

# Can ecotypic differences in male courtship behaviour be explained by visual cues provided by female threespine stickleback?

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## ABSTRACT

**Background:** Research on the evolution of reproductive isolation concentrates on an evaluation of the probability of mating within and between species with little attention to the role of male and female mate choice in the process. Male threespine stickleback, as well as females, select mates but male choice behaviour is poorly understood. Male threespine stickleback use visual cues in courting females, and have been shown to prefer females with more distended abdomens, which may indicate higher fecundity.

**Question:** Do male threespine stickleback from divergent allopatric populations prefer females of their own ecotype using visual cues from live females?

**Hypothesis:** Males will court females of their own ecotype more vigorously than they do females of the other ecotype. Males will also prefer females who are more fecund.

**Organisms:** Allopatric populations of anadromous, benthic, and limnetic threespine stickleback.

**Methods:** We presented males with the opportunity to court two females, one from a benthic population and one from a limnetic population. During 5-min trials, we recorded the following behaviours: zigzags, direct approaches, and the time a male spent following each female.

**Results:** Visual cues were not sufficient to elicit male courtship differences towards females of different ecotypes. However, contrary to expectations, males reduced their courtship toward females with a higher estimated fecundity.

*Keywords:* allopatric differentiation, ecological speciation, male mate choice, parallel evolution, threespine stickleback.

## INTRODUCTION

Natural selection is thought to be responsible for most cases of speciation, either through ecological or mutation order speciation (Schluter, 2001, 2009). Ecological speciation can occur when populations invade novel environments, exposing a portion of the original population

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to new conditions. As the population adapts, reproductive isolation can arise as an indirect consequence of natural selection (Schluter, 2000, 2001; Rundle and Nosil, 2005; Nosil, 2012).

Parallel ecological speciation provides some of the strongest support for ecological speciation (Schluter, 2000, 2009; de Busschere *et al.*, 2012; Nosil, 2012; Ostevik *et al.*, 2012). In parallel ecological speciation, populations independently derived from an ancestral population, that subsequently adapt to divergent habitats are expected to repeatedly evolve greater reproductive isolation than those adapting to similar habitats. Thus, one expects to observe greater reproductive isolation between populations of different ecotypes than among populations of the same ecotype. In many well-studied cases, populations adapted to divergent environments exhibit incipient speciation, whereas populations adapted to similar environments do not (e.g. Rice and Hostert, 1993; Schluter and Nagel, 1995; Bernatchez *et al.*, 1996; Rundle *et al.*, 2000; Vines and Schluter, 2006; Langerhans *et al.*, 2007; Nosil *et al.*, 2008; Schwartz *et al.*, 2010).

Research on the evolution of reproductive isolation has often been limited to evaluating the probability of mating within and between ecotypes, with limited attention to the relative roles of males and females in the process. This omission is likely to have a considerable impact on our understanding of reproductive isolation for species in which mate choice is partially the outcome of complex behavioural interactions. The contributions of male mating preference are less well explored in studies of reproductive isolation. However, male mate choice is thought particularly likely to evolve when there is parental investment by the male, there is a skewed operational sex ratio, or female quality varies (for a review, see Edward and Chapman, 2011).

Here we take advantage of an unusual opportunity to explore the possibility that male mating preferences have diverged over the course of parallel, ecotypic differentiation in the threespine stickleback (*Gasterosteus aculeatus*). The threespine stickleback is a holarctically distributed species with large, oceanic populations and derivative freshwater populations that are restricted to coastal areas. Oceanic populations may be either anadromous, coming into freshwater to breed, or entirely marine, spending their entire lives in the ocean. Oceanic threespine stickleback repeatedly colonized freshwater habitats along the northwest coast of North America, particularly during the last glacial retreat, 15,000–20,000 years ago (reviewed in Bell and Foster, 1994). Freshwater populations of this species have repeatedly, and independently, diverged into benthic (bottom-feeding) and limnetic (plankton-feeding) ecotypes from oceanic ancestors, giving rise not only to divergent, replicated ecotypes but also to sympatric species pairs (McPhail, 1994; Rundle *et al.*, 2000; Schluter, 2000; Taylor and McPhail, 2000; Gow *et al.*, 2008). Differences in ecology have driven behavioural differences between the ecotypes, some of which are manifested in divergent courtship behaviour (Foster, 1994, 1995; Foster *et al.*, 1998, 2008).

