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Olfactory perception of mates in ecologically divergent stickleback: population parallels and differences

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ABSTRACT

Background: The independent evolution of sympatric species pairs of threespine sticklebacks has provided a natural system to explore how divergent ecologies shape mating preferences. Research has shown that both limnetic and benthic females discriminate against heterospecific males, but not against populations of the same species from different lakes, at least when visual cues are available (Rundle *et al.*, 2000). It is known that olfaction is used in species discrimination by benthic but not limnetic sticklebacks in one of the species pairs (Rafferty and Boughman, 2006), but differences across populations are unknown.

Hypotheses: Females from benthic habitats make use of olfactory cues to distinguish between species but not lakes of potential mates. Limnetic females will not show preferences for males of different species or lakes when limited to only olfactory cues.

Organisms: Benthic and limnetic populations of threespine stickleback (*Gasterosteus aculeatus*) from Paxton and Priest Lakes, British Columbia.

Methods: We exposed gravid females from each population to chemical stimuli from nesting males in a Y-maze, and recorded which stimulus a female chose and how much time was taken to make a decision.

Results: We did not find significant differences between female populations in the preference for conspecific over heterospecific male odours. There was also no preference for odours of males from the same or a different lake. In all populations of females, the preference for male odours of different lakes differed between the two species of male odours: benthic male odours from a different lake were selected over limnetic male odours. The amount of time taken to make a decision differed between female populations, but only when benthic females ultimately chose a limnetic male odour over a benthic one.

Conclusions: The preference for conspecific over heterospecific odours, although not strong, may still contribute to reproductive isolation in sympatric sticklebacks, particularly through interactions with other senses and environmental properties.

Keywords: *Gasterosteus aculeatus*, mate choice, olfaction, parallel evolution, threespine stickleback.

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INTRODUCTION

Perceptual systems evolve under the influence of multiple biotic and abiotic factors of the niche a species occupies (Dangles *et al.*, 2009). Through adaptation, the senses may become specialized for particular tasks, as in the detection of conspecific sex pheromones in moths (Schneider, 1992), and auditory prey localization in owls (Knudsen and Konishi, 1979). The nature of such specializations is particularly apparent when they arise repeatedly in different populations due to parallel evolution. The use of model animals and sensory systems has been critical for a detailed understanding of the proximate mechanisms of individual sensory modalities adapted to specific types of environments (Krogh, 1929). The focus on specializations in a single modality has in some cases led to the neglect of the contributions of additional modes of perception. However, in ecological contexts, animals may employ a number of senses to make behavioural decisions, and focusing on a single specialized modality prevents a complete understanding of behaviour (Elias *et al.*, 2005).

The threespine stickleback (*Gasterosteus aculeatus*) has long been used to study the importance of specialized sensory modalities. Tinbergen's early work demonstrated the importance of vision to the fish's ecology (Tinbergen, 1952), and stickleback vision has remained an active field of research (McDonald and Hawryshyn, 1995; Boughman, 2001; McLennan, 2007; Rick and Bakker, 2008; Novales Flamarique *et al.*, 2013). In contrast, the stickleback olfactory system, although demonstrated to have a necessary function in mating behaviours (Segaar *et al.*, 1983), was overlooked for many years, partly in light of anatomical research, which concluded that sticklebacks had a poor sense of smell (Wootton, 1976). This view of the olfactory system would change dramatically in the twenty-first century, as researchers began studying the role of olfaction in species recognition and mate choice in multiple members of the Gasterosteidae family (Aeschlimann *et al.*, 2003; McLennan, 2003, 2004; Milinski *et al.*, 2005; Rafferty and Boughman, 2006; Mehlis *et al.*, 2008; Kozak *et al.*, 2009; Hiermes *et al.*, 2015). These studies have not only demonstrated the importance of a historically de-emphasized sensory modality in these fish, but have also led to new studies on the effects of the ecology of perception and reproductive behaviour.

