Olfactory mate recognition in a sympatric species pair of three-spined sticklebacks

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Mate recognition is critical to the maintenance of reproductive isolation, and animals use an array of sensory modalities to identify conspecific mates. In particular, olfactory information can be an important component of mate recognition systems. We investigated whether odor is involved in mate recognition in a sympatric benthic and limnetic species pair of three-spined sticklebacks (*Gasterosteus* spp.), for which visual cues and signals are known to play a role in premating isolation. We allowed gravid females of each species to choose between water scented by a heterospecific male and water scented by a conspecific male. Benthic females preferred the conspecific male stimulus water significantly more often than the heterospecific male stimulus water, whereas limnetic females showed no preference. These species thus differ in their odor and may also differ in their use of olfaction to recognize conspecific mates. These differences are likely a consequence of adaptation to disparate environments. Differences in diet, foraging mode, habitat, and parasite exposure may explain our finding that odor might be an asymmetric isolating mechanism in these sympatric stickleback species. *Key words: Gasterosteus aculeatus*, mate recognition, odor, olfaction, reproductive isolation, three-spined sticklebacks. *[Behav Ecol 17:965–970 (2006)]*

Mate recognition is critical to the maintenance of reproductive isolation, particularly when gene flow is still possible between recently evolved sympatric species. Identifying the mechanisms of mate recognition can thus further our understanding of speciation as the traits that species use to recognize conspecific mates may underlie species divergence (Andersson 1994). If, for instance, the traits that coexisting species rely on to identify conspecific mates differ in conjunction with the environment each inhabits, then ecological speciation is implicated (Schluter 2001).

Isolating traits that provide a means of discriminating between conspecifics and heterospecifics may evolve through several different mechanisms, including reinforcement (Noor 1995; Sætre et al. 1997; Rundle and Schluter 1998; Nosil et al. 2003), adaptation to different ecological niches (Rundle et al. 2000; Nosil et al. 2002, 2003), and direct selection (Albert and Schluter 2004). The ability to recognize conspecific mates can thus be under strong selection, and animals accomplish this task with a wide array of sensory modalities, including behavioral, visual, olfactory, auditory, and tactile cues and signals.

Evidence for the importance of olfaction in conspecific recognition comes from a diversity of taxa, including salamanders (Dawley 1984), mice (Heth et al. 2003), and many insects (Singer 1998). In various *Drosophila* species, cuticular hydrocarbons serve as pheromones that function in olfactory-based mate recognition (Coyne et al. 1994; Blows and Allan 1998). Several species of fish are known to use odor to discriminate between conspecifics and heterospecifics (e.g., McKinnon and Liley 1987; McLennan and Ryan 1997; Strecker and Kodric-Brown 1999). In fact, olfactory mechanisms of mate recognition are likely widespread, as many animals, including almost all vertebrate taxa, use chemical stimuli in their interactions with conspecifics (Sorensen and Stacey 1999).

In the stickleback family (Gasterosteidae), olfaction plays a role in mate detection and mate choice within species.

Female fifteen-spined sticklebacks (*Spinachia spinachia*) preferred water scented by a nesting male over water without a male (Ostlund 1995). Similarly, female three-spined sticklebacks (*Gasterosteus aculeatus*) can distinguish between water scented by males with nests and water scented by males without nests (Häberli and Aeschlimann 2004). Some evidence suggests that they may also be able to distinguish between water scented by a displaying male and water scented by a nondisplaying male (Waas and Colgan 1992). Compelling evidence that odor is involved in mate choice comes from three-spined sticklebacks in Germany: gravid females are able to discriminate between conspecific males with different numbers and diversities of alleles at the major histocompatibility complex (MHC) loci by odor alone (Reusch et al. 2001; Aeschlimann et al. 2003; Milinski et al. 2005).

Several members of the Gasterosteidae also use odor in mate recognition to discriminate between conspecific and heterospecific mates. Male brook sticklebacks (*Culaea inconstans*) favored the odor of conspecific females more than that of three-spined stickleback females (McLennan 2004). Similarly, female brook and three-spined sticklebacks preferred conspecific more than heterospecific male odor (McLennan 2003). Thus, brook, fifteen-spined, and three-spined sticklebacks are sensitive to olfactory stimuli, and there is evidence that they rely, in part, on these stimuli to recognize conspecifics.

