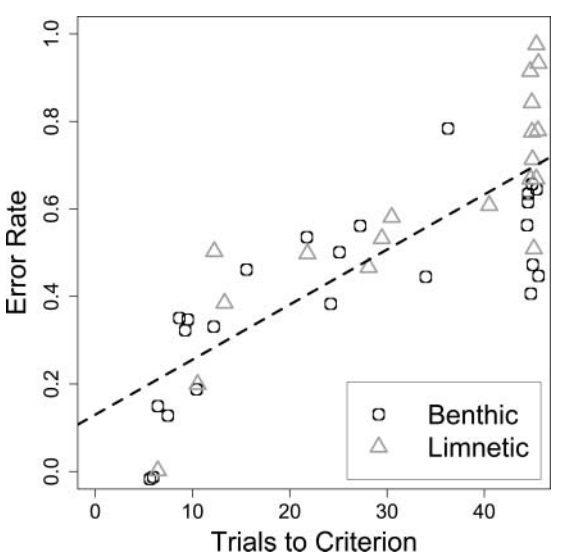


**Fig. 5.** Correlation between error rate and trials to criterion. The dotted line indicates the regression line. Points have been jittered slightly in both the x- and y-directions to enable all points to be viewed. The points on the far right indicate those fish that never reached criterion. The error rate distribution of the benthic fish that did not reach criterion is very different from that of the limnetic fish ($t_{16} = -3.80$, $P = 0.0016$).

between landmark and the side with the reward. Importantly, our results support the hypothesis that there is a genetic basis underlying the difference in spatial learning ability between benthic and limnetic fish. In addition, it suggests that this species difference has evolved in response to selection from the different environments in which the two species tend to forage. However, we found no significant differences between fish raised in environments that differed in spatial complexity, mirroring results previously found with anadromous sticklebacks that are likely similar to the ancestors of benthic and limnetic sticklebacks (Park, 2013).

Our study specifically focused on the use of local visual landmark cues to learn the location of the reward. Global (external to the T-maze) cues were non-informative because of the randomization of the starting and reward arms. Algorithmic strategies (e.g. always turn right) would also not work in our experiment because of this randomization. Previous work has demonstrated that wild-caught benthic and limnetic fish from the same lakes tend to use local landmark cues over algorithmic strategies (Odling-Smee *et al.*, 2008). Park (2013) conducted experiments where global cues were reliable, but local cues were not. Under these conditions, both wild-caught benthic-like and limnetic-like fish from Alaskan single-population lakes were able to learn which arm held the reward, but benthic-like populations (with a few interesting exceptions) learned faster.

We did not find an environmental effect on spatial learning ability in benthic or limnetic sticklebacks. This is similar to results from anadromous sticklebacks (Park, 2013) and Scottish sticklebacks (Brydges and Braithwaite, 2009), but contrasts with similar studies in other species of fish (Spence *et al.*, 2011; Salvanes *et al.*, 2013). Threespine sticklebacks do show (probably adaptive) developmental and behavioural plasticity in other traits, including body morphology (Day *et al.*, 1994; Day and McPhail, 1996; Wund *et al.*, 2008), brain size (Park *et al.*, 2012), mate preferences (Tinghitella *et al.*, 2013), and courtship behaviour (Kozak *et al.*, 2009). If the anadromous sticklebacks studied by Park (2013) that show no plasticity in spatial learning are representative of the anadromous sticklebacks ancestral to freshwater sticklebacks, then perhaps there was no genetic variation for plasticity, limiting its ability to evolve. It is also possible that the environmental manipulations done by us, Park (2013), and Brydges and Braithwaite (2009) were not severe enough to manifest in a plastic response. Alternatively, the degree to which these responses are plastic may be much smaller than the genetic contribution, and difficult to discover with the smaller sample sizes typical of labour-intensive spatial learning assays.

