Learned conspecific mate preference in a species pair of sticklebacks

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Sexual isolation between species often depends on preferences for conspecific mates. Recent models suggest that whether conspecific preference is determined genetically or learned will affect the process of speciation: Learned conspecific preferences might make speciation more likely. However, we understand little about how often and for which taxa, conspecific preferences are learned. Some species learn conspecific preference by imprinting on conspecifics; others learn from experience with heterospecifics. Even when one sex learns conspecific preference, the other may not. We tested whether conspecific mate preference is learned through social experience in males and females from 2 three-spined stickleback species that show strong sexual isolation (benthics and limnetics: \textit{Gasterosteus} spp.). We reared fish with either mostly conspecifics or mostly heterospecifics and measured how this experience affected conspecific preference. In both sexes, experience enhanced conspecific mate preference but the sexes differed in the outcome. Females learned to prefer their own species through experience with conspecifics; males learned to discriminate through experience with heterospecifics. We also found species differences in the effect of social experience related to differences in sociality. Our results suggest that learned conspecific mate preference may have facilitated rapid speciation in the post Pleistocene radiation of sticklebacks. \textit{Key words:} learning, mate recognition, sexual isolation, speciation, sticklebacks. [\textit{Behav Ecol} 20:1282–1288 (2009)]
encountered members of the opposite sex (Hebets 2003; Fincke et al. 2007). In sticklebacks, fathers care for the eggs and after hatching guard the offspring for several days (Tulley and Huntingford 1987; Rowland 1994). After this short period of parental care, sticklebacks associate in groups with siblings early in life and form larger groups with other juveniles as they age (FitzGerald and Morrissette 1992; Frommen et al. 2007; Kozak and Boughman 2008). Thus, experience with siblings and other juveniles could be an important source of information for the development of mate preferences. Fish raised with their own species we term majority fish because the majority of the fish in their tanks were conspecifics (mean number of conspecifics = 87%). Accordingly, minority fish are those raised with the other species (mean number of conspecifics = 16%). When fish were adults, we tested conspecific mate preferences and compared them between majority and minority fish.

If preference was learned through experience, we predicted that rearing environment would alter the strength of conspecific mate preference. If fish learned through imprinting on siblings, they would prefer familiar phenotypes (conspecifics when raised with conspecifics). Alternatively, if fish learned to discriminate, they would learn to distinguish through experience with heterospecifics and reject heterospecifics more when they were raised with them. However, if preference was genetically inherited then rearing environment would have no effect on preference: conspecifics would always be preferred over heterospecifics as in wild fish (Rundle et al. 2000). Furthermore, we know that morphological cues such as body size and male nuptial color are important to conspecific mate preference in these species (Nagel and Schluter 1998; Boughman 2001; Boughman et al. 2005). Thus, we estimated the influence of rearing environment on mate preference along with the influence of body size and male color.

We also predicted that the effect of experience on conspecific mate preference could differ between the sexes and the species. In the stickleback species pairs, females determine whether heterospecific matings occur (Kozak et al. 2009). Females strongly prefer conspecifics. Males will court both species of females but alter courtship based on a female’s species. Thus, both males and females recognize the differences between conspecific and heterospecific preferences. The selective pressures that have led to sex differences in conspecific preference might also lead to differences in how experience influences recognition and preference of conspecifics. The effect of experience could also differ between species. Limnetics and benthics differ in sociality: limnetics are social; benthics are not (Vamosi 2002). We predicted that a more social species would be more affected by an alteration in the social environment.