Threespine stickleback occupy a number of aquatic systems throughout the northern hemisphere, and have undergone several, and in some cases rapid, adaptive radiations (Wootton, 1976; Boughman, 2007). In multiple systems, populations have diverged into paired ecotypes such as anadromous/stream, stream/lake, and benthic/limnetic (Hagen, 1967; Lavin and McPhail, 1985; Thompson *et al.*, 1997; McKinnon and Rundle, 2002; Boughman, 2007). Benthic–limnetic species pairs have become a focus of speciation research, as the two members of such pairs constitute biological species. Although genetically compatible, benthics and limnetics rarely mate in the wild, and ecological and sexual selection reduces the fitness of hybrids (Vamosi and Schluter, 1999; Gow *et al.*, 2007; Hendry *et al.*, 2009). In addition, benthic–limnetic pairs have arisen independently in multiple lakes (Taylor and McPhail, 2000), and experimental work has shown that mating preferences hold across lakes: benthics spawn with other benthics from their own or another lake more readily than with any population of limnetics, and limnetics similarly discriminate against benthic populations while accepting limnetics from other populations as mates (Rundle *et al.*, 2000).

It is perhaps not surprising that the behavioural mechanisms of benthic–limnetic isolation have predominantly been studied from the visual perspective, since conspecific recognition has been found to rely heavily on visually mediated traits such as body size and colour (Boughman, 2001; Boughman *et al.*, 2005). However, increased attention to the role of olfactory systems in the Gasterosteidae has prompted studies on the relative importance of olfaction

in benthic and limnetic sticklebacks. In Paxton Lake, the species have been well studied. Benthic females there use olfaction to discriminate between species of potential mates (Rafferty and Boughman, 2006). Furthermore, benthic females imprint on their father's odour and will show a preference for the odour of males they are exposed to shortly after hatching, whether they are conspecific or heterospecific males (Kozak *et al.*, 2011). Limnetics do not demonstrate such reliance on olfaction (Rafferty and Boughman, 2006; Kozak *et al.*, 2011).

These results indicate important differences in the sensory biology of the two species in Paxton Lake. Rafferty and Boughman (2006) suggest that these differences may be a product of the different ecological niches of benthics and limnetics. Bottom-dwelling benthics have less access to light due to attenuation and denser vegetation, and feed on organisms that may be hidden or camouflaged by the substrate. The relatively enclosed environment in the structured benthic habitat may make chemical stimuli more readily available and traceable than in open habitat. Limnetics, on the other hand, primarily inhabit the open water column where light is more readily available, and it requires acute vision to detect the small planktonic organisms they feed on. Thus benthic fish occupy a habitat where visual acuity may be less important, and olfactory cues may have more utility. This may promote the use of olfaction in benthic mate choice, while limnetics' reliance on vision may suppress dependence on olfaction in a perceptual trade-off. Such habitat differences have recently been linked to olfactory recognition by stickleback from lakes with different photic conditions (Hiermes *et al.*, 2015). The intrinsic differences in benthic and limnetic habitats, coupled with the evolutionary independence of benthic–limnetic pairs in different lakes allows for further exploration of how sensory systems evolve and enforce reproductive isolation.

Here we test patterns of mate discrimination across populations of benthic and limnetic sticklebacks when fish only have access to olfactory information. By exploring these behaviours in animals that have evolved in parallel, we ask if selection has resulted in fish from the same type of habitat using olfaction in a similar fashion, as they do vision (Boughman, 2001), as an adaptation to their similar ecologies. Females were allowed to choose between the odour stimuli of two males: either males of the same species but different lakes, or males of the same lake but different species. We predicted that neither species of female would discriminate between males from different lakes, as long as the two males were of the same species. We further predicted that limnetic females would not show a preference for males of either species because visually biased limnetics should not make substantial use of olfactory cues. However, benthic females were expected to show a preference for benthic over limnetic male odour, regardless of lake of origin.