Here, we focus on sympatric species pairs of benthic and limnetic three-spined sticklebacks (*G. aculeatus* species complex). These pairs evolved from the marine three-spined stickleback, likely after 2 colonization events in multiple lakes in British Columbia, Canada, 10 000–12 000 years ago (McPhail 1993, 1994). Although benthics and limnetics are capable of hybridizing, they do so at a low, stable rate and maintain separate gene pools (McPhail 1984, 1992; Gow et al. 2006). Mate recognition is likely critical to their reproductive isolation. Among benthic and limnetic species pairs of three-spined sticklebacks, visual cues and signals, such as body size and color, are known to play a role in premating isolation and the identification of conspecific mates (Nagel and Schluter 1998; Boughman 2001). Both traits have diverged between benthics and limnetics because of differences in their ecology (Boughman et al. 2005).

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Ecological differences between benthics and limnetics may also affect odor and odor perception. Whereas benthics feed mainly on invertebrates in the littoral zone (McPhail 1984, 1992) and might use olfactory cues to detect prey, limnetics, which are smaller (Schluter and McPhail 1992), feed on zooplankton in the pelagic zone (McPhail 1984, 1992) and are likely visual foragers (Bell and Foster 1994). Diet has been shown to affect odor in sticklebacks (Ward et al. 2004, 2005). Furthermore, the MHC influences three-spined stickleback odor (Reusch et al. 2001), and there is reason to expect that benthics and limnetics might have different MHC alleles.

The role of olfaction in conspecific mate recognition has not previously been investigated in sympatric species pairs of three-spined sticklebacks. We sought to determine whether females of a benthic and limnetic species pair of three-spined sticklebacks are able to discriminate between conspecific and heterospecific males by odor alone and thus whether odor might function as a premating isolating mechanism. We measured the preference of gravid females of each species for water scented by conspecific and heterospecific males. We predicted that if female three-spined sticklebacks can identify conspecific males by odor, they should choose to spend more time in conspecific-scented stimulus water than in heterospecific-scented stimulus water. If, on the other hand, females are unable to recognize their species by odor, we predicted that they would show no preference for water scented by conspecific males.

MATERIALS AND METHODS

Experimental animals

Reproductively mature three-spined sticklebacks from Paxton Lake (49°43′N, 124°30′W), British Columbia, Canada, were caught in minnow traps and transported to Madison, Wisconsin, USA, in April and June 2005. Benthics and limnetics were held in separate 110-l glass aquaria and maintained on a 12:12 h light:dark cycle at 16 °C. Males and females were housed together. Fish were fed frozen *Artemia* in excess once a day.

From the holding aquaria, we chose males that were expressing breeding coloration to place individually in visually isolated 76-l nesting aquaria. The same type of nesting material was added to each aquarium, and males were allowed to build nests. Every few days until they built nests, males were enticed with conspecific gravid females that were placed in jars outside of each nesting aquarium. Females were not used again that day.

Olfactory choice trials

To test whether females could distinguish between conspecific and heterospecific males by odor alone, dichotomous choice trials were conducted in a flow channel (122 × 33 cm) through which deionized water flowed continuously (Figure 1). Deionized water was supplied by 2 plastic hoses (1.0 cm in diameter) attached to the inside wall of the inlet compartment. A constant flow rate and a water level of 4.5 cm were maintained during all trials. The inlet compartment was divided into half longitudinally by a partition (76 × 52 cm). The female test area was enclosed by 2 screens, and the middle dividing line was marked from above. Water flowed out of the tank through 3 plastic hoses (1.0 cm in diameter) inserted into holes in the outlet compartment. A bridge spanned the tank above the inlet and held a peristaltic precision pump (Watson-Marlow Bredel 401U/DM2) and 2 trays. The pump was used to add stimulus water from each tray to different halves of the flow channel via 2 marprene tubes (Watson-Marlow, 1.85 mm in diameter). The flow channel was tested
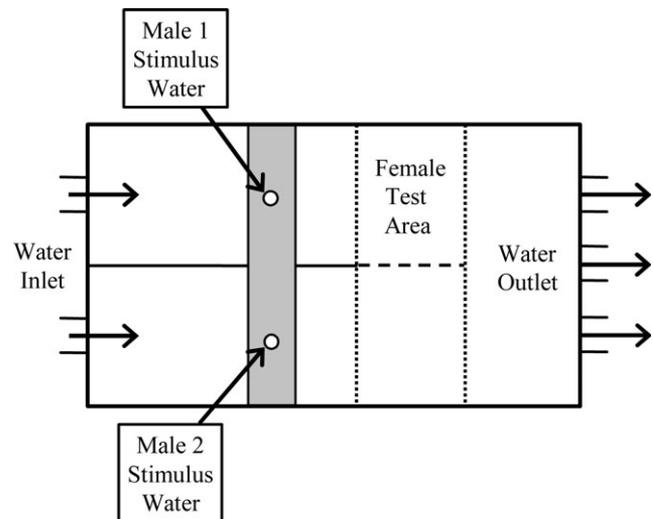

