

## Sequential mate choice and sexual isolation in threespine stickleback species

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### Abstract

Sequential mate choice strategies predict how females should alter their choosiness based on the availability of attractive males. There are many studies on sequential mate choice within species, but few have asked whether females apply these strategies to interactions between species and how these strategies may affect hybridization. We tested how previous interactions with conspecific and heterospecific males affect mate preference and sexual isolation in two threespine stickleback species (benthics and limnetics: *Gasterosteus* spp.). Consistent with previous work, we found that within species, stickleback females gauge male attractiveness relative to previously encountered males. If females extend these decision rules between species, we predicted that previous interactions with conspecifics should make heterospecifics less attractive, whereas interactions with heterospecifics should make conspecifics more attractive. However, females found heterospecifics less attractive after prior experience, largely independent of the species of male first encountered. Thus, sequential mate choice strategies are used within but not between species in sticklebacks. Further, learning from prior courtship interactions acts to enhance existing sexual isolation between species.

### Introduction

Mate preference can be influenced by prior encounters in species that mate multiple times and in species where individuals sample multiple potential mates before selecting a single mate (Bakker & Milinski, 1991; Shelly & Bailey, 1992; Jennions & Petrie, 2000; Wong *et al.*, 2004; Kokko & Mappes, 2005). Both the nature and frequency of prior encounters with potential mates are key in determining sexual selection within species where females encounter males sequentially. There are numerous strategies that females can use during sequential mate choice and how females employ these strategies often depends on the costs and benefits of

searching (Janetos, 1980; Real, 1990; Milinski & Bakker, 1992; Wiegmann *et al.*, 2010). For example, the distribution of potential mates in the population strongly influences female sequential mate choice strategies and females often become less choosy when males are sparse (Wagner *et al.*, 2001; Hebets & Vink, 2007; Bonachea & Ryan, 2011; Fowler-Finn & Rodriguez, 2012). If females apply similar sequential strategies during within- and between-species mate choice, then these strategies could also affect sexual isolation in sympatric species. When females use sequential mate choice strategies between species, the strength of sexual isolation will depend on the order and frequency of encounters with heterospecific and conspecific males. However, speciation research has not considered the effects of sequential mate choice strategies on sexual isolation. This is despite much work on sequential mate choice within species (Jennions & Petrie, 2000; Pitcher *et al.*, 2003; Wong *et al.*, 2004; Chaine & Lyon, 2008; Rebar *et al.*, 2011), avid interest in how sexual selection within species may produce isolation between species

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(Boughman, 2001; Ritchie, 2007; Kraaijeveld *et al.*, 2011; Maan & Seehausen, 2011), and a growing number of studies on how other types of prior experience alter sexual isolation (Servedio *et al.*, 2009; Verzijden *et al.*, 2012).

Within species, the quality of previously encountered mates can strongly influence female mate choice and in many species, females 'trade-up' – preferring only males that are more attractive than those that have been previously encountered (Jennions & Petrie, 2000; Uy *et al.*, 2000; Pitcher *et al.*, 2003; Bailey & Zuk, 2009; Rebar *et al.*, 2011). In this way, females are able to ensure mating when only low-quality males are present but are also able to avoid mating with low-quality mates when high-quality mates are available. If conspecific and heterospecific mates are evaluated along a continuum of quality with heterospecifics treated as exceedingly unattractive conspecific mates (Mendelson & Shaw, 2012), then females may extend mate choice strategies used within species to interactions with heterospecifics. The direction of change in female behaviour during sequential interactions might be similar when selecting mates within and between species; however, the magnitude of response may differ because the costs of mating with heterospecifics are likely to be greater than the costs of mating with unattractive conspecifics due to reduced fitness of hybrid offspring (Butlin *et al.*, 1989; Coyne & Orr, 2004). Females might 'trade-up' between species, and prior experience with heterospecific males should make conspecific males more attractive to females, whereas experience with conspecifics should make heterospecifics less attractive (Izzo & Gray, 2011). Understanding how sequential strategies are used between species would allow us to predict patterns of hybridization and potentially explain why females seem more likely to accept heterospecific mates when conspecifics are scarce (Wirtz, 1999; Veen *et al.*, 2001; Randler, 2002).

No previous work has contrasted sequential mate choice strategies within and between species. A few studies have measured the effect of a single type of prior experience on sexual isolation, leading to conflicting results. Prior experience with conspecific males reduces female preference for heterospecifics in crickets and swordtails (Izzo & Gray, 2011; Willis *et al.*, 2011). Conversely, prior experience with heterospecifics makes heterospecific mates less attractive in male fruit flies, male guppies, female damselflies and female hamsters (Dukas, 2004; Magurran & Ramnarine, 2004; Dukas, 2008; Svensson *et al.*, 2010; delBarco-Trillo & Johnston, 2011). The relationship of these patterns to sequential mate choice strategies is not clear. Only one study has explicitly tested sequential mate choice between species. This study found that one cricket species, *Gryllus texensis*, uses sequential strategies between species, whereas the closely related species *Gryllus rubens* does not (Izzo & Gray, 2011).