METHODS

Fish collection and housing

Fish were collected from Priest and Paxton Lakes in British Columbia using minnow traps at the beginning of the 2013 and 2014 breeding seasons. Males were identified by the presence of nuptial colours, while females were identified by the appearance of gravidity. Traps were collected, and any animals that were not the target species were released. Species-specific characteristics – specifically, coloration, body size, and shape – were used to distinguish between limnetic and benthic species (Boughman *et al.*, 2005). The fish, separated by species, lake, and sex, were transported to Michigan State University. Once in the laboratory, the fish were housed in aquaria at a density not exceeding one fish to 4 litres of

water. Fish were fed each morning *ad libitum*. Because the species have distinct feeding styles and diets in the wild that may impact their olfactory signature, benthics and limnetics were given different diets (Bentzen and McPhail, 1984). The benthics from each lake were fed only frozen bloodworms; the limnetics from each lake were fed only frozen brine shrimp. As such, our design focuses on the ability of species to recognize olfactory cues, rather than to determine the nature of odour differences between species.

Reproductive males were placed in individual tanks (nesting tanks) and given time and materials to construct a nest. Males were identified as reproductive by their bright red throats, blue eyes, and dark coloration. Tanks for nesting males were equipped with a plastic plant and half of a clay pot (for cover), and a 900-mL tray of sand in which to construct a nest. Males were given grass and *Chara* to use as nesting material. Nesting tanks were cleaned and plant material was replenished every 2 weeks as part of routine maintenance. The sides of nesting tanks were covered to prevent aggression between neighbouring males, and to minimize disturbances from the laboratory.

Males were enticed daily to encourage nest building. A gravid female of the same lake and species was placed into a transparent glass jar filled with water and covered with a transparent mesh. The mesh allowed the male and female to receive olfactory and visual cues from each other without coming into physical contact. The jar was placed into a male's tank and left for 10 minutes while observers noted whether the male had built a nest. Each male was enticed once daily, but females were used two to three times. After every male had been enticed, all tanks were covered, and the females were returned to their home tank.

Experimental apparatus

Preference tests were conducted in a Y-maze with a main arm of 71 cm, choice arms of 56 cm, and walls 9 cm high. The maze was filled to a depth of 4 cm with reverse osmosis (RO) water. A 38-litre tank, elevated 75 cm above the maze, supplied the maze with RO water by gravity through 0.6-cm diameter polyvinyl hoses connected to each choice arm. Two additional hoses at the base of the maze drained the maze, generating a current. The rate of flow from the header to the maze was approximately 1300 $mL \cdot min^{-1}$ (i.e. 650 $mL \cdot min^{-1}$ into each arm). Containers that supplied stimuli (see below) were elevated above the maze, and connected to each choice arm by gravity-fed hoses. The rate of flow from the stimulus containers was approximately 17 $mL \cdot min^{-1}$ into each arm. Marked PVC airline control valves, connected to each hose, controlled flow rates. Tests with dye confirmed that flow from each choice arm was laminar and did not mix in the main arm. Between trials the Y-maze and associated materials were cleaned with ethanol and rinsed thoroughly with RO water.

Stimulus preparation

Chemical stimuli were taken from two different reproductively active males (i.e. those that had built a nest, responded to enticement, and tended their nest on the trial day) for each trial. For each male in the trial, a plastic container was filled with 500 mL of water from the vicinity of the male's nest to use as a stimulus, and the nest itself was placed in the container. We then placed the male that produced the nest into the stimulus container for 10 minutes to add additional odours to the stimulus water. Although a small amount of female odour may have been introduced to nesting tanks during enticements, the brief

exposure time, overall volume, and filtration of the tank make it unlikely that female odours influenced the stimulus. Males were returned to their nesting tanks before trials. For each trial, we used males who built nests within 7 days of each other.

Behavioural trials

We performed two types of behavioural experiments. In the species discrimination experiment ($n = 61$), the stimuli used came from males of the same lake but different species (i.e. a Paxton limnetic paired with a Paxton benthic male, or a Priest limnetic paired with a Priest benthic male). In the lake discrimination experiment ($n = 57$), the two stimuli came from males of the same species but different lakes (i.e. a Paxton limnetic paired with a Priest limnetic male, or a Paxton benthic paired with a Priest benthic male). Due to limitations on the availability of fish from each population, we did not perform tests using males from the same population, or males of different lakes and species.