When assortative mating was assessed within and between ecotypes from different sympatric species pairs, species of the same ecotype mated more readily than did those of alternative ecotypes (Rundle *et al.*, 2000) and appeared to discriminate primarily on the basis of size and colour (Boughman *et al.*, 2005). Assortative mating between species pairs is strongly correlated with body size, a trait that is divergent between the two ecotypes, and benthic and limnetic stickleback prefer to mate with individuals whose body size is similar to their own (Nagel and Schluter, 1998; Conte and Schluter, 2013). Body size appears to be diverging due to natural selection, as laboratory studies indicate that it is not diverging due to sexual selection (Head *et al.*, 2009). Females are thought to contribute primarily to reproductive isolation between the species pairs, but males do recognize conspecifics (Kozak *et al.*, 2008). Females from allopatric benthic and limnetic populations prefer mates of their own ecotype, suggesting incipient

ecological speciation in allopatry (Vines and Schluter, 2006). Thus there is evidence in the adaptive radiation of the threespine stickleback of extensive parallelism along a benthic–limnetic continuum, and this has played out in both ecotypic variation and in the evolution of species pairs.

We focus upon allopatric benthic and limnetic populations, and ask whether males exhibit preferences for females of their own ecotype, but from a different population. Mutual mate choice has been demonstrated in threespine stickleback (for reviews, see Rowland *et al.*, 1994; Kraak and Bakker, 1998), and thus might contribute to reproductive isolation among ecotypes. Sympatric male stickleback alter their behaviour based on the ecotype of the female (Kozak *et al.*, 2008). However, males from sympatric limnetic populations have demonstrated a preference for females of their own ecotype, whereas males from allopatric limnetic populations have demonstrated a preference for larger, benthic females (Albert and Schluter, 2004). We sought to examine male mate preference among allopatric populations using a larger number of populations than has previously been examined. Our design allowed males to assess only visual cues, as visual cues alone have been shown to elicit male mating preferences in threespine stickleback (Rowland, 1982; Albert and Schluter, 2004). In our study, allopatric benthic, limnetic, and anadromous laboratory-reared males were simultaneously presented with a benthic and limnetic female, to help determine whether male preference matched the pattern predicted by ecological speciation. We used multiple populations for each ecotype to assess whether male preference has evolved in parallel. In addition, we sought to understand whether anadromous males, a surrogate for the ancestral form that gave rise to post-glacial populations of threespine stickleback (Hohenlohe *et al.*, 2010), exhibited a preference for either derived ecotype.

## METHODS

### Test animals

All fish used in experiments were laboratory reared, produced from crosses between wild-caught adults. Gravid females and males exhibiting nuptial coloration were collected using hand nets and mesh minnow traps from four limnetic populations, four benthic populations, and two anadromous (ancestral) populations in May and June of 2009 and 2010. The limnetic populations included those from North (49°42'N, 124°54'W), Garden Bay (49°38'N, 124°01'W), and Ambrose lakes (49°43'N, 124°0'W) on the Sechart Peninsula, British Columbia, and that in Lynne Lake (61°44'N, 150°02'W) in the Matanuska-Sustina Valley, Alaska. Benthic populations included that in Crystal Lake (49°02'N, 123°53'W) on Vancouver Island, British Columbia and those in Beverly (61°36'N, 149°34'W), Stepan (61°34'N, 149°49'W), and Willow lakes (61°44'N, 150°03'W) in the Matanuska-Sustina Valley, Alaska. Fish from two anadromous populations were collected at Rabbit Slough (61°53'N, 149°22'W) in the Matanuska-Sustina Valley, Alaska, and Resurrection Bay (60°03'N, 149°09'W) on the Kenai Peninsula, Alaska.

Live fish were brought to a laboratory where crosses were made. Males were anaesthetized with MS-222, and their testes removed and macerated in a sterile embryo medium (0.5 ppt Instant Ocean in sterile water). This solution was poured over clutches of eggs that had been gently extruded from ovulated females from the same population, into a sterile Petri dish. Five minutes were allowed for fertilization, after which the eggs were washed with sterile embryo medium. After a day, the eggs in each clutch were separated and washed

with iodine. Embryo medium was changed daily as the eggs developed. Developing embryos were shipped within 3 days, overnight in sealed test tubes, to Clark University. Embryos were then transferred back to Petri dishes and the embryo medium changed as needed.

Full-sibling families were housed in tanks at a rearing facility at Clark University, Worcester, MA. Fish did not have access to nesting material in these tanks. Adults and juveniles were fed frozen bloodworms (*Chironomid* spp.) and Cyclop-Eze (Argent Chemical Co., Redmond, WA) once daily, and kept on an 8 h/16 h light/dark cycle during the winter. In April, the light cycle was changed to provide 16 h of light daily, to bring adults into reproductive condition, mimicking light conditions in the northern hemisphere.