In this study, we compare the use of female sequential mate choice strategies when selecting mates within

and between species in two species of threespine stickleback: benthics and limnetics (*Gasterosteus* spp.). Stickleback females evaluate multiple males before laying their entire clutch of eggs in a single male's nest (Foster *et al.*, 1994). Previous work has shown that in some threespine stickleback populations, females do use sequential mate choice strategies when selecting mates within species, because females respond less to low-quality males with dull red coloration after previous experience with high-quality bright red males (Bakker & Milinski, 1991; Milinski & Bakker, 1992). Benthics and limnetics are sympatric species that live in the same lakes, but occupy different habitats (Bentzen & McPhail, 1984; McPhail, 1984, 1992; McPhail *et al.*, 1994). There is spatial and temporal overlap in breeding between species, and males of each species can nest in close proximity to one another (Ridgway & McPhail, 1987). Benthic and limnetic females discriminate against heterospecific mates in no-choice trials (Nagel & Schluter, 1998; Rundle *et al.*, 2000; Boughman, 2001; Boughman *et al.*, 2005), and hybrid offspring have reduced fitness (Schluter, 1993, 1995; Rundle, 2002; Gow *et al.*, 2007). Furthermore, benthic and limnetic females contribute more to premating isolation than males, who court both species of female equally (Kozak *et al.*, 2009).

We tested benthic and limnetic female mate preferences using sequential mating trials. To verify that female benthics and limnetics exercise sequential mate choice within species, we measured changes in female behaviour over two sequential trials with conspecific males that varied in red nuptial coloration. We predicted that females would prefer a second male that had greater red coloration than their first male (Bakker & Milinski, 1991; Boughman, 2001; Pitcher *et al.*, 2003). To look at sequential mate choice between species, we compared female preference for conspecific and heterospecific males when females had prior experience either with a conspecific male or with a heterospecific male. If within-species sequential mate choice strategies extend between species and heterospecific males are treated as unattractive mates, we can make a number of predictions. First, we predicted that females who have been courted by heterospecific males would subsequently find conspecific males more attractive than females who had previously seen conspecific males (Rebar *et al.*, 2011). Second, we predicted that prior experience with conspecifics would lead females to find subsequent heterospecifics less attractive (Izzo & Gray, 2011). Third, we predicted females who previously seen heterospecific males should find subsequent heterospecifics as attractive as the first, because on average both males should be relatively unattractive. Alternatively, if females always showed stronger preferences for conspecifics over heterospecifics, it would suggest that females do not apply sequential strategies to between-species mate choice.

## Materials and methods

We collected benthic and limnetic sticklebacks using minnow traps from Paxton Lake (49°43' N, 124°31' W), Texada Island, British Columbia, at the beginning of the breeding season (March–April) in 2006, 2008 and 2009. Sticklebacks were in reproductive condition, but nesting males were not observed in the lake at the time of collection, indicating that breeding had not yet commenced. Fish were transported from British Columbia to the University of Wisconsin and housed in tanks by species and sex for at least a week prior to testing to insure that females used in the experiment had no recent prior mating experience. Fish were used in mating trials the year they were caught in the wild. The laboratory was maintained at 16 °C and 16 h of light. All fish were fed a mixture of frozen brine shrimp (*Artemia* spp.) and bloodworms (*Chironomus* spp.).

In sequential no-choice trials, females were courted by one male and then given 2 h to rest before a second mating trial. For each courtship trial, a female was placed in an opaque holding container in an aquarium with a single male and his nest (tank size: 101–142 L). We released the female from the holding container after she had acclimated to the tank (2–5 min) and began the trial after the male began courtship. Trials lasted for 20 min or until the female entered the nest and attempted to spawn. Actual spawning of eggs by the female was prevented, so females could be used in multiple trials. All behaviours of the female and male were recorded using Observer event recorder (Noldus Information Technology, Leesburg, VA, USA). Between trials, females were held in individual holding tanks (19–40 L). After the second trial, we measured female weight and standard length (with vernier callipers). We used a pair of stimulus males only once and each individual male up to three times. Male standard length was measured with callipers.

We quantified female preference at different stages of stickleback courtship. Female latency to respond to courtship was the amount of time (in min) from when the male began courting a female to when she performed her first behaviour indicating interest: approaching the male, following him or performing a head-up [a behaviour that has previously been found to indicate interest in a male (Milinski & Bakker, 1990)]. If the female did not respond during the trial, the time value was set at the total trial length (20 min). To be included in our sample, females had to respond to at least one of the males. We measured responsiveness (the number of times a female approached or followed a male when he led her to the nest) and nest inspection rate (the number of times a female examined a male's nest after he showed it) (Kozak *et al.*, 2009). Preference score measured how far courtship progressed [ranging from no response to attempted spawning: 0–4: (Kozak & Boughman, 2009)]. Male aggression towards the female was

also measured. We classified male bites and chases as aggressive behaviours and calculated the rate of male aggression per minute of the trial (Kozak *et al.*, 2009).