MATERIALS AND METHODS

Rearing treatments

We collected adult benthic and limnetic three-spined sticklebacks (Gasterosteus spp.) from Paxton Lake, British Columbia in 2005. All experimental procedures received approval from the University of Wisconsin—Madison Animal Care and Use Committee (Protocol no. L00317). In petri dishes, we fertilized eggs stripped from females with male sperm separately for each species (Kozak and Boughman 2008). To manipulate experience with conspecifics, we placed 35 eggs in an eggcup and manipulated the proportion of benthic and limnetic eggs. Benthic and limnetic eggs were placed on either side of a window screen divider in the eggcup, so we could track hatching separately. In minority treatments, 2–5 of 35 eggs were conspecific. In majority treatments, 30–33 of 35 eggs were conspecific. Additionally, we had 2 tanks with more equal ratios (15–20 of 35 eggs) and 4 that received only conspecific eggs. When the fish hatched, fry fell to the bottom of the 101-l tank. After hatching, we counted the number of benthic and limnetic fry in the tank and calculated the ratio of conspecifics to the total number of fish of each species. In minority tanks, the ratio = 0.04–0.24 (limnetics n = 7 tanks, benthics n = 8 tanks). In equal tanks, the ratio = 0.43–0.57 (limnetics n = 2 tanks, benthics n = 2 tanks). In majority tanks, the ratio = 0.76–0.96 (limnetics n = 11 tanks, benthics n = 10). In all conspecific tanks, the ratio = 1.0 (limnetics n = 2 tanks, benthics n = 2). Ratios varied slightly due to egg mortality. Based on the final distribution of ratios, we reclassified all tanks as either minority or majority. Tanks were classified as minority if the ratio was between 0.04 and 0.43 (mean ratio ± standard error [SE] = 0.16 ± 0.11: limnetics n = 9 tanks, benthics n = 8 tanks) or majority if the ratio was between 0.57 and 1.0 (mean ratio = 0.87 ± 0.11: limnetics n = 13 tanks, benthics n = 14 tanks). Using ratio as a continuous factor in statistical models yields similar results to those reported below. All individuals in the tank interacted freely. Densities in the tanks were adjusted before 9 weeks of age to below 45 fish, whereas maintaining the ratio of conspecifics to heterospecifics. Tanks were visually and chemically isolated from one another. Males and females are indistinguishable until sexual maturity and no courtship or mating occurred prior to testing.

Female conspecific preference test

Stickleback female mate choice is expressed during complex courtship interactions with males. Females spawn with preferred males and terminate courtship with unpreferred males (Rowland 1994). We measured preference for each female in 2 no-choice tests: one with a conspecific male and one with a heterospecific male (in random order) (Albert 2005). Females were placed in a 101-l tank with a male (wild-caught in 2006) that had a nest. We recorded all behaviors of the male and female for 20 min or until spawning occurred using an event recorder (Observer: Noldus Technologies, Wageningen, The Netherlands). Females were held in small tanks for 30 min before the first trial (to acclimate to the room) and 2 h between first and second trials (resting time). Trial number had no effect on preference. After the final test, we measured female length with vernier calipers and verified she had eggs ready to be released. We measured male length and throat color (color index = red area + intensity score [Boughman 2007]). We tested 1–4 females from each family (each species in each tank). We included only responsive females (which showed interest in at least one of the 2 males). We tested a total of 39 limnetic females (from 22 tanks: 9 minority, 15 majority) and 38 benthic females (from 21 tanks: 7 minority, 14 majority). Each pair of stimulus males was used for both a benthic female and a limnetic female (order random with respect to female species). A male could be re-paired for an additional trial with a female from a different experience level. We calculated a female preference score for each male: ranging from no behavioral interest (score = 0) to spawning (female entered the nest to lay her eggs, score = 4). Intermediate scores represented behaviors of moderate interest: approaching the male (score = 1), following the male toward the nest (score = 2), and investigating the male’s nest (score = 3). We analyzed female preference score using family means for cons- and heterospecific males in repeated measures analysis of covariance (ANCOVA) (mixed model with compound symmetry) in SAS statistical software v.9.1 (SAS Institute Inc 2007). We conducted post hoc tests within each species (Bonferroni corrected: 12 total) to determine significant differences in female preference.
depending on experience levels (majority vs. minority) and male species (conspecific vs. heterospecific).