This study adds spatial learning differences to the long list of genetically based differences between benthic and limnetic sticklebacks from British Columbia. There has been great success at exploring the genetic basis of morphological traits that have diverged between freshwater and anadromous sticklebacks (Shapiro *et al.*, 2004; Miller *et al.*, 2014) or benthic and limnetic sticklebacks (Malek *et al.*, 2012; Arnegard *et al.*, 2014; Conte *et al.*, 2015). Although dissecting the genetic basis of behavioural differences is notoriously difficult, Greenwood *et al.* (2013, 2015) recently found that behavioural differences in schooling propensity between benthic and anadromous sticklebacks were associated with several quantitative trait loci (QTL). Given our confirmation of a genetic basis for spatial learning differences, the possibility exists for future genetic mapping work. However, for this to be truly feasible, some method for automating presentation of spatial learning trials must be developed to make gathering data on large numbers of individuals practical.

Several factors other than cognitive ability could have affected spatial learning performance, including motivation, boldness, propensity for exploration, or activity levels. Not feeding the fish for roughly 48 hours prior to trials was done to make them highly

motivated to find the food reward. In addition, we have found that lab-reared sticklebacks are very highly motivated to eat bloodworms, reaching satiation at only very high numbers ($n = 75+$). We have not found differences between wild-caught benthic and limnetic fish from Paxton and Priest Lakes in motivation to feed on bloodworms [measured as speed to eat a bloodworm reward off of the bottom of the tank (J. Keagy and J.W. Boughman, unpublished data)]. Wild-caught limnetic sticklebacks typically forage in shoals more than wild-caught benthic sticklebacks (Larson, 1976), so it is possible the lab-reared limnetic fish were more motivated by the shoal than the lab-reared benthic fish and/or more comfortable foraging on the food reward in the presence of the shoal. However, if this difference created a motivational bias in our experiment, we would have expected the opposite pattern of species differences in spatial learning from that which we observed. We have not found differences in hiding propensity between wild-caught benthic and limnetic fish from Paxton and Priest Lakes (J. Keagy and J.W. Boughman, unpublished data), and Odling-Smee *et al.* (2008) found no differences in a combined measure of boldness and exploration between benthic and limnetic fish from Paxton and Priest Lakes. Park (2013) collected extensive data on exploration, boldness, and activity levels and found no evidence of any differences between wild-caught benthic-like or limnetic-like fish from single-population lakes in Alaska. Therefore, it is very likely that the species differences in spatial learning performance we detected are due to differences in spatial cognitive ability.

We used a novel experimental design that controlled for odour of the food reward because several lines of evidence suggest that benthic sticklebacks rely more on odour than limnetic sticklebacks in guiding their behaviour. First, Rafferty and Boughman (2006) found that benthic females distinguished between the odour of benthic versus limnetic males, whereas limnetic females did not. Second, Mobley *et al.* (2016) also found that benthic and limnetic females use olfactory information differently. Finally, volumetric data from high-resolution magnetic resonance imaging (MRI) show large differences in olfactory bulbs, the sensory processing region for olfaction (J. Keagy and J.W. Boughman, unpublished data). By controlling for bloodworm odour, we confirmed that spatial learning differences do exist between benthic and limnetic fish.

In conclusion, we confirmed a genetic basis for differences in spatial learning abilities between benthic and limnetic fish, but found no effect from our environmental manipulation. That benthic fish learn faster and with fewer errors than limnetic fish in Priest Lake (this study; Odling-Smee *et al.*, 2008), Paxton Lake (Odling-Smee *et al.*, 2008), and Alaskan single-population lakes (Park, 2013) strongly implicates response to selection by the divergent environmental and ecological niches that benthic and limnetic fish occupy. These fish have responded to selection in a myriad of other ways such as their morphology and behaviour. It remains to be seen what other cognitive traits [e.g. social learning (*sensu* Coolen *et al.*, 2003); associative learning (*sensu* Roche *et al.*, 2012); memory (*sensu* Mackney and Hughes, 1995)] have diverged between these incipient species.

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