### Within-species sequential mate choice

For sequential mate choice within species, we tested 94 females (48 benthics, 46 limnetics) with two conspecific males in 2006. We looked at the change in female preference relative to the difference in male red coloration. First and second males were randomly selected. Male red throat coloration was quantified immediately after courting the female. A trained observer scored male red coloration using a red index score (range = 0–10) which takes into account both the area of the throat that is red (scored from 0 to 5) and the intensity of that red (scored from 0 to 5). This standardized colour scoring method has been developed by our laboratory group to measure colour during behavioural interactions (Boughman, 2001, 2007; Lewandowski & Boughman, 2008; Kozak *et al.*, 2011) and yields similar results to reflectance data (Albert *et al.*, 2007; Boughman, 2007).

We calculated the difference between a female's preference for second and first conspecific males and the difference in red coloration between the two males (second minus first). For each of our preference measures: latency to respond, responsiveness, inspection, and preference score, we tested the relationship between relative preference and relative red coloration using an ANCOVA including species, relative red coloration and the interaction between species and relative coloration in our model.

### Between-species experience and sequential mate choice

For sequential mate choice between species, we compared the mate preferences of 80 females across four male presentation orders: conspecific–conspecific, conspecific–heterospecific, heterospecific–conspecific and heterospecific–heterospecific. Conspecific–conspecific trials were run in 2006 (10 females) and 2009 (15 females) for a total of 25 females (11 benthics, 14 limnetics). Conspecific–heterospecific (20 females: eight benthics, 12 limnetics), heterospecific–conspecific (18 females: seven benthics, 11 limnetics) and heterospecific–heterospecific (17 females: five benthics, 12 limnetics) trials were run in 2008 and 2009. We analysed both species together and tested for differences between species by including it as a factor in our statistical models.

To determine whether female behaviour changed based on the current male or general courtship experience (with any male), we analysed female behaviour across first and second trials using a repeated measures ANCOVA (with compound symmetry). Females had no recent mating experience prior to first trials, but in

second trials they had previous experience. Therefore, trial was equivalent to experience, and we used experience as a factor that was repeated within female: none (first trial) or experienced (second trial). In addition to female experience, we included in our model current male (conspecific or heterospecific), female species, current male red coloration and all interactions. To specifically test female use of sequential mate choice strategies between species, we analysed female preference in the second trial using an ANCOVA with previous male identity (conspecific, heterospecific), current male (conspecific or heterospecific), female species (benthic, limnetic), the difference in red coloration between the second and first male (as a covariate) and all interactions. In this analysis, sequential mate choice would be evident from a significant interaction between the identity of the previous and current male. We included year of testing as a factor in our statistical models, but it was nonsignificant in all analyses and removed (repeated measures: all  $F_{2,76} < 2.56$ ,  $P > 0.08$ ; second trial only: all  $F_{2,71} < 1.78$ ,  $P > 0.18$ ). Nonsignificant interactions were also removed to simplify our models.

Differences in aggression between species alone might alter subsequent female preference. We tested how aggression from a particular type of male changed female response towards that male type, by analysing preference for the second male in an ANCOVA with aggression rate of the first male as a covariate (using only females that had seen two conspecifics or two heterospecific males: 42 females; 25 con–con, 17 het–het; 16 benthics, 26 limnetics). We also included previous male, female species and relative red coloration (second minus first) and all interactions in the model. All statistical analyses were performed in SAS v.9.3 (SAS Institute Inc., Cary, NC, USA). Least-squared means and

slope estimates are presented plus or minus standard error throughout.