The side of the maze for each stimulus was randomly assigned in each trial. A ready-to-spawn female, verified by lightly squeezing the abdomen after trials to ensure the presence of ripe eggs, was used for trials. Females were used at most two times (13 of 106 females) and never in the same experiment (e.g. a female that was used in the lake discrimination experiment could only be re-used in the species discrimination experiment).

The female was placed into the Y-maze once flow in the maze and the stimuli had been started. She was placed into an acclimation area behind a gate at the base of the maze and left undisturbed for 5 minutes. After 5 minutes, the gate was slowly raised. During the subsequent 10 minutes, an observer verified that the fish was exposed to both stimulus streams by her position in the main arm. The choice arm she swam up was recorded, along with how quickly she chose an arm. The trial was considered complete once the fish fully entered either choice arm of the Y-maze, or once 10 minutes had elapsed. Trials that were not completed in the time frame, or where the female was not exposed to both stimulus streams, were not included in analyses.

Once the trial was over, the female was tagged with elastomer (Northwest Marine Technology, Inc., Shaw Island, WA) to allow identification in future experiments, and was weighed, measured, and photographed before being placed back in her tank of origin. The males used were also marked, weighed, measured, and photographed at the end of trials. All procedures for handling the fish and their use in experiments were approved by the MSU Institutional Animal Care and Use Committee (Protocol #04/13-092-00).

Statistical analysis

Statistical analyses were performed in R v.3.2.1 (R Development Core Team, 2015). We used generalized linear models ('glm') to assess female preference. Preference was scored as a binomial response of females' selection of either conspecific (1) or heterospecific (0) male odour in the species discrimination experiment, and selection of either a male odour from the same (1) or different (0) lake in the lake discrimination experiment. Models used a logit-link function. Female species, lake, and the pairing of males in each trial (i.e. if the two male odours were from Paxton Lake or Priest Lake in the species discrimination experiment, and if the male odours were limnetic or benthic in the lake discrimination experiment), as well as all two- and three-way interaction terms were used as independent variables. Because very few trials were performed in the first year, year is not included in the models. Tests of

significance were based on chi-square (χ^2) tests, given the binomial nature of our data. An effect size [the odds ratio: the proportional odds of a dichotomous outcome between two groups (Nakagawa and Cuthill, 2007)] was determined to estimate the magnitude of differences between female lakes, and female species, for each experiment.

Since there were no differences between lakes, in each experiment the data were pooled across female lakes (that is, we combined benthics from both lakes, and limnetics from both lakes to test the species effect). Binomial tests were used to determine if preferences within a species of female differed from chance. For species discrimination, a directed test (Rice and Gaines, 1994) was used with benthic females, as they were expected to show a preference for conspecifics. For limnetic females in species discrimination, and both species of female in lake discrimination, two-tailed tests were used, as there were no *a priori* expectations of preference.

In addition, a two-tailed binomial test was performed on the individual levels of variables that significantly differed to determine if either level itself differed from chance. This test was only applied to the pairing of males in the lake discrimination experiment.

The amount of time taken to complete trials was our measure of latency. The response variable was square-root-transformed to meet assumptions of normality, and fitted to a linear model with female lake, species, and the type of male odour selected, together with all two- and three- way interaction terms as factors, for analysis in both the species and the lake discrimination experiments. In the event of significant effects, *post-hoc* comparisons were performed using Tukey's HSD on *a priori* comparisons of biological interest. We performed eight comparisons for the species discrimination experiment:

1. Paxton benthic females vs. Priest benthic females when each chose a conspecific odour;
2. Paxton benthic females vs. Priest benthic females when each chose a heterospecific odour;
3. Paxton benthic females that chose a conspecific odour vs. Paxton benthic females that chose a heterospecific odour;
4. Priest benthic females that chose a conspecific odour vs. Priest benthic females that chose a heterospecific odour;
5. Paxton limnetic females vs. Priest limnetic females when each chose a conspecific odour;
6. Paxton limnetic females vs. Priest limnetic females when each chose a heterospecific odour;
7. Paxton limnetic females that chose a conspecific odour vs. Paxton limnetic females that chose a heterospecific odour; and
8. Priest limnetic females that chose a conspecific odour vs. Priest limnetic females that chose a heterospecific odour.