Male conspecific preference test

We used dichotomous choice tests to measure male conspecific mate preference. Individual sexually receptive males were placed in a 1014 tank with a nesting tray filled with sand and filamentous algae. To stimulate nest building and maintenance, we presented males with a sexually mature conspecific female inside a glass jar for 10 min every other day. This exposure allowed only minimal courtship interaction. When a male had built a nest, we tested him, modifying a protocol from Albert and Schluter (2004). We presented wild-caught females in a clear Plexiglas 3-sided container divided into 2 compartments (each 17.5 cm long × 13 cm wide × 17.5 cm high) and suspended from the top of the tank. The rear wall of the tank acted as the rear wall of the container as well, allowing some exchange of water between the container and the tank. A black plastic divider extended 11 cm down and 15 cm out from the front of the container, forcing males to only direct courtship to one female at a time. We placed a benthic female in one randomly selected compartment and a limnetic female in the other. After an acclimation period, we removed an opaque plastic divider and the male approached the females from the front of the tank. We recorded all courtship behaviors the male directed to each female over 10 min in Observer. Each male was tested again on the following day with a different pair of females (female species position reversed relative to the first test). We tested 1–4 males from each family and included only males that performed at least 1 behavior to each female. We tested 43 limnetic males (from 20 tanks: 8 minority, 12 majority) and 48 benthic males (from 21 tanks: 8 minority, 13 majority). We reused a pair of females for a male of one species if they had been previously seen by a male of the other or for a second trial if they had been used in another male’s first trial. We measured 3 key elements of courtship: zigzags, bites, and leads (Rowland 1994), and we summed these 3 behaviors to estimate courtship vigor (Kozak et al. 2009). For each behavior, we calculated the difference in the number of behaviors to con- and heterospecific females out of the total number behaviors in a trial (e.g., [number of zig conspecific – number of zig heterospecific]/[number of behaviors conspecific + number of behaviors heterospecific]). We took the mean of a male’s 2 trials and analyzed family means for each score using ANCOVA in SAS.

Our design allows us to identify sex differences in the outcome of experience, despite differences in male and female tests. Previous work in our species suggests that measures of male behavior are comparable between no-choice and choice tests (e.g., limnetic males zigzag more to limnetic females than to benthic females [Albert and Schluter 2004; Kozak et al. 2009]). More generally, no-choice and choice tests have been shown to differ in power but measure preference similarly (Phelps et al. 2006). Thus, we can compare the direction of the effects of experience on conspecific preference between the sexes.

RESULTS

Female conspecific mate preference

Benthic and limnetic females differed in how social experience affected preference for conspecifics (Table 1). Limnetic females only preferred conspecifics when raised with them (Figure 1a). Majority limnetics showed a strong preference for limnetic males (conspecific vs. heterospecific male least squares mean ± SE = 1.31 ± 0.31, \( t_{65} = 4.10, P = 0.0002 \), Bonferroni corrected \( P = 0.0024 \)), but minority limnetics showed no preference for either type of male (conspecific vs. heterospecific male = \( -0.15 ± 0.35, t_{65} = -0.36, P = 0.7184 \)), Bonferroni corrected \( P = 0.99 \)). We found that minority limnetics were willing to mate with males of both species. The decrease in conspecific preference between majority and minority limnetics was significant (majority vs. minority = \( 1.44 ± 0.46, t_{65} = 3.14, P = 0.0034, \) Bonferroni corrected \( P = 0.041 \)). Thus, only majority limnetics showed a strong preference for their own species. Therefore, experience with conspecifics leads to a strong preference for cons- over heterospecific males; if experience is confined to heterospecifics this preference does not develop.