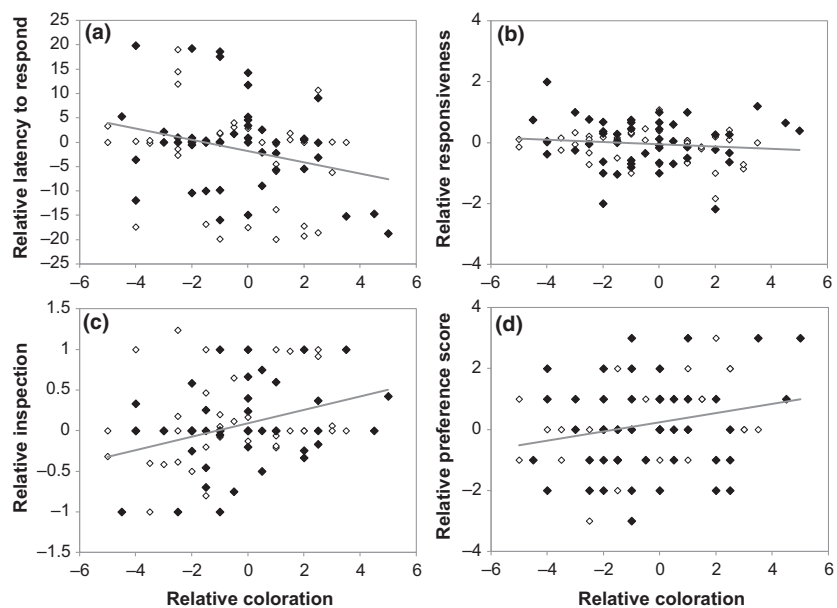
## Results

### Within-species sequential mate choice

Similar to other stickleback species, benthic and limnetic females did show sequential mate choice within species. We did not find any species differences in relative preference (all  $F_{1,90} < 1.25$ ,  $P > 0.27$ ) or the effects of relative red coloration on preference (species by relative red coloration interaction: all  $F_{1,90} < 0.31$ ,  $P > 0.58$ ), so we present the results of both species together. There was a positive relationship between the difference in red coloration between males and the difference in female preference for first and second males (Fig. 1). As the second male's red coloration increased relative to the first male, female response time decreased ( $\beta = -1.15 \pm 0.41$ ,  $t_{92} = -2.80$ ,  $P = 0.0063$ ), female nest inspection increased ( $\beta = 0.085 \pm 0.027$ ,  $t_{92} = 3.09$ ,  $P = 0.0027$ ) and preference score increased ( $\beta = 0.15 \pm 0.064$ ,  $t_{92} = 2.34$ ,  $P = 0.021$ ). Female responsiveness (follows per lead) did not change ( $\beta = -0.057 \pm 0.07$ ,  $t_{92} = -0.81$ ,  $P = 0.41$ ). We also analysed the effect of the absolute value of the red coloration of the second male, rather than his coloration relative to the first male, but it did not explain a significant amount of variation in female preference (all  $t_{92} < 2.80$ ,  $P > 0.11$ ).

### Between-species experience and sequential mate choice

When comparing female behaviour between first and second trials, we found courtship experience in and of



**Fig. 1** Sequential mate choice within species. Female preference for second male relative to the first based on red coloration of second male relative to the first for benthics (filled symbols) and limnetics (open symbols). Line indicates slope estimate from regression with species pooled. (a) Latency to respond in minutes ( $R^2 = 0.078$ ,  $P = 0.0063$ ), (b) Responsiveness ( $R^2 = 0.015$ ,  $P = 0.41$ ), (c) Inspection rate ( $R^2 = 0.094$ ,  $P = 0.0027$ ), (d) Preference score ( $R^2 = 0.056$ ,  $P = 0.021$ ).

itself substantially altered sexual isolation. Females had stronger preferences for conspecific over heterospecific males when they had previously experienced courtship (i.e. second trials) than prior to any experience of courtship (i.e. first trials) (Table 1; Fig. 2). Experienced females took longer to respond to heterospecifics (mean difference second vs. first:  $4.58 \pm 1.11$ ,  $t_{76} = 4.10$ ,  $P = 0.0001$ ) and had lower preference scores for heterospecifics (second vs. first:  $-0.44 \pm 0.17$ ,  $t_{76} = -2.50$ ,  $P = 0.015$ ). Females did not change their inspection rate between first and second trials and always inspected the nests of conspecific males more than those of heterospecifics (mean first trials:  $0.39 \pm 0.14$ ,  $t_{76} = 2.84$ ,  $P = 0.0058$ ; mean second trials:  $0.33 \pm 0.14$ ,  $t_{76} = 2.43$ ,  $P = 0.017$ ). Responsiveness towards both species of males decreased with experience (mean difference second vs. first:  $-0.45 \pm 0.21$ ,  $t_{76} = 2.17$ ,  $P = 0.03$ ).

In contrast, female preference in their second trial did not depend on the order in which females saw conspecific and heterospecific males (Table 2; Fig. 3). There was a significant interaction between the current male and the previous male in only one of our preference measures: nest inspection. Females with heterospecific experience inspected the nests of conspecific males slightly more than females with conspecific experience (mean difference het vs. con prior male:  $0.31 \pm 0.17$ ,  $t_{73} = -1.77$ ,  $P = 0.08$ ) and inspected the nests of heterospecific males slightly less than those with conspecific experience (het vs. con prior male:  $-0.30 \pm 0.19$ ,  $t_{73} = -1.59$ ,  $P = 0.12$ ), although these differences were not significant. Overall, females with heterospecific experience inspected the nests of conspecific males more than heterospecific males (mean difference con vs. het male =  $0.63 \pm 0.19$ ,  $t_{73} = 3.34$ ,  $P = 0.0013$ ), whereas females with conspecific experience did not (con vs. het male =  $0.027 \pm 0.17$ ,  $t_{73} = 0.16$ ,  $P = 0.87$ ). However, these differences are not entirely in the direction we would predict if females were using sequential mate choice strategies. We also found that prior experi-

ence with heterospecific males tended to reduce benthic female preference in second trials, independent of the current male. Experience with heterospecifics increased latency to respond and tended to decrease responsiveness in benthic females (latency: mean difference con vs. het experience =  $-5.27 \pm 2.31$ ,  $t_{73} = -2.28$ ,  $P = 0.025$ ; responsiveness: con vs. het experience =  $0.74 \pm 0.40$ ,  $t_{73} = 1.88$ ,  $P = 0.065$ ), and this change was unique to benthics (latency: mean difference benthic vs. limnetic het experience =  $6.90 \pm 2.23$ ,  $t_{73} = 2.10$ ,  $P = 0.0028$ ; responsiveness: benthic vs. limnetic het experience =  $-1.01 \pm 0.38$ ,  $t_{73} = 2.64$ ,  $P = 0.01$ ).