### Choice trials

Glass aquaria (75.7 litres; 76.8 × 31.8 × 32.4 cm) were covered on three sides with tan paper, with one long side left open for observation. A dish filled with sand and with a vertical stick attached as a landmark was placed at the centre of the aquarium, and nesting material (dried and washed grass clippings) was added to each tank. A single, sexually mature male was placed in an aquarium and allowed to build a nest overnight. If males did not nest overnight, a clear jar with a gravid female was placed in front of the aquarium for approximately 15 min. This was done daily, until the male either built a nest or was removed from the tank. Males that did not nest within a week were returned to the rearing facility and replaced with new males.

The day after a male nested, two clear Plexiglas tubes (12.5 cm in diameter) were inserted in the aquarium, in line and on opposite sides of the nest at a distance of approximately 30 cm. The cylinders were covered in opaque plastic before the start of the trial. Pairs of ovulated benthic and limnetic females were assigned randomly to the left or right tube, and allowed to acclimate for 10 min before the trial. Anadromous females were never used. The females were paired to be of similar standard length, and they were typically similar in size to females found in the male's population; however, we did not attempt to match the female lengths to the male's length. Males were never shown females of their own population. Video recording began when the plastic coverings on the cylinders were gently raised and the male was allowed to view the females. The trial was recorded for 5 min after the male had oriented towards both females.

After the trial, males and females were digitally photographed in a 9-cm Petri dish to assess standard length. Males were spine-clipped to avoid testing the same individuals twice, and then returned to their family aquaria in the rearing facility for use in fertilizing eggs of tested females. After trials, females were moved to a separate laboratory, where their eggs were eventually stripped and fertilized to produce embryos for other studies.

A total of 171 males were successfully tested. Twelve of these males represented marine populations for which we had only two families each; these males were not considered further. The final data set included 32 anadromous males (16 each from Resurrection Bay and Rabbit Slough), 73 benthic males (16 from Beverly, 18 from Crystal, 22 from Stepan, and 17 from Willow), and 54 limnetic males (14 from Ambrose, 12 from Garden Bay, 10 from Lynne, and 18 from North). Female pairings were not evenly distributed among populations, as gravid females were used as available. The number of times a female from each of the main populations appeared in a trial is presented in Table 1. The choice of female pairs was dependent upon availability of females with newly ovulated eggs on the day of testing. Tested females were eventually stripped of their eggs (see above) and

**Table 1.** The number of times females of each study population were used in male behaviour trials

Female ecotype	Population	Left side	Right side
Limnetic	Ambrose Lake	13	13
	Garden Bay Lake	17	13
	Lynne Lake	15	11
	North Lake	34	33
Benthic	Beverly Lake	10	8
	Crystal Lake	27	43
	Stepan Lake	12	16
	Willow Lake	21	18

*Note:* Females were randomly assigned to be presented to the male on either the left or right side of the test tank.

returned into their home tanks. We did not mark females in a way that allowed us to identify them individually (as marks such as spine clips or tags may have affected them, or affected how males perceived them) but females within each family tank (only 5–8 fish per tank) were usually distinguishable based on size and other physical features. Of 342 females used (324 of which participated in successful trials), we re-used only 13.

All methods involving live fish followed Clark University IACUC protocol 05R. Collection permits were issued to S.A.F. and J.A.B. by the Alaska Department of Fish and Game in 2009 (SF2009-66) and 2010 (SF2010-98), by the Province of British Columbia (NA-SU10-61420) in 2010, and Massachusetts import permits were issued in both years (2009, 041.09LP; 2010, 067.10LP).

### Estimation of female clutch size

Despite carefully length-matching females in each trial, we had no control over the size of the clutch produced by individual females. This could have been a confounding factor that affected male preference, and thus we needed to account for any differences. In addition, we were also interested in exploring any effects of female gravidity itself. To disentangle this ‘gravidity’ effect from the effect of our factor of interest (ecotype identity), we use reproductive data (available for all of our study populations) to calculate a measure that estimated the difference in clutch size for each of the pairs of females we used in our trials. Using the allometric relationship between clutch size and standard length for each population independently, we estimated the number of eggs each female would have held at her observed size. Standard length was measured in ImageJ 1.440 using the still photos of females; standard length is strongly predictive of clutch size (Baker, 1994; Baker *et al.*, 2008).