RESULTS

Species discrimination was not influenced by whether odours of male species pairs came from Paxton or Priest Lake ('glm', $n = 61$, $\chi^2_1 = 0.82$, $P = 0.37$). We did not find differences in the selection of conspecific over heterospecific male odours between female lakes, or female species ('glm', $n = 61$, χ^2_1 tests, all $P > 0.05$). The odds ratio for Paxton–Priest female selection of conspecific over heterospecific male odours was 0.59 (95% CI = 0.20–1.70).

Although not statistically significant, benthic females selected conspecific over heterospecific odours more frequently than limnetic females. The odds ratio for benthic–limnetic female selection of conspecific over heterospecific male odours was 1.84 (95% CI = 0.66–5.13). Collectively, benthic females selected conspecific over heterospecific odours in 63% of trials, although this did not differ from chance (directed binomial test, $n = 30$, 95% CI = 0.46–1.00, $P = 0.13$; Fig. 1a). Limnetic females selected conspecific over heterospecific odours in 48% of trials, which did not differ from chance (two-tailed binomial test, $n = 31$, 95% CI = 0.30–0.66, $P = 1.00$; Fig. 1a).

In lake discrimination, the selection of male odours from different lakes did not differ between female lake or species origin ('glm', $n = 57$, χ^2_1 tests, all $P > 0.05$; Fig. 1b). The odds ratio for Paxton–Priest female selection of male odours of the same lake over a different lake was 1.01 (95% CI = 0.35–2.95). The odds ratio for benthic–limnetic female selection of male odours from the same lake over the different lake was 0.90 (95% CI = 0.38–3.03). Collectively, benthic females selected the male odour from the same lake in 46% of trials, which did not differ from chance (two-tailed binomial test, $n = 28$, 95% CI = 0.28–0.66, $P = 0.85$). Limnetic females selected male odour from the same lake in 45% of trials, which did not differ from chance (two-tailed binomial test, $n = 29$, 95% CI = 0.27–0.65, $P = 0.71$).

However, we found the species of male odour did influence selection of lakes: in assessing benthic and limnetic females of both lakes, females selected the benthic male odour from a different lake more often than they selected limnetic male odours from a different lake ('glm', $n = 57$, $\chi^2_1 = 4.32$, $P = 0.03$; Fig. 2). Females selected benthic male odours from their own lake over the other lake in only 31% of trials, a rate marginally different than chance (two-tailed binomial test, $n = 26$, 95% CI = 0.14–0.51, $P = 0.08$; Fig. 2). The limnetic male odours from the same lake were selected in 59% of trials, which did not differ from chance (two-tailed binomial test, $n = 31$, 95% CI = 0.39–0.75, $P = 0.47$; Fig. 2).