Differences in relative red coloration between males did not explain female behaviour towards heterospecifics. We found no consistent differences in red coloration between conspecific and heterospecific males (benthic females: conspecific vs. heterospecific  $t_{72} = 0.17$ ,  $P = 0.87$ , limnetics females: conspecific vs. heterospecific  $t_{104} = 0.05$ ,  $P = 0.96$ ). In contrast to our findings within species, differences in red coloration between first and second males did not have a significant effect on female preference between species (Table 2; latency  $\beta = -0.53 \pm 0.31$ ,  $F_{1,73} = 2.84$ ,  $P = 0.096$ ; responsiveness  $\beta = 0.013 \pm 0.05$ ,  $F_{1,73} = 0.06$ ,  $P = 0.80$ ; inspection  $\beta = 0.033 \pm 0.03$ ,  $F_{1,73} = 1.42$ ,  $P = 0.24$ ; preference score  $\beta = 0.10 \pm 0.05$ ,  $F_{1,73} = 3.71$ ,  $P = 0.058$ ).

Females did not receive more aggression from heterospecific males than conspecific males during courtship (conspecific aggression rate =  $3.70 \pm 0.32$ , heterospecific aggression =  $3.88 \pm 0.37$  behaviours per minute, conspecific vs. heterospecific:  $t_{176} = -0.36$ ,  $P = 0.72$ ). When we looked at the relationship between the aggression females had previously received from the first male and subsequent female behaviour, we found that aggression significantly altered female latency to respond, but effects differed depending on male species (aggression by species interaction:  $F_{1,36} = 8.36$ ,  $P = 0.0065$ ). Females took longer to respond when heterospecific males were more aggressive (Fig. 4). In contrast, conspecific male aggression

**Table 1** Courtship experience and female preference. *F*-values from repeated measures ANCOVA. Significance of terms indicated by symbols. Interactions between species and other factors were nonsignificant and removed from the model.

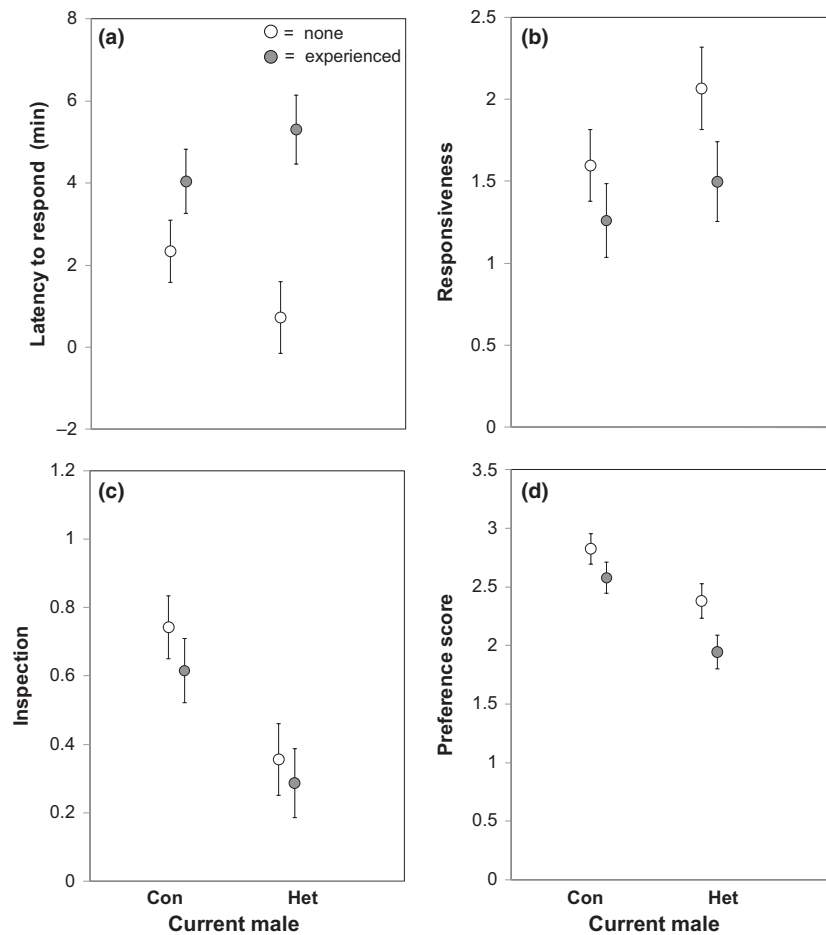
	d.f.	Latency to respond	Responsiveness	Inspection	Preference score
Current male (con, het)	1,37	0.05	2.35	13.76**	17.46**
Experience (first, second)	1,76	19.76**	4.72*	1.25	10.40**
Current male × Experience	1,76	3.36†	0.26	0.09	0.56
Female species (B, L)	1,78	6.70*	1.89	31.23**	42.15**
Current male colour	1,76	1.73	2.44	2.56	4.90*
Within female covariance‡		2.25* (7.09 ± 3.16)	1.99* (0.51 ± 0.26)	1.80† (0.08 ± 0.05)	3.79** (0.40 ± 0.11)
Error (mean square)		19.68	1.68	0.30	0.43

† $P < 0.10$ .