### Behaviour scoring

Video was scored using Noldus XT The Observer 7. The scorer was blind to the identity of both males and females. The number of zigzags, direct approaches, and time spent following each female along her tube were scored. Zigzags are commonly used as a measure of male preference (Rowland, 1989; Kraak and Bakker, 1998; Albert and Schluter, 2004), but direct approaches are also

used when recording male courtship behaviour (Ridgway and McPhail, 1984; Ishikawa and Mori, 2000). Zigzags were rarely observed during trials, and as a result a combination of zigzags and direct approaches to the female was used in the statistical analysis.

### Statistical analyses

To analyse male preference, independent, nested analyses of covariance (ANCOVA) were performed on two measures of male behaviour: (1) the combined number of direct approaches and zigzags to a female, and (2) the time spent following the female along the tube. Combining the direct approach and zigzag values was supported by a principal components analysis that placed these variables on a single component. Individual populations (a random effect) were nested within male 'type' (benthic, limnetic, or anadromous ancestor). Although family identity was recorded, there was insufficient balance in the data to include this in any analyses. Because two data points were generated for each male (responses to the two females), the statistical analysis needed to account for this so as to disentangle the effect of male-to-male variation from error variation. We performed this analysis two ways, first attempting to account for male individuality by using trials as an additional random effect. This analysis employed Satterthwaite's Procedure for estimating  $F$ -tests, and thus can produce fractional denominator degrees of freedom. For the second analysis, we treated the two data points for each male as repeated measures. Both analyses had the same goal – to estimate the within-male variation in order to construct more sensitive  $F$ -tests. We note that this is not a traditional use of repeated measures, as the two 'measures' for each male were made simultaneously; nevertheless, it does produce the desired effect of partitioning within-male variation from error variation. Using a simple difference score would not have achieved this goal. In each analysis, female standard length, and the difference between the estimated clutch sizes for the limnetic and benthic female, were used as covariates. Covariates were centred prior to analysis (Kraemer and Blasey, 2004).

The same analyses were used for the time males spent following a female, which was log-transformed. Finally, we also performed a generalized ANCOVA in which the preference of each male was scored as a two-state outcome – prefers own type vs. prefers alternative type. Because we used only benthic and limnetic females (never any anadromous females), this analysis included only benthic and limnetic males. All statistical analyses were performed using STATISTICA version 10 (StatSoft, Inc., 2011).

We further explored the relationship between the relative distention of the two females used in each trial and the courtship vigour (combined zigzags and straight approaches) by calculating the major axis regression between the variables. We performed this analysis for each male ecotype separately, pooling males from all populations of each ecotype.

## RESULTS

The two parametric analyses produced very similar outcomes, and thus we present only results from the analysis in which trials constituted a random effect (Table 2). First, based on combined zigzags and direct approaches, males of the three ecotypes showed no overall preference for one female ecotype over the other (female ecotype term:  $F_{1,155} = 0.001$ , N.S.), and this held for all three male ecotypes (male ecotype  $\times$  female ecotype interaction term:  $F_{2,154.4} = 0.37$ ,  $P = 0.69$ ; Fig. 1). There was modest variation among populations within ecotypes ( $F_{7,145.5} = 3.14$ ,  $P = 0.004$ ); however, in no population was there a substantial

**Table 2.** Nested, factorial ANCOVA results for the analysis of courtship vigour (combined zigzags and direct approaches) by males

Model term	Effect type	SS	d.f.	MS	Error d.f.	Error MS	<i>F</i>	<i>P</i>
Intercept	Fixed	5055.5	1	5055.5	6.73	7.840	644.7	0.0001
Distention difference	Fixed	22.024	1	22.02	84.42	2.813	7.820	0.0064
SL difference	Fixed	0.111	1	0.111	113.98	2.685	0.041	0.8393
Male ecotype	Fixed	13.794	2	6.89	6.87	7.670	0.899	0.4499
Female ecotype	Fixed	0.002	1	0.002	154.98	1.258	0.001	0.9716
Interaction	Fixed	0.924	2	0.462	154.45	1.257	0.367	0.6930
Population within ecotype	Random	52.589	7	7.513	145.53	2.395	3.136	0.0040
Trial within population	Random	351.843	148	2.377	150.00	1.248	1.904	0.0001
Error		187.262	150	1.248				