In contrast, both majority and minority benthics responded slightly more strongly to benthic over limnetic males (Figure 1b) but in neither case was this preference significant (minority conspecific vs. heterospecific = \( 0.77 ± 0.42, t_{65} = 1.84, P = 0.0741 \)), Bonferroni corrected \( P = 0.99 \); majority conspecific vs. heterospecific = \( 0.83 ± 0.30, t_{65} = 2.78, P = 0.0086, \) Bonferroni corrected \( P = 0.10 \)). Because there was no effect of experience (majority vs. minority = \( 0.05 ± 0.50, t_{65} = 0.10, P = 0.9181 \)), Bonferroni corrected \( P = 0.99 \), we pooled treatments and found that all benthic females show a weak but not quite significant preference for conspecific males (all conspecific vs. heterospecific = \( 0.71 ± 0.24, t_{65} = 3.00, P = 0.0049, \) Bonferroni corrected \( P = 0.059 \)). This preference is not altered by experience with either species.

In addition to experience, female preferences were affected by male characteristics. Males of both species develop red color in our study populations (Boughman 2001), and all females preferred males with redder throats (\( \beta = 0.147 ± 0.05, t_{65} = 2.96, P = 0.0056; \) Table 1). In wild populations, mating between species occurs primarily when males and females are close in size (Nagel and Schluter 1998; McKinnon et al. 2004; Boughman et al. 2005). Minority limnetic females strongly preferred males close in size. They mated with all but the largest fish, which were often very large benthic males (Figure 2). Majority limnetic females preferred limnetic over benthic males regardless of size. This suggests that minority limnetics may rely on extreme body size difference to reject heterospecific males, whereas majority limnetics rely on species identity, always rejecting heterospecifics. In benthics, majority females appeared to have a slight preference for males different in size, whereas minority females did not appear to use size. We do not know why majority benthics preferred males larger or smaller than themselves, as previous evidence of this pattern in sticklebacks is lacking. However, it is clear

### Table 1

<table>
<thead>
<tr>
<th>Test</th>
<th>Degrees of freedom</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experience (minority vs. majority)</td>
<td>1,35</td>
<td>2.92</td>
<td>0.0957</td>
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<tr>
<td>Female species (benthic vs. limnetic)</td>
<td>11.65</td>
<td>0.0015</td>
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<td>Experience × female species</td>
<td>1,35</td>
<td>1.64</td>
<td>0.2078</td>
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<tr>
<td>Male type (conspecific vs. heterospecific)</td>
<td>1,35</td>
<td>14.80</td>
<td>0.0005</td>
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<td>Experience × male type</td>
<td>1,35</td>
<td>4.06</td>
<td>0.0516</td>
</tr>
<tr>
<td>Male type × female species</td>
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<td>0.35</td>
<td>0.5669</td>
</tr>
<tr>
<td>Experience × male type × female species</td>
<td>1,35</td>
<td>5.13</td>
<td>0.0298</td>
</tr>
<tr>
<td>Absolute body size difference (M – F)</td>
<td>2,35</td>
<td>&lt;0.01</td>
<td>0.9945</td>
</tr>
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<td>Experience × body size difference</td>
<td>1,35</td>
<td>4.86</td>
<td>0.0342</td>
</tr>
<tr>
<td>Female species × body size difference</td>
<td>1,35</td>
<td>9.07</td>
<td>0.0048</td>
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<tr>
<td>Male color index</td>
<td>1,35</td>
<td>8.74</td>
<td>0.0056</td>
</tr>
</tbody>
</table>

Results from repeated measures ANCOVA. Absolute body size difference calculated as absolute value of difference between male (M) and female (F) length.

* Effects evaluated between subjects as opposed to within.
that preferences for con- or heterospecific males are not simply the result of differences in body size or color between male species.