\* $P < 0.05$ .

\*\* $P < 0.01$ .

‡Covariance measure significance determined by Wald Z test; covariance estimate listed in parentheses.



**Fig. 2** Female preference for conspecific and heterospecific males before and after courtship experience. Least-squared mean preference ( $\pm$  SE) shown for conspecific and heterospecific males in first (open) vs. second trials (shaded). (a) Latency to respond in minutes, (b) Responsiveness, (c) Inspection rate, (d) Preference score.

**Table 2** Effect of prior experience with conspecific and heterospecific males on subsequent female preference. *F*-values from ANCOVA of preference in second trial shown. Significance of terms indicated by symbols. Nonsignificant interactions between species and other factors were removed from the models.

	d.f.	Latency to respond	Responsiveness	Inspection	Preference score
Current male (con, het)	1	0.59	0.41	6.81*	9.47**
Previous male (con, het)	1	2.10	0.77	0.01	1.24
Current $\times$ Previous male	1	2.30	0.44	5.79*	0.02
Female species (B, L)	1	6.61*	3.76†	19.60**	26.49**
Female species $\times$ Previous male	1	4.66*	4.37*	0.06	0.76
Relative male colour	1	2.84†	0.06	1.42	3.71†
Error (mean square)	73	38.77	1.14	0.31	1.01

† $P < 0.10$ .

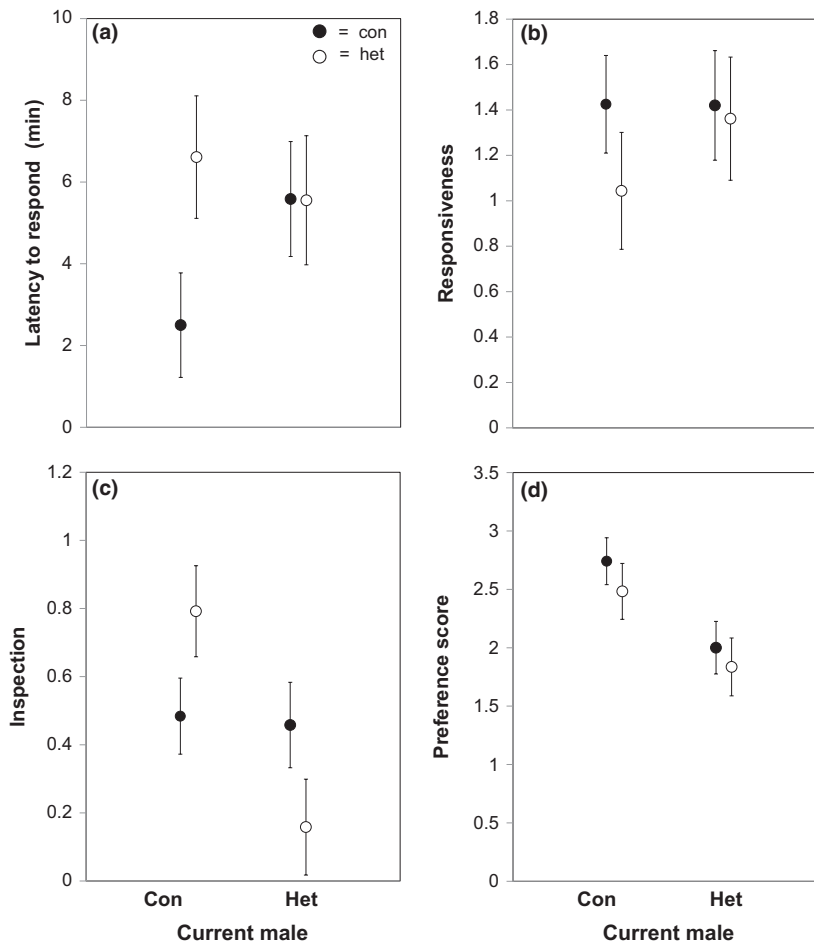
\* $P < 0.05$ .

\*\* $P < 0.01$ .

tended to reduce female time to respond. Aggression did not affect any other measures of preference (all  $F_{1,36} < 2.67$ ,  $P > 0.11$ ). Benthics are known to have more aggressive courtship, but we did not find any differences in the responses of benthic and limnetic females to aggression (all  $F_{1,33} < 0.52$ ,  $P > 0.47$ ).