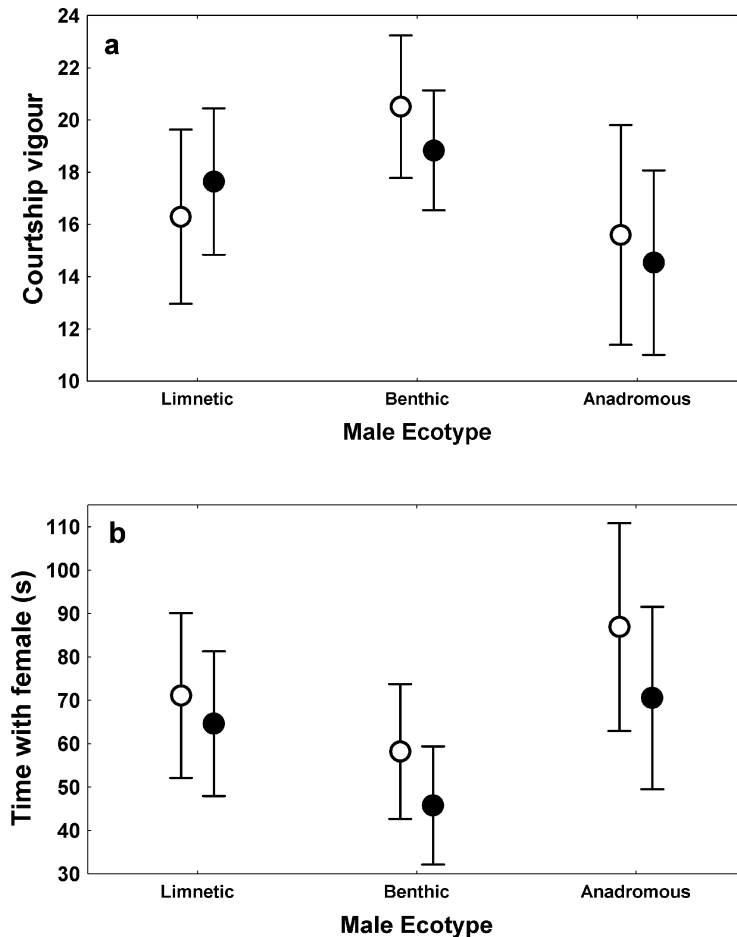
preference for one female type over the other (compare male population means between the left and right top panels of Fig. 2). Similarly, our second measure of courtship preference, the amount of time a male spent following a female, indicated no overall preference (Table 3) for either female ecotype (female ecotype term:  $F_{1,150} = 2.15$ ,  $P = 0.14$ ). Males spent no more time following females of their own ecotype than females of the other ecotype (interaction term:  $F_{2,150} = 0.69$ ,  $P = 0.50$ ).

Even though we size-matched females, we could not completely eliminate differences, and thus we included the size difference as a covariate. The relative size of the two females in a trial was not important in predicting male preference in terms of combined zigzags and direct approaches ( $F_{1,114} = 0.04$ ,  $P = 0.89$ ). However, the relative clutch size of a female did affect the number of zigzags and direct approaches a male exhibited towards her ( $F_{1,84.4} = 7.83$ ,  $P = 0.006$ ). Surprisingly, across all three ecotypes males directed more zigzags and direct approaches towards the female with the smaller estimated clutch size, regardless of the female ecotype (Fig. 3). The amount of time a male spent following a female was not predicted by either female size or clutch size differences (Table 3).

The generalized analysis comparing the categorical preferences of benthic and limnetic males (preferred 'own' or 'other' female ecotype) largely mirrored the parametric analyses, indicating no significant overall difference in preference for 'own type' by either limnetic or benthic males ( $\chi^2_1 \leq 1$ ,  $P = 0.99$ ). Although there was some population-to-population preference differences (Fig. 4), the population-level variation was not quite significant ( $\chi^2_6 = 9.77$ ,  $P = 0.13$ ).

## DISCUSSION

In parallel ecological speciation, reproductive isolation evolves independently and repeatedly between populations exposed to differing environments, but not between those evolving in similar environments. Thus, we expect to see at least incipient reproductive

