Genetic and plastic components of divergent male intersexual behavior in Misty lake/stream stickleback

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The extent of variation in mating signals between environments can be an important determinant of reproductive isolation. We tested divergence in male courtship behavior between lake and stream three-spined stickleback (*Gasterosteus aculeatus* L.) in the Misty system, Vancouver Island. Presumably, genetically based divergence was assessed by comparing male behavior (aggressive, display, and nest activities) among Lake, Inlet, and Outlet ecotypes that were reared from fertilization to maturity in a common environment. Plastic components of behavior were assessed by comparing male behavior in presence of females from each of the 3 ecotypes. We found that Inlet males had less aggressive elements than Lake and Outlet males in their behavior, whereas Lake and Outlet males were similar in their behavior. We further found that males of all ecotypes performed less display and nest activities in the presence of Lake and Outlet than Inlet females and that the male mating behavior was influenced by the relative difference in male and female body sizes. We suggest that divergent selection has driven genetic divergence in aggressive behaviors, whereas other behavioral components respond more plastically to variation in female phenotype. We discuss the implications of these results for reproductive isolation. *Key words:* adaptive divergence, courtship behavior, mating traits, natural selection, plastic divergence, reproductive isolation, sexual selection, three-spined stickleback. *[Behav Ecol 19:1217–1224 (2008)]*

The ecological theory of adaptive radiation predicts that reproductive isolation can arise as a by-product of adaptation to different environments (Schluter 2000, 2001). One way this process can occur is when the traits that undergo adaptive divergence also influence mating signals. To date, most studies of ecologically based divergence in mating signals have focused on nonbehavioral traits, such as color and morphology (see examples below). However, courtship behaviors will also likely contribute to mating isolation (Miller et al. 1998; Hurt et al. 2004; Podos and Hendry 2006). We here take the first step toward understanding to what extent mating isolation is influenced by genetic and plastic divergence in male courtship behavior.

Divergent environments might cause "genetic" divergence in mating signals in several different ways, of which we here mention 2. First, mating signals may initially diverge as a pleiotropic consequence of adaptation outside a mating context. As one example, the adaptation of beak sizes of Darwin's finches to different food types has caused correlated divergence in male songs (Podos 2001; Podos and Hendry 2006). Second, mating signals may diverge owing to direct selection for increased signal transmission to females (Endler 1992; Schluter and Price 1993; Boughman 2001). As one example, selection for increased visibility to females in the local photic environment may drive divergence in male color between fish populations (Boughman 2001; Maan et al. 2006).

Divergent environments may, however, also cause "plastic" divergence in mating signals, which might subsequently influence mating isolation (Price 2006). As an example, the progeny of anadromous (ocean going) fish sometimes remain in freshwater where growth opportunities are limited and hence mature at a much smaller size. Mating may then be limited between anadromous and freshwater individuals under a "mate with your own size" rule (Wood and Foote 1996; McKinnon et al. 2004). Another possibility is that ecologically based divergence in the mating traits of one sex (e.g., body size), whether genetic or plastic, may lead to plastic mate choice differences in the other sex. For example, male stickleback can be more aggressive toward larger females—because these are more likely to cannibalize the eggs already in a male's nest (Albert and Schluter 2004).

Few studies have explicitly evaluated the genetic and plastic contributions to divergence in mating signals. Yet, this should have important implications for mating isolation in nature. For example, plastic differences in mating signals may mean that individuals moving between environments are not selected against—if they are able to produce the appropriate signals in the new environment. This realization has motivated our interest in determining to what extent male three-spined stickleback (*Gasterosteus aculeatus*) from divergent environments (i.e., "ecotypes") differ genetically or plastically in their courtship behavior.

Study system

Three-spined stickleback are renowned for their repeated and independent postglacial adaptation to particular freshwater environments (Bell and Foster 1994; McKinnon and Rundle 2002). Some repeated axes of divergence include benthic–limnetic, freshwater–anadromous, mud–lava, and lake–stream. Previous work has demonstrated divergence in mating traits that influence mating isolation for benthic–limnetic

(Foster 1995; Foster and Baker 1995; Nagel and Schluter 1998; Boughman 2001; Albert and Schluter 2004; Boughman et al. 2005; Vines and Schluter 2006), freshwater–anadromous (Hagen 1967; McPhail and Hay 1983; McKinnon et al. 2004), and mud–lava contrasts (Olafsdottir et al. 2006). Our work asks similar questions for lake–stream contrasts, starting here with an analysis of genetic and (some) environmental influences on male mating behavior.

We specifically focus on stickleback in the Misty Lake and its inlet and outlet streams (Northern Vancouver Island, British Columbia, Canada). Misty Lake and Inlet stickleback differ dramatically in genetically based, and apparently adaptive, morphological traits (Lavin and McPhail 1993; Hendry et al. 2002; Moore and Hendry 2005; Moore et al. 2007). They have also diverged in traits that stickleback use in mate choice: Inlet fish are small and Inlet males have drab nuptial coloration, whereas Lake fish are large and Lake males have blue–black nuptial coloration (Lavin and McPhail 1993; Millar NP, unpublished data). In contrast to Inlet fish, Outlet fish are similar to Lake fish both in morphology and color, presumably owing to intermediate selection in the outlet and high gene flow from the lake (Hendry et al. 2002; Moore et al. 2007). We here ask whether these 3 ecotypes differ also in male courtship behavior and in the effects of female ecotype on male courtship behavior. Note that although the Outlet fish, due to their intermediate phenotypes, arguably do not represent a different ecotype, the population inhabits a distinct location, and we therefore use ecotype for convenience of presentation.

The genetic and environmental contributions to mating behavior and mating isolation are not currently known for stickleback. The reason is that existing studies have either used wild-caught fish (e.g., Nagel and Schluter 1998; Rundle and Schluter 1998; Hay and McPhail 2000; Ishikawa and Mori 2000; Albert and Schluter 2004; Boughman et al. 2005) or not explicitly evaluated both environmental and genetic effects (Hatfield and Schluter 1996; Albert 2005). We investigate (presumably) genetically based and plastic components of behavior by studying Lake, Inlet, and Outlet stickleback reared from fertilization to maturity in a common-garden environment. We then compare the courtship behavior of each male ecotype toward females of each ecotype. This design allowed us to test for genetic (main effect of male ecotype), plastic (main effect of female ecotype), and genotype-by-environment (interaction between male and female ecotypes) effects on male courtship behavior. We then discuss the implications of these effects for mating isolation in nature.

MATERIALS AND METHODS

Collections and rearing

In June 2004, parental fish were collected using unbaited minnow traps from 3 sites in the Misty system on Vancouver Island, Canada: Misty Lake (lake site 1 in Moore et al. 2007), Misty Inlet (inlet site 4 in Moore et al. 2007), and Misty Outlet (outlet site 4 in Moore et al. 2007). Standard artificial crossing methods (Hatfield and Schluter 1996) were used to generate 8 full sibling families for the Lake, 7 for the Outlet, and 4 for the Inlet (fewer mature females were available for the Inlet during the study period). Fertilized eggs were transferred to our laboratory at McGill University, Montréal, Canada, where rearing proceeded in 20- to 100-L tanks. Members of each family