Figure 1
Flow channel (122 × 33 cm) used to conduct dichotomous olfactory choice trials (viewed from above). Water entered the channel through 2 hoses at the inlet end, flowed through the tank, and exited through 3 hoses at the outlet end. A plastic partition (solid line; 76 × 52 cm) divided the tank into half longitudinally from the inlet end to the edge of the female test area. The female test area was enclosed by 2 mesh screens (dotted lines), and the middle was marked from above (dashed line). A bridge (gray bar) spanned the channel and held a peristaltic precision pump and 2 trays with stimulus water from 2 males. The pump drew the water from each tray into 2 tubes that were positioned to drip into the channel through 2 holes in the bridge.

multiple times with colored water to ensure that the water in the 2 halves did not mix. In general, we followed the flow tank design used by Aeschlimann et al. (2003).

For each trial, a 500-ml sample of water was taken from near the nests of both a benthic and a limnetic male. Each male was then placed in the water sample taken from his aquarium for 20 min, after which the males were returned to their nesting aquaria. The presence or absence of red and blue breeding coloration was noted for each male, and paired males were matched roughly for color intensity and the date they were placed in nesting aquaria. Although all males were in nesting aquaria for similar periods, benthics consistently took longer to construct their nests and thus necessarily were paired with limnetics that had constructed nests several days earlier. We allowed at least 3 days to elapse from the time a male was placed in a nesting aquarium and when he was used in a trial. The stimulus water from each pair of males was used in 2 trials, once with a benthic female and once with a limnetic female. In total, 12 trials were conducted with 6 benthic and 6 limnetic females, using stimulus water from 6 different pairs of benthic and limnetic males.

Half (250 ml) of the stimulus water sample from each male was placed in a tray on the bridge. A gravid female who was ready to spawn was placed in the female test area and allowed to acclimatize in neutral water for 7 min. During this time, the female was required to cross the middle of the test area to indicate that she was aware of both sides of the tank and not overly stressed. Stimulus water from each male was then added (18 ml min^{-1}) for 7 min, followed by 7 min of neutral water, and 7 additional min of stimulus water. Intervals of 7 min were chosen to ensure that all of the stimulus water was removed from the tank during the periods of neutral water. During the second 7 min of stimulus water, the sides were switched to control for a female side preference. In every

trial, females crossed the dividing line during both 7-min periods of stimulus water.

The female's behavior was recorded by an observer who was blind to the source of the stimulus water, which was coded by another person. The observer was visually screened from the female and used an event recorder to record which side of the test area the female was on.

The second half (250 ml) of the stimulus water sample, which was kept covered with parafilm, was used in a second trial within 1.5 h of the first trial in all but one case. In this case, fresh samples were taken from nesting aquaria, and the males were placed in the samples for 20 min. After each trial, the flow channel, pump tubing, and trays were drained, wiped with 100% ethanol, and dried.

Data analysis

Because trials varied in duration by a few seconds, we calculated the proportion of time that females spent on each side of the flow channel during the first 7-min stimulus period, the second 7-min stimulus period, and for both stimulus periods combined, and we used proportions in all statistical tests. Each period and the total trial were analyzed separately. Data were checked for normality with a Kolmogorov–Smirnov test and were found not to be normally distributed (KS $= 0.42$, $P = 0.001$). Based on this result and because sample size was small, we performed nonparametric tests.