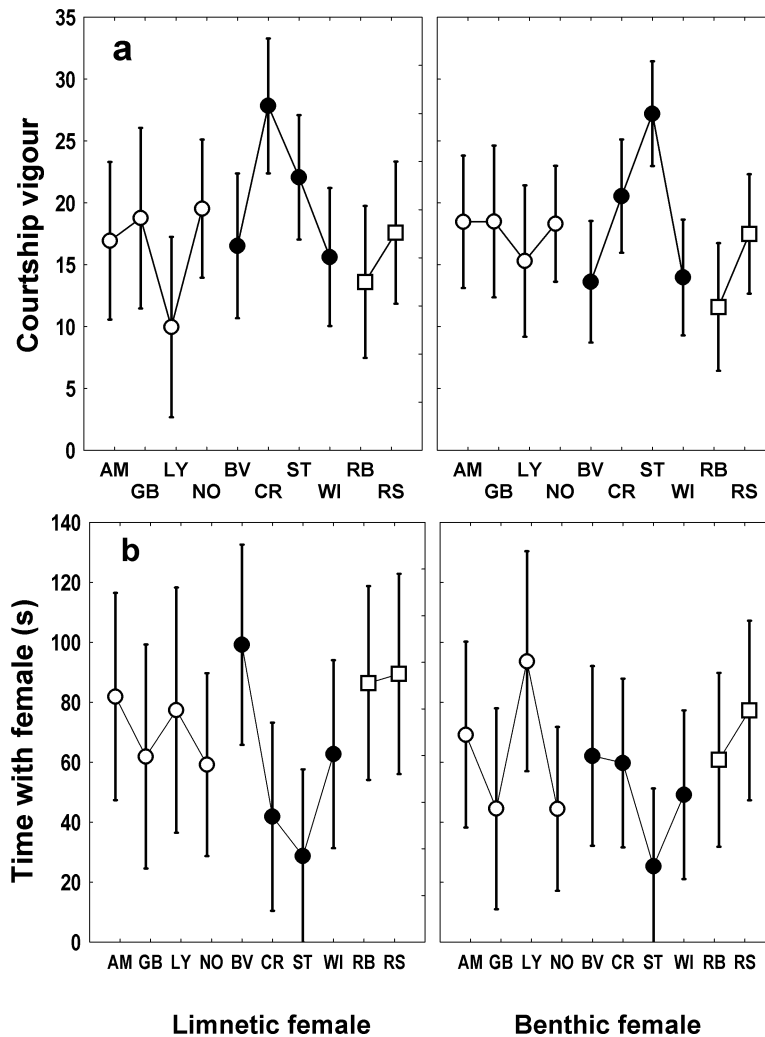


**Fig. 1.** Differences among the ecotypes in male responses to females: panel (a) compares combined zigzags and direct approaches by males towards a female, while panel (b) compares the time spent following a female. Open circles indicate responses to limnetic females and solid circles responses to benthic females. Raw data are displayed although data were transformed for statistical analyses as described in the text. Symbols are means; whiskers are 95% confidence bounds.

isolation between populations of differing ecotypes, but not between those of the same ecotype. Using allopatric benthic and limnetic populations, we evaluated whether male mate choice contributes to reproductive isolation in stickleback. We found no evidence that male stickleback distinguished between benthic and limnetic females during courtship, based on female visual cues. Unexpectedly, males did prefer females with a smaller estimated clutch size. This is contrary to the prediction that males should prefer more fecund females (for a review, see Edward and Chapman, 2011), and to previous work showing that male threespine stickleback court larger, more fecund females more intensively than they do smaller, apparently less fecund females (Rowland, 1982; Kraak and Bakker, 1998).

Visual cues have been used successfully to evaluate male mating preferences in stickleback. Dummy females, which can only provide visual cues, were presented to males who