Male conspecific mate preference

In contrast to females, experience with heterospecifics led males to discriminate between the species better (Table 2). The species differ in typical courtship behavior: benthic courtship consists mainly of bites, whereas limnetic courtship consists mainly of zigzags (Ridgway and McPhail 1984; Boughman et al. 2005). Minority males performed more of their species-typical courtship behaviors to conspecifics than majority males (Figure 3). Thus, experience with heterospecifics led males to court conspecifics more appropriately. Minority limnetic males zigzagged more to conspecific females than did majority males (Figure 3a), but the amount of bites, leads, or vigor directed to conspecifics did not differ between experience levels. Minority benthic males bit conspecifics more than majority males (Figure 3b). In addition, they zigzagged and courted conspecifics more as well; thus, benthics raised with heterospecifics showed increased conspecific preference in all measures except leads. Differences between the species existed in overall preference across measures: benthic males tended to prefer conspecifics and limnetic males tended to prefer heterospecifics (Figure 3). Unlike for female preference, body size differences did not influence male preference in either species (Table 2).

**DISCUSSION**

We provide the first evidence that both female and male conspecific mate preference is learned in recently diverged species and show that the sexes learned through different types of experience. Female limnetics learned to prefer conspecific mates through experience with siblings. Male conspecific mate preference is not simply the result of differences in body size or color between male species.
Evidence is accumulating that there may be substantial sex differences in how conspecific mate preference is learned. Our results suggest that stickleback males are better at recognizing and appropriately courting females when they have substantial social experience with heterospecifics. In other taxa, males also learn to discriminate through experience with heterospecifics during courtship (Dukas 2004, 2008; Magurran and Ramnarine 2004). In contrast, limnetic females preferred conspecific mates only when they had social experience with conspecifics, suggesting that they may imprint on siblings. In several other species, females imprint and learn to prefer mates of the same species as their parents (Grant PR and Grant BR 1997; ten Cate and Vos 1999; Verzijden and ten Cate 2007). However, males do sometimes imprint on parents as well (Immelmann 1972), but even when males imprint sex differences exist. Males and females imprint on different morphological features in zebra finches (Burley 2006), and in some Lake Victoria cichlids, females base mate preferences on their mothers, whereas males base rival preferences on their siblings (Verzijden et al. 2008).

Why might sex differences exist in how experience affects conspecific mate preference? Further work is certainly needed; however, differences between males and females might stem from their roles during mating and the different selective pressures these create. For instance, females are the searching sex in sticklebacks and thus could benefit from using experience to form a search image. In support of this idea, Engallagma damselfly males are the searching sex and learn to prefer familiar female morphs (Fincke et al. 2007). In contrast, males that maintain mating territories (as stickleback males do) do not search for mates but instead make decisions about which to court. If there is more than one female in their territory, males would benefit from being able to discriminate among females and choose the most receptive or fecund female. When female encounter rates are high, males which learn the qualities of receptive females can be favored over indiscriminate males (Dukas et al. 2006). Another difference between the sexes is that males usually have a greater need to identify and be aggressive to rivals. If rival recognition and mate recognition are similar processes in males, then male

### Table 2

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Zigzags</th>
<th>Bites</th>
<th>Leads</th>
<th>Vigor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_{1,36}$</td>
<td>$P$</td>
<td>$F_{1,36}$</td>
<td>$P$</td>
</tr>
<tr>
<td>Experience (minority vs. majority)</td>
<td>10.98</td>
<td>0.0021</td>
<td>3.13</td>
<td>0.0853</td>
</tr>
<tr>
<td>Male species (benthic vs. limnetic)</td>
<td>55.31</td>
<td>&lt;0.001</td>
<td>91.69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Experience $\times$ male species</td>
<td>0.03</td>
<td>0.8656</td>
<td>9.03</td>
<td>0.0048</td>
</tr>
<tr>
<td>Body size difference (BF $-$ LF)</td>
<td>2.23</td>
<td>0.1439</td>
<td>0.18</td>
<td>0.6766</td>
</tr>
</tbody>
</table>