## Discussion

Courtship experience and sequential mate choice can be important in determining within-species mate preferences (Jennions & Petrie, 2000; Pitcher *et al.*, 2003; Wong *et al.*, 2004; Chaine & Lyon, 2008; Rebar

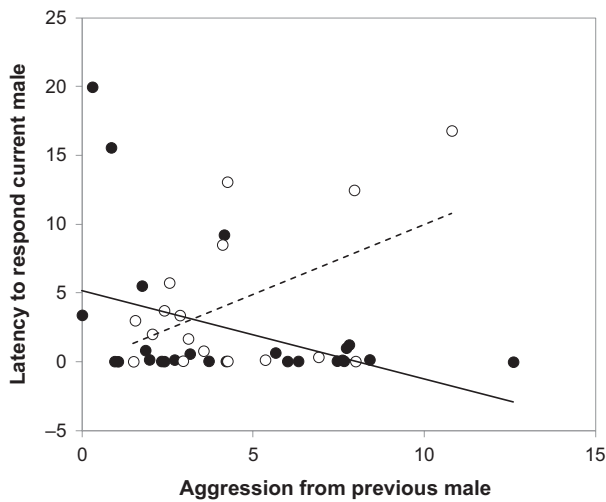


**Fig. 3** Female preference for conspecific and heterospecific males in second trials based on male experienced in the first trial. Least-squared mean preference ( $\pm$  SE) shown for conspecific and heterospecific males in second trials based on female prior experience: conspecific experience (filled), heterospecific experience (open). (a) Latency to respond in minutes, (b) Responsiveness, (c) Inspection rate, (d) Preference score.

*et al.*, 2011). If these mediators of mate choice also operate between species, then they may alter sexual isolation between sympatric species. Here, we show that within-species, benthic and limnetic females use a sequential mate choice strategy. Similar to other species of stickleback (Bakker & Milinski, 1991; Milinski & Bakker, 1992), females preferred males with greater red coloration when presented with males sequentially. However, during between-species mate choice, the species identity and coloration of the first male encountered had few effects on female preference. After courtship experience with either species of male, females took longer to respond to heterospecifics and performed fewer behaviours indicating preference for heterospecifics. We expected preference for heterospecifics to remain unchanged when females saw two heterospecific males sequentially, but instead we found that heterospecific experience increased female aversion to heterospecifics. During nest inspection, heterospecific experience improved sexual isolation through the combined effect of increased preference for conspecifics and decreased preference for heterospecifics. Heterospecific aggression also increased female latency to respond to

heterospecifics. These results are not consistent with use of sequential strategies between species and suggest that prior courtship experience aids in female discrimination between species and strengthens sexual isolation, possibly through learned aversion to heterospecific males.

Our results suggest that hybridization costs may have selected against use of sequential mate choice strategies for heterospecifics, resulting in females that use different decision rules when interacting with conspecific and heterospecific males. These results are in contrast with previous work on field crickets, which suggests that females can apply sequential mate choice between species (Izzo & Gray, 2011). Differences in the costs and frequency of hybridization or degree of divergence between species may underlie these species differences (Ord *et al.*, 2011). In particular, there is little evidence for natural hybridization between *Gryllus* cricket species that use sequential strategies (Gray *et al.*, 2008), whereas there is ample evidence for past and ongoing hybridization between benthics and limnetics (McPhail, 1992; Gow *et al.*, 2006). Further work on the application of sequential mate choice strategies to heterospecific interactions is needed to test this idea that



**Fig. 4** Effect of aggression on female latency to respond. Latency to respond to current male plotted against aggression received from previous male (number of aggressive behaviours per minute) for females that saw two conspecific males (filled symbols; solid line), or two heterospecific males (open symbols; dotted line).  $\beta_{\text{con}} = -0.69 \pm 0.35$ ,  $t_{36} = -1.99$ ,  $P = 0.054$ ,  $\beta_{\text{het}} = 1.03 \pm 0.48$ ,  $t_{36} = 2.15$ ,  $P = 0.038$ .

hybridization may select against using sequential mate choice strategies between species. It may be that sequential mate choice strategies are used between diverging populations during the initial stages of speciation or during initial secondary contact. It seems likely that early in divergence, rejection of heterospecifics may be the outcome only of sexual selection (Ryan & Rand, 1993), but as divergence proceeds natural selection against hybrids through reinforcement should strengthen the rejection of heterospecifics. This idea could be tested by comparing the use of sequential mate choice strategies within and between species using females from sympatric and allopatric populations; sympatric female choice will be shaped by the costs of hybridization but allopatric female choice will not.

Differences in female strategies within and between species suggest that conspecific and heterospecific males are not assessed along a continuum; conspecifics and heterospecifics are treated qualitatively differently. This difference may make mating decisions in these sympatric species cognitively complex. Stickleback females need to recognize heterospecifics, rate mate attractiveness and weigh prior experience differently depending on the species of male encountered. The degree of cognitive complexity will depend on the types of decision rules that females use and whether decision-making is a hierarchical or a multiplicative process (Castellano, 2010; Ryan, 2011; Mendelson & Shaw, 2012). Future work could vary the quality or species-specificity in male traits known to be important to mate choice and species recognition (red coloration, shape and odour) to

better understand how females make mating decisions. Additionally, the effect of male encounter rate could be investigated by looking at female sequential choice when male–female encounters occur over shorter or longer time periods. Previous work indicates that stickleback females become less selective as time between encounters increases, but females can remember previously encountered males for more than an hour (Milinski & Bakker, 1992). Our finding of a difference in strategy during mate choice within and between species will likely be robust to time between sequential male presentations, because learned female aversion to heterospecific males was quite strong after two hours, although these effects may be of greater magnitude at shorter time scales.