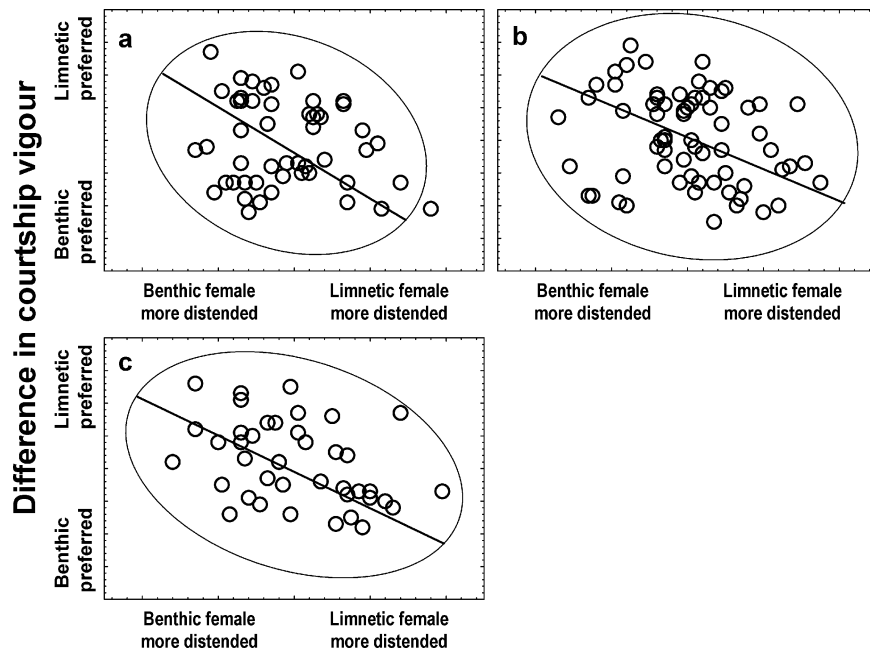


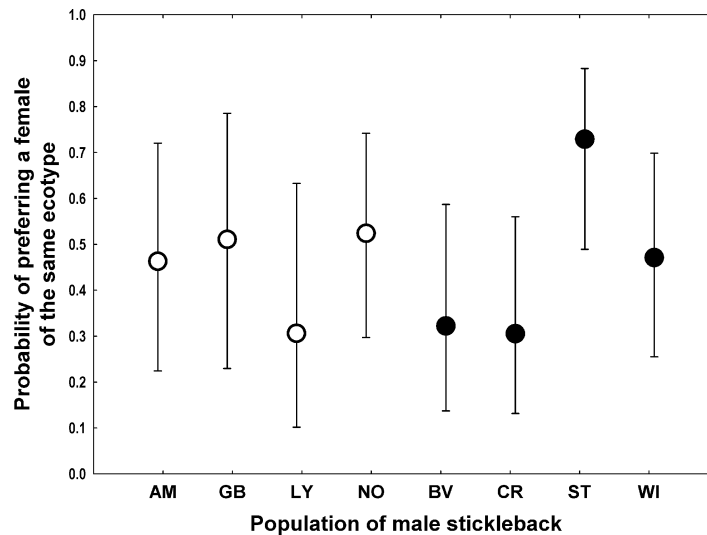
**Fig. 2.** Population differences in (a) courtship vigour (combined zigzags and direct approaches to females) and (b) time spent following a female. The left-hand panels show the responses of males from the individual populations to the limnetic female in a trial, while the right-hand panels show the responses of the males to the benthic female in a trial. Open circles indicate responses by limnetic males, solid circles by benthic males, and squares by anadromous males. Symbols are means; whiskers are 95% confidence bounds. Limnetic populations are in Ambrose (AM), Garden Bay (GB), Lynne (LY), and North (NO) lakes, while benthic populations are in Beverly (BV), Crystal (CR), Stepan (ST), and Willow (WI) lakes. Anadromous populations are from Resurrection Bay (RB) and Rabbit Slough (RS). Additional details are given in the text.

responded preferentially to those which were largest or had the most distended abdomens (Rowland, 1982, 1989). Similarly, live females confined in Plexiglas containers, which preclude the exchange of olfactory cues, have been used to document differential body size preferences between sympatric and allopatric limnetic males (Albert and Schluter, 2004) and to demonstrate a general preference for larger females (Sargent *et al.*, 1986). The results of these previous studies

**Table 3.** Nested, factorial ANCOVA results for the analysis of time spent by males near females of one of two ecotypes

Model term	Effect type	SS	d.f.	MS	Error d.f.	Error MS	<i>F</i>	<i>P</i>
Intercept	Fixed	712.95	1	712.9	6.674	0.789	902.9	0.0001
Distention difference	Fixed	0.008	1	0.008	103.101	0.341	0.024	0.877
SL difference	Fixed	0.065	1	0.065	128.678	0.327	0.199	0.656
Male ecotype	Fixed	2.125	2	1.062	6.836	0.773	1.373	0.315
Female ecotype	Random	5.319	7	0.759	141.862	0.297	2.552	0.017
Interaction	Random	44.282	148	0.299	150.000	0.394	0.758	0.954
Population within ecotype	Fixed	0.847	1	0.847	150.000	0.394	2.146	0.145
Trial within population	Fixed	0.544	2	0.272	150.000	0.394	0.690	0.503
Error		59.209	150	0.394				

**Fig. 3.** Relationship between courtship vigour (combined zigzags and direct approaches) and female distention (based on estimations of female clutch sizes). The major axis regression is significant for anadromous males (a: slope =  $-0.51$ ,  $P = 0.02$ ) and approaching significance for limnetics (b: slope =  $-0.70$ ,  $P = 0.06$ ) and benthics (c: slope =  $-0.51$ ,  $P = 0.08$ ). Ellipses are 95% bivariate confidence bounds.