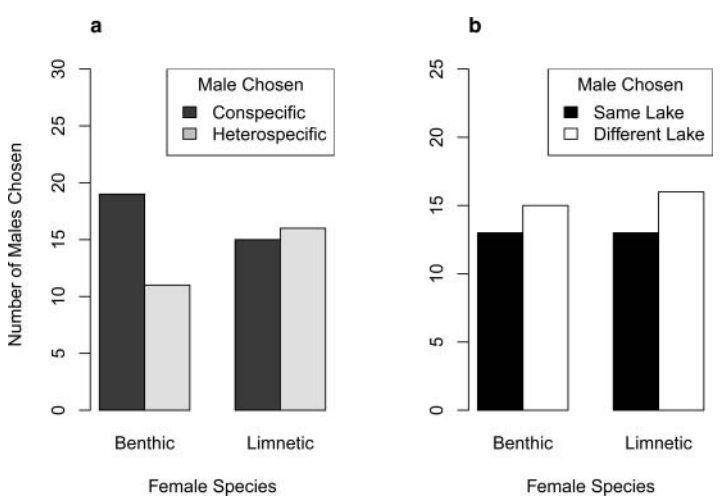

Fig. 1. Bar plots showing the number of trials in which benthic and limnetic females chose males from (a) different species or (b) different lakes. In choosing between the odours of male species, benthic females select conspecific over heterospecific odours more frequently than limnetic females, but the difference is not significant ($\chi^2_1 = 1.43$, $P = 0.23$).

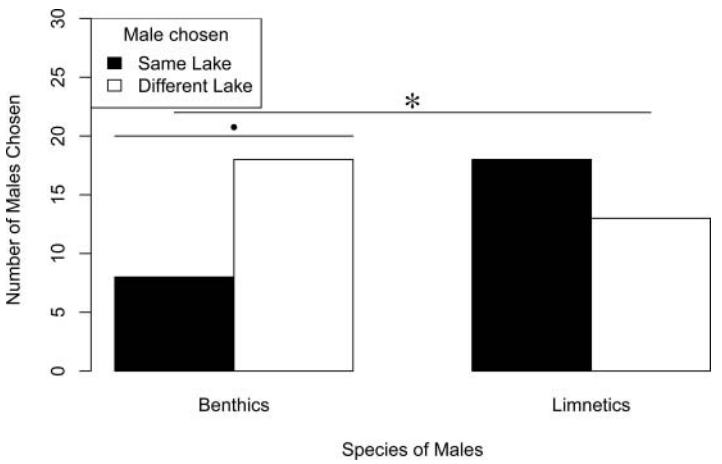

Fig. 2. Bar plots showing the number of trials in which females from all populations selected a male from their own or the other lake, depending on male species. The proportion of males selected from a different lake was higher when choosing between benthic male odours than when choosing between limnetic male odours ($\chi^2_1 = 4.41$, $^*P = 0.03$). The number of trials in which the benthic male from a different lake was chosen over a benthic from a female's own lake is nearly significant (two-tailed binomial test: probability of success = 0.69, $^\bullet P = 0.08$).

The interaction of female species, lake, and the choice of a conspecific or heterospecific male odour significantly affected decision times in species discrimination (three-way interaction, $F_{1,53} = 8.52$, $P = 0.005$; Fig. 3a). Within each female population, the time to choose benthic or limnetic male odours did not differ (Tukey's HSD, all $P > 0.05$). Significant differences in latency between females from different lakes occurred only when limnetic male odours were selected. The time Priest benthic females took when they ultimately chose a limnetic male odour was greater than the time taken by Paxton benthic females that chose limnetic odours (Tukey's HSD = 11.17, $n = 30$, $P = 0.009$; Fig. 3a). The difference in latency between Priest and Paxton limnetic females that chose a limnetic male odour approached statistical significance (Tukey's HSD = 8.67, $n = 31$, $P = 0.05$; Fig. 3a). In lake discrimination trials, the time to make a decision did not differ between female species or lakes, or the lake of the male odour chosen (ANOVA F-tests, all $P > 0.05$; Fig. 3b).

DISCUSSION

Although we did not find between-population differences in odour discrimination for male species or lake, our results suggest that odour may contribute to mate choice. Previous findings indicate that only Paxton benthic females discriminate between benthic and limnetic odour (Rafferty and Boughman, 2006), and prefer conspecific odours (estimated odds ratio: 13, 95% CI = 0.48–349.52). Our effect size ranged from 0.66 to 5.13, which overlaps with these earlier findings, and tends towards stronger conspecific odour preferences by benthic females. Moreover, when able to use all sensory modalities, Rundle *et al.* (2000) found the probability of a stickleback spawning with a conspecific to be about 2.5 times the