Results from ANCOVAs for each behavior. Body size difference calculated as difference in benthic female (BF) minus limnetic female (LF) length.
mate recognition may be dependent on the type of experience needed to develop rival recognition. Rival recognition can be learned: Males direct aggression toward rivals of the same species as their parents in species of fish (Hansen and Slager 2003) and their siblings in cichlids (Verzijden et al. 2008). However, we understand little about the relationship between rival recognition and mate recognition. Finally, sex differences in selection against hybridization might also determine how experience shapes preference. In the stickleback species pairs, differences in strength of conspecific preference suggest selection to avoid heterospecific matings may be strongest on females (Kozak et al. 2009). Despite these emerging patterns, we remain ignorant of what factors might favor learned conspecific preference in each sex and why they rely on different types of experience. Nor do we have a good understanding of how differences in learned conspecific mate preference between the sexes might enhance or impede speciation. We hope future studies will consider learned mate preferences in both males and females to explore these issues.

Species differences in how experience influences conspecific mate preference also give us insight into when learned conspecific preference might be favored. In our study, the effect of social experience differed between the species: Experience with conspecifics influenced limnetic but not benthic females. Limnetics are a more social species and under natural circumstances may have more opportunity to learn from social interactions. Therefore, they may depend more on these interactions as a source of information. Benthic females may instead rely on unlearned preferences or preferences learned through interactions with parents or via self-referent phenotype matching (Mateo and Johnston 2000). Species differences also existed in benthic and limnetic male conspecific preference. Minority benthic males zigzagged and bit conspecifics more than majority males and had a strong preference for conspecifics. Minority limnetics zigzagged more to conspecifics than majority limnetics, but this did not result in an overall preference for conspecifics. It may be that limnetic males require some additional experience (such as experience with a father) to develop a strong preference for conspecific females. We are currently investigating how experience with parents might influence conspecific preference in both sexes in these species.

The nature of imprinting may be key to how it affects speciation. Maternal and paternal imprinting produce different dynamics in theoretical models (Verzijden and ten Cate 2007; Verzijden et al. 2008). Maternal imprinting is more likely to lead to specification, but paternal imprinting evolves more rapidly within a species. We show here for the first time that experience throughout life can lead to conspecific mate preference rather than simply influencing the phenotypes that females prefer in males of their own species (Hebets 2003, 2007). Sticklebacks learned conspecific preferences from individuals in their own generation. In the wild, sticklebacks group with siblings early in life and then group with other juveniles (FitzGerald and Morrissette 1992). Previous work suggests sticklebacks learn to prefer familiar kin through early experience with siblings (Frommen et al. 2007); perhaps a similar mechanism leads to conspecific preference in our species. Given the different effects of maternal and paternal imprinting, it will be important to explore how imprinting on those of the same generation influences the evolution of imprinting and its role in speciation.

Does learned conspecific mate preference facilitate rapid speciation? Learned conspecific preferences appear to have contributed to rapid sympatric speciation in at least some Lake Victoria cichlids (Verzijden and ten Cate 2007; Verzijden et al. 2008). But, learning may also contribute to speciation by enhancing the rate of preference divergence when populations are in allopatry or over longer time periods, as in Darwin’s finches (Grant PR and Grant BR 1997; Grant BR and Grant PR 1998). Our results show that conspecific preference learned through social experience may be important in a system where reinforcement is at work and where speciation has been extraordinarily rapid. Benthic and limnetic sticklebacks are estimated to have diverged in less than 15,000 years (Taylor and McPhail 1999, 2000). In contrast, collared and pied flycatchers diverged approximately 2 million years ago due in part to reinforcement but have genetically based, sex-linked conspecific preference and no evidence of imprinting (Saetre et al. 2001; Saether et al. 2007). Whether there is a link between time since divergence and learned conspecific preference is unknown and would be worth investigating. However, our study contributes to increasing evidence that conspecific mate preferences are learned in young species pairs that have undergone rapid speciation.

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University of Wisconsin—Madison Graduate Fellowship (to G.M.K.); National Science Foundation (NSF) Graduate Research Fellowship (to G.M.K.); NSF Grant IBN0416808 (to J.W.B.).

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