To determine if females preferred conspecific male odor, we tested if the proportion of time that females spent on the conspecific male side was greater than the random expectation of 0.5 with Wilcoxon signed-rank tests. To exceed the random expectation, females must be able to discriminate between conspecific and heterospecific odor and prefer conspecific odor. We also performed exact binomial tests to determine if females of each species preferred the conspecific male side in more than the expected number of trials. To test whether the species differed in the proportion of time that they spent on the conspecific male side when presented with the same pair of males, we used 2-tailed Wilcoxon signed-rank tests.

Values are reported as means $\pm$ standard error (SE). All statistical tests were conducted in S-Plus (Mathsoft, Seattle, Washington), and statistical significance was set at $\alpha = 0.05$.

RESULTS

During the first stimulus period, benthic females consistently preferred the conspecific male side (Wilcoxon signed-rank test: $Z = 2.21$, $P = 0.01$; Figure 2a). Limnetic females, however, did not demonstrate a preference for the side of the flow channel with conspecific male stimulus water in the first stimulus period (Wilcoxon signed-rank test: $Z = 0.73$, $P = 0.77$; Figure 2a). Benthic females chose the conspecific male side all 6 times (exact binomial test: $N = 6$, $P = 0.02$). Limnetic females favored the conspecific side only twice (exact binomial test: $N = 6$, $P = 0.89$). Benthic females spent significantly more time than limnetic females on the conspecific male side in the first stimulus period (Wilcoxon signed-rank test: $Z = 1.99$, $P = 0.046$).

In the second stimulus period, neither benthic (Wilcoxon signed-rank test: $Z = 1.15$, $P = 0.12$) nor limnetic (Wilcoxon signed-rank test: $Z = 0.10$, $P = 0.46$) females spent significantly more time on the conspecific male side (Figure 2b). Benthic females chose the conspecific side 4 out of 6 times (exact binomial test: $N = 6$, $P = 0.34$), and limnetic females chose the limnetic male side only 3 times (exact binomial test: $N = 6$, $P = 0.66$). The difference between the proportion of time that benthic and limnetic females spent on the
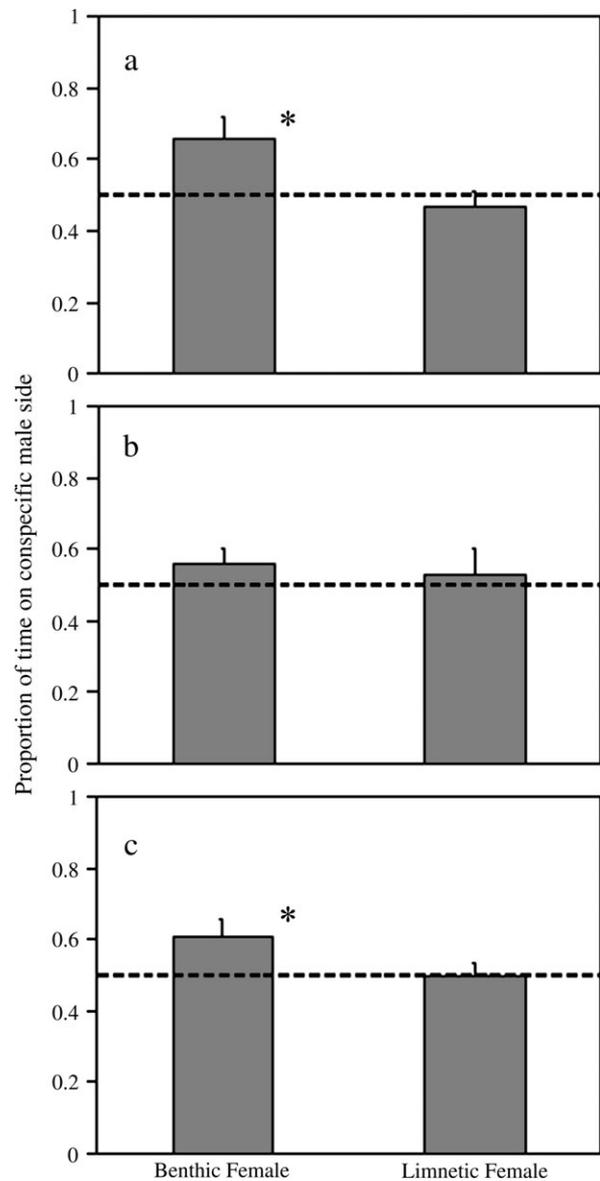

Figure 2
Mean proportion of time $\pm$ SE that benthic and limnetic females spent on the conspecific male side of the flow channel during (a) the first stimulus period, (b) the second stimulus period, and (c) both stimulus periods combined for 6 trials. *$P < 0.05$.

conspecific male side in the second stimulus period was not significant (Wilcoxon signed-rank test: $Z = -0.11$, $P = 0.92$).