Our study adds to growing evidence that experience during or immediately prior to mating leads to learned aversion of heterospecifics (Dukas, 2004; Magurran & Ramnarine, 2004; Dukas, 2008; Svensson *et al.*, 2010; delBarco-Trillo & Johnston, 2011). Courtship interactions with either species of male enhanced existing stickleback conspecific mate recognition. Females discriminated against heterospecifics even during their first exposure, consistent with previous work in these species (Nagel & Schluter, 1998; Rundle & Schluter, 1998; Rundle *et al.*, 2000; Boughman, 2001; Boughman *et al.*, 2005; Kozak *et al.*, 2009). Courtship experience could allow females to pick up on subtle differences in shape, colour or odour between species and make it easier for females to identify and reject heterospecifics (Boughman, 2001; Rafferty & Boughman, 2006). Learning from both con- and heterospecific experience may also occur in other species (Dukas, 2009) and may potentially explain why some studies have found that conspecific experience improves discrimination, whereas others found an effect of heterospecific experience (Dukas, 2004; Magurran & Ramnarine, 2004; Dukas, 2008; Svensson *et al.*, 2010; delBarco-Trillo & Johnston, 2011; Willis *et al.*, 2011). Therefore, understanding how courtship experience alters conspecific mate preference requires experimental designs that include multiple types of experience.

Our results suggest sexual isolation in sympatric benthics and limnetics should not be easily disrupted by fluctuations in encounter rates between species. Based on our findings, we would predict that increased encounters with heterospecifics should strengthen isolation between benthics and limnetics rather than weaken it. This is in contrast to previous work that suggests that hybridization rates increase when encounters with heterospecifics increase (Wirtz, 1999; Veen *et al.*, 2001; Randler, 2002; Willis *et al.*, 2011). It may be that only at extreme skews in density, when females rarely encountered any conspecifics, will females accept heterospecifics as mates. When females encounter intermediate ratios of conspecifics and heterospecifics, discrimination against heterospecifics may be strong due to learned aversion.



Increasing attention has been paid to the role of learning in generating sexual isolation but has focused primarily on sexual imprinting, when individuals learn to prefer mates similar to their parents by memorizing a set of cues during a critical period early in life (Bischof, 2003; Verzijden *et al.*, 2005; Verzijden & ten Cate, 2007; Price, 2008; Servedio *et al.*, 2009; Kozak *et al.*, 2011; Verzijden *et al.*, 2012). Learned aversion of heterospecifics during courtship may be a different way in which learning can contribute to sexual isolation between species. Unlike imprinting, learning during courtship may depend on associating certain phenotypes with positive experiences (mating) and others with negative experiences (rejection) (Dukas, 2009; Kujtan & Dukas, 2009). For instance, our results suggest that aggressive courtship from heterospecific males is a negative experience, which can increase female latency to respond. Learned aversion further differs from imprinting because it is frequency dependent and therefore might be strongest when females frequently encounter heterospecifics. Such differences between learned aversion and imprinting may lead to different evolutionary outcomes; however, this has not yet been tested mathematically.

Reinforcement of premating isolation in sympatry has primarily been attributed to genetic changes in preference between populations, but it is increasingly clear that learned preferences may also play some role (Irwin & Price, 1999; Servedio & Noor 2003; Servedio *et al.*, 2009; Verzijden *et al.*, 2012). Sympatric benthics and limnetics have stronger conspecific mate preferences than allopatric populations (Rundle & Schluter, 1998; Albert & Schluter, 2004). In this study, we provide evidence that sympatric females learn aversion during courtship and we have previously shown that females also imprint on fathers and prefer mates of their father's species (Kozak *et al.*, 2011) and males raised with heterospecific siblings learn to avoid heterospecific females (Kozak & Boughman, 2009). Therefore, experience both early in life and during courtship may contribute substantially to conspecific preference in these sympatric stickleback populations, although if this learning is unique to sympatric populations remains unknown. In damselflies, sympatric females learn to avoid heterospecific males, but allopatric females show much weaker learned responses (Svensson *et al.*, 2010). However, in guppies, allopatric males show strong learned aversion to heterospecifics, but sympatric males have innate discrimination (Magurran & Ramnarine, 2005). Additional studies are clearly needed to determine the frequency with which learning contributes to preference in sympatric populations.

## Conclusions

Our results show that female sticklebacks use different decision rules when choosing whether to mate with

conspecifics and heterospecifics. When encountering multiple males within the same species, females used a sequential mate choice strategy showing increased preference depending on the relative attractiveness of males encountered. When encountering both conspecific and heterospecific males, however, females showed enhanced discrimination following previous courtship experience. In these scenarios, females had increased preference for conspecific males (regardless of the species of male first encountered) and discriminated earlier in the courtship sequence. Such fine-tuning of female preferences based on prior courtship experience is likely to enhance sexual isolation in sympatric species.

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