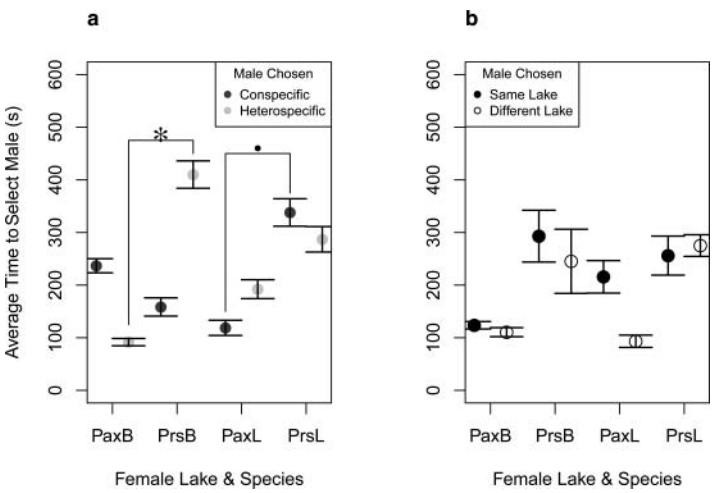

Fig. 3. Time taken by females from the two populations to select between males of (a) different species and (b) different lakes. Females from Paxton Lake took less time than females from Priest Lake when choosing a limnetic male over a benthic male. Untransformed means are shown, but analyses used a square-root transformation of the response variable to fit normality assumptions (Tukey's HSD; •$P = 0.05$; *$P = 0.009$). Symbols denote means, while error bars denote standard errors. PaxB = Paxton benthic female; PrsB = Priest benthic female; PaxL = Paxton limnetic female; PrsL = Priest limnetic female.

probability of spawning with a heterospecific. Kozak *et al.* (2009) found similar degrees of conspecific preference reported by Rundle and colleagues, not only in spawning probabilities but measures of female behaviours that precede spawning [estimated odds ratio from Kozak *et al.* (2009): 1.04, 95% CI = 0.70–1.53]. It is interesting to note that the preference of both species of females for conspecifics over heterospecifics is more similar when all sensory modalities are available, but is asymmetric when only olfaction is used. Because the probability of heterospecific spawning is low (Rundle *et al.*, 2000; Kozak *et al.*, 2009), even slight differences in preference may contribute to the strong reproductive isolation that exists in these species (Lackey and Boughman, 2014).

Olfactory perception is influenced by the local habitat (Heuschele and Candolin, 2007; Heuschele *et al.*, 2009), and there is good evidence that the use of olfaction in sticklebacks for discrimination tasks has evolved in response to the environment, and is inherited. Fish bred and reared in common laboratory conditions show population-level differences in olfactory use (Hiermes *et al.*, 2015). Furthermore, the population differences Hiermes and colleagues found appear to correlate to the visual environment; offspring of fish from tea-stained lakes are more prone to use olfaction than offspring of fish from clear lakes, which indicates that there is selection for olfaction when visual perception is constrained.

This leaves several questions about the interaction of senses and environments in decision-making. Chemical cues may interact with other cues to modulate information about the sender (Partan and Marler, 1999; Munoz and Blumstein, 2012), or be used at particular stages of mating to draw attention to the signaller (Rowe, 1999). Thus, chemical communication may interact with known differences between benthic–limnetic pairs in a number of traits, such

as body shape, size, colour, visual perception (reviewed in McKinnon and Rundle, 2002), and the lateral line system (Wark and Peichel, 2010).