**Fig. 4.** Predicted probability that a male from a specific population would show a preference for a female of his own ecotype. Symbols are estimated probabilities, and whiskers indicate 95% confidence bounds. Open circles indicate limnetic populations, solid circles benthic populations. Acronyms indicating specific populations are as in Fig. 2.

suggest that visual cues do provide sufficient information for males to use in mate choice. It is possible that much of the choice detected previously was based upon some measure of body size difference (e.g. length, distention, or projection area), and our close matching of standard length removed these cues. Previous research has found that probability of mating between sympatric benthic and limnetic stickleback increases as size disparity decreases (Nagel and Schluter, 1998).

Alternatively, males may also use tactile and olfactory cues when choosing among females, and it may be that these additional cues distinguish ecotypes. Dorsal pricking is a common courtship behaviour in benthic and oceanic populations (Foster, 1994, 1995; Foster *et al.*, 1998, 2008) where it may be favoured because it is relatively inconspicuous and allows the courting pair to monitor risk posed by foraging groups (Sargent, 1982; Foster, 1995). Females and males were not able to perform this courtship behaviour, as the females were confined. Olfaction plays a role in mate choice in sympatric populations of stickleback (Eizaguirre *et al.*, 2011), and females can imprint on their father's olfactory cues (Kozak *et al.*, 2011). The role of tactile and olfactory cues should be explored to determine whether some combination of these cues allows males from allopatric ecotypes to distinguish between females from allopatric ecotypes.

Other studies of assortative mating in threespine stickleback that used laboratory-reared fish also failed to detect positive assortative mating between ecotypes (Raeymaekers *et al.*, 2010; Räsänen *et al.*, 2012). Lack of assortative mating among laboratory-reared individuals may support the hypothesis that plastic or environmentally influenced traits play a role in assortative mating (Räsänen *et al.*, 2012). One possibility is that learning influences mating preferences in stickleback more strongly than was initially suspected. In 1994, McPhail suggested that imprinting could play a role in speciation in threespine stickleback. Recently, imprinting and social learning have been shown to influence mate choice and courtship

behaviour in stickleback (Kozak and Boughman, 2009; Kozak *et al.*, 2011). As most other studies examining male mate choice in threespine stickleback have used wild-caught fish (Albert and Schluter, 2004; Boughman *et al.*, 2005; but see Räsänen *et al.*, 2012), the difference between our results and those studies could reflect reduced opportunities for learning. In particular, the opportunity to imprint upon the father is missing, although the potential for imprinting upon siblings is retained. It would appear in this case that social learning has little influence upon male mating preferences.

Across all ecotypes, when there was a large difference in clutch size between the two females, males preferred the less fecund female. This contradicts the prediction that males should prefer larger, more fecund females in general (Edward and Chapman, 2011) and in stickleback specifically (Rowland, 1982; Kraak and Bakker, 1998). The benthic and limnetic females did not differ systematically in length, as the experimental design controlled for size, but benthic females had a greater mean estimated clutch by about nine eggs. One possibility is that benthic and anadromous males avoid courting larger females, as larger females might be more likely to cannibalize eggs from a male's nest (Albert and Schluter, 2004). Under this scenario, limnetic males would presumably have retained the ancestral tendency to avoid large females. As our populations are allopatric, there is no opportunity for reinforcement of this tendency, whereas reinforcement likely enhanced isolation in the sympatric benthic–limnetic populations (Albert and Schluter, 2004). Other research has shown that males courted smaller females and that the relationship between male and female body size can affect a male's courtship behaviour (Delcourt *et al.*, 2008; Räsänen *et al.*, 2012). However, these studies used stickleback from a lake–outlet system that can still experience gene flow, whereas the allopatric populations that we used do not. Since female behaviour was not scored during the trials, it is possible that there was a behavioural difference between females that also affected the male's response.

Reproductive isolation between divergent ecotypes is one of the defining characteristics of ecological speciation. Mating isolation can develop as a consequence of adaptation to divergent environments, and often this can be the product of mate choice. Although males can evolve mate preferences, the contribution of this factor to reproductive isolation has rarely been evaluated. As a species with mutual mate choice, the threespine stickleback is an ideal candidate in which to explore the role of male mate preference in incipient ecological speciation. The results we present here suggest that visual cues provided by females did not elicit male behaviour indicative of a preference for females similar in ecotype. This does not preclude a role for male mate preference in the evolution of incipient reproductive isolation between divergent allopatric ecotypes, but suggests that further studies involving additional cues and gene  $\times$  environment interactions are needed to fully explore the causes of incipient reproductive isolation that has been shown to exist between allopatric benthic and limnetic ecotypes. Further examination could provide novel insights into the process of ecological speciation.

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