When both stimulus periods were combined, benthic females spent a significantly greater proportion of time on the side of the flow channel with benthic male stimulus water (Wilcoxon signed-rank test: $Z = 2.21$, $P = 0.01$; Figure 2c). This was not the case for limnetic females (Wilcoxon signed-rank test: $Z = 0.11$, $P = 0.46$; Figure 2c). The mean proportion of time that females spent on the side with conspecific stimulus water was 0.61 ± 0.05 for benthics and 0.50 ± 0.03 for limnetics. Overall, benthic females chose the conspecific male side 6 out of 6 times (exact binomial test: $N = 6$, $P = 0.03$; Figure 3). Limnetic females chose the conspecific male side only 3 out of 6 times (exact binomial test: $N = 6$, $P = 1$; Figure 3). On the whole, benthic females spent significantly more time on the conspecific male side than did limnetic females (Wilcoxon signed-rank test: $Z = 1.99$, $P = 0.046$).
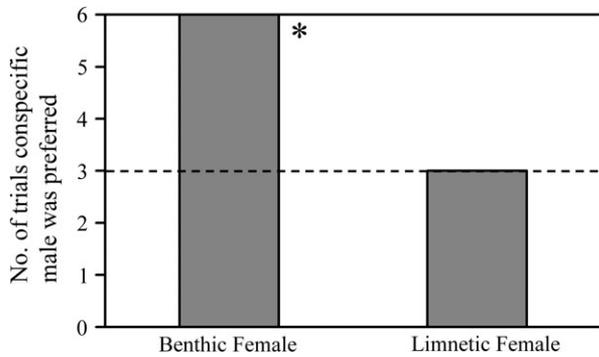
Figure 3
Number of trials (out of 6 total) in which benthic and limnetic females spent more than 50% of the time on the conspecific male side. *$P < 0.05$.

DISCUSSION

Our results reveal that benthic females recognize males of their own species by odor, whereas limnetic females may not. Female benthic three-spined sticklebacks preferred conspecific male odor, spending more time in water scented by conspecific males in all 6 trials and a significantly greater proportion of time in water scented by conspecific males overall. We did not detect a preference on the part of limnetic females; thus, they may have been unable to discriminate between conspecific and heterospecific male odor or may have no preference based on odor. Alternatively, our negative result could be due to a lack of statistical power. Yet, despite small sample sizes, we have fairly strong evidence that benthic females responded more strongly to conspecific male odor than did limnetic females. This was particularly true during the first stimulus period, when female response was less likely to be affected by complicating factors, such as decreased interest. Our findings support the hypothesis that odor can play a role in mate recognition for benthics, advancing our understanding of premating reproductive isolation in this species pair.

Previous work has shown that body size and color contribute to premating isolation in the benthic–limnetic species pairs. Body size differences are used by both species to recognize conspecific mates (Nagel and Schluter 1998; Boughman et al. 2005) and also play a role in premating isolation among anadromous-stream pairs worldwide (McKinnon et al. 2004). Conversely, differences in color and color preference contribute in an asymmetric way to premating isolation between benthics and limnetics. Benthics mate in deeper water, where illumination is predominantly at the red end of the spectrum and where red signals have low contrast. The result is that benthic males have reduced nuptial color, and benthic females have weak preferences for red. Limnetics, on the other hand, mate in shallower water where red males are more visible. Consequently, limnetic males have more red color, and limnetic females have a strong preference for red males (Boughman 2001). Limnetic females discriminate against benthic males on the basis of color, and thus, color contributes to premating isolation in this direction. In contrast, color undermines premating isolation in the other direction, as benthic females are more likely to mate with brightly colored limnetic males. Therefore, color alone is not sufficient for premating reproductive isolation in this system (Boughman et al. 2005).

We now have evidence that, like color, odor may be an asymmetric isolating mechanism. Because males glue their nests together with an adhesive protein called spiggin that they secrete from their kidneys (Jakobsson et al. 1999; Jones et al. 2001), odor may be particularly important when females

inspect nests, one of the final phases of courtship and mate choice. Together with body size, odor, and color provide benthics and limnetics each with 2 reliable mechanisms of mate recognition: benthics rely on odor and size, whereas limnetics may rely on color and size. Our data thus provide further evidence that the way by which benthics and limnetics recognize conspecific mates may differ, possibly as a consequence of ecology.

In these species pairs, body size and breeding color have diverged in parallel as a consequence of adaptation to different environments (Nagel and Schluter 1998; Rundle et al. 2000; Boughman 2001; Boughman et al. 2005). Body size and color differences reflect adaptations to different foraging niches (Schluter and McPhail, 1992; Schluter 1994) and light environments (Boughman 2001), respectively. Similarly, ecological differences can potentially explain our finding that benthics and limnetics differ in their odor and, possibly, in their use of olfaction in mate recognition.

What might cause odor differences? Both environmental and genetic factors may be involved. Diet affects odor in sticklebacks (Ward et al. 2004, 2005), as well as in other fish species (Bryant and Atema 1979, 1987; Olsen et al. 2003). Thus, the different diets of benthics and limnetics are likely to influence their odors. Interestingly, our findings point to an additional, genetic component to odor differences among stickleback species. The wild-caught sticklebacks used in this study were fed on a common diet in the lab, which may have reduced diet-based odor differences (Ward et al. 2005). Thus, it is unlikely that the benthic females in our study are relying solely on diet cues to discriminate between conspecific and heterospecific males. The genetic component of odor differences could lie in the MHC. Parasite communities tend to differ in littoral and pelagic zones (Dorucu et al. 1995; Knudsen et al. 1997). Thus, benthics and limnetics are likely to be exposed to different parasites, which might select for different MHC alleles (Wegner, Kalbe et al. 2003; Wegner, Reusch, and Kalbe 2003) and result in odor differences (Milinski et al. 2005).

Different foraging strategies may also cause benthics and limnetics to differ in their dependence on olfaction. Benthics feed on larger prey that may require less visual resolution (Bell and Foster 1994) and may use chemical and tactile cues in foraging, whereas limnetics have the larger eyes (McPhail 1984; Baumgartner et al. 1988) and visual acuity that are necessary for feeding on zooplankton (Bell and Foster 1994). Furthermore, zooplankton use chemical cues to detect predators (Larsson and Dodson 1993); thus, limnetics may be selected to be relatively odorless.

If benthics are more sensitive to odor because they are more reliant on olfactory cues for foraging, then benthic males might exploit this enhanced perception to attract females. Similarly, if limnetics are more reliant on vision for foraging, then males might employ visual signals to attract females. That limnetic females have a stronger preference for colorful males than do benthic females (Boughman 2001) supports this possibility. Furthermore, each species might be expected to be more discriminating in the modality to which they are most attuned. Such sensory bias has been implicated in mate choice in several taxa and modalities (e.g., Basolo 1990; Ryan et al. 1990; Proctor 1991; McClintock and Uetz 1996; Rodd et al. 2002;), and a bias for red color has been shown for three-spined sticklebacks (Smith et al. 2004).

Research in recent years has revealed much on the role that divergent natural selection has played in adaptive evolution and speciation (Schluter 2001; Nosil et al. 2002; Allender et al. 2003; Rundle and Nosil 2005; Funk et al. 2006), and work on three-spined sticklebacks has been at the hub of these advances (Rundle et al. 2000; Schluter 2001; McKinnon et al. 2004). However, this work has focused primarily on morphological

traits such as trophic morphology, body armor, and shape (Bentzen and McPhail 1984; McPhail 1984; Schluter and McPhail 1992; Schluter 1993; Vamosi and Schluter 2004). Much less is known of evolved differences between the stickleback species pairs in behavioral traits, including the criteria by which they select mates and avoid mating with heterospecifics—traits that are likely due to a combination of divergent sexual selection and natural selection. Our results suggest that future work investigating habitat-induced differences in odor and odor perception will add to our understanding of how reproductive isolation has evolved in these species. Directly relating the potentially asymmetrical use of odor in mate recognition to differences in the ecology of benthic and limnetic species could strengthen the already well-supported case for ecological speciation in the divergence of these stickleback species pairs (Schluter, 1994; Rundle et al. 2000; Boughman et al. 2005).

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