That all populations of females were more likely to choose benthic male odours than limnetic male odours from a different lake is surprising. One use of olfaction in a number of vertebrates, including sticklebacks, is to evaluate the major histocompatibility complex (MHC) (Aeschlimann *et al.*, 2003; Milinski *et al.*, 2005). The MHC encodes a number of proteins important to immune system functioning; by selecting a mate with an optimal complementary set of MHC alleles, the heterozygote offspring produced may be protected from a broader array of pathogens (Aeschlimann *et al.*, 2003; Milinski *et al.*, 2005). Benthic females may be selecting male odour from the other lake because males from different lakes have more dissimilar MHC alleles. Although differences exist between lakes and species in MHC diversity (Matthews *et al.*, 2010), additional data on the alleles themselves are needed to see how this would affect interspecific preferences. For limnetic females, selecting a benthic male from a different lake may be a product of reproductive isolation evolving independently in each lake. Reinforcement increases pre-zygotic mating barriers between hybridizing populations due to selection against heterospecific mating (Servedio and Noor, 2003). In benthic–limnetic pairs this is manifested in sexual and ecological selection against hybrid offspring (Vamosi and Schluter, 1999; Gow *et al.*, 2007; Hendry *et al.*, 2009). But the species pairs in Paxton and Priest Lakes have arisen independently of one another (Taylor and McPhail, 2000), and discrimination against heterospecifics due to reinforcement is a product of selection only within lakes. Females may be especially likely to reject a heterospecific from their own lake, and discrimination against heterospecifics from other lakes may be but an extension of this bias. Discrimination against heterospecifics within lakes is stronger than that between lakes when fish are able to use multiple types of cues in mate choice (Rundle and Schluter, 1998; Rundle *et al.*, 2000). It would be interesting to see if these patterns persist in the absence of olfactory cues.

These results further indicate that although odour is more similar within than between species, populations of species from different lakes still differ in their chemical cues. Odour is comprised of multiple components and derived from a number of ecological factors, and may even be altered due to individual activity and changes over seasons (Sommerfeld *et al.*, 2008). In addition to the MHC complex, populations may differ in the glue (i.e. spiggin) used to make a nest, as there are multiple spiggin alleles and these differ between some populations of stickleback (Seear *et al.*, 2015). Dietary differences also influence individuals' olfactory signature and can affect responses in social context (Ward, 2004), and might also be used to distinguish species. Furthermore, responses to odour cues can also be plastic, with exposure to common resources altering association preferences in only a few hours (Webster *et al.*, 2007). As our aim was not to determine the nature of genetic or environmental effects, but to see how females use olfaction during the breeding season, we reflected natural differences between populations by using wild-caught fish sustained on species-specific diets, and housed in population-specific tanks (Larson, 1976). While this would accentuate odour differences between the species, it means we cannot determine the role of diet in odour discrimination.

When choosing between options that are equally preferable, organisms may take longer to make a decision when information is available on each option than when no information is present (Bernays and Wcislo, 1994). In both species, Paxton females were quicker to choose a limnetic male odour, which indicates there is a difference in how benthic and limnetic scents are compared in each lake. The quick selection of heterospecific odour by Paxton benthic

females may be a speed–accuracy trade-off (Wickelgren, 1977). Priest benthics may be more opposed to the scents of limnetics. As such, Priest benthics spend more time evaluating odours used to discriminate species than do their Paxton counterparts, where the accuracy in choosing a conspecific over a heterospecific odour is impaired by choosing quickly. However, the longest latencies were by Priest benthic females that chose the limnetic odour over the benthic odour, an apparently inaccurate decision, and one that rarely occurred. Why fish that took longer would make inaccurate decisions is unclear, but determining the neural mechanisms that underlie these decisions may enable their explanation (Chittka *et al.*, 2009).

Olfaction is recognized as an important modality to facilitate speciation in a number of taxa (Smadja and Butlin, 2008), and many stickleback populations have olfactory preferences (McLennan, 2003, 2004; Hiermes *et al.*, 2015). Although this suggests that the marine ancestor of the benthic–limnetic pairs used olfaction in mate choice, vision may take precedence in light environments that transmit nuptial colour, and olfaction may only become important following the invasion of new habitats, which impose new sensory demands and constraints. Has the behavioural incorporation of odour in mating decisions been lost repeatedly in the limnetics, or has it appeared multiple times in the benthics and photically constrained populations? The behavioural role and evolution of olfactory perception in this system remains a promising avenue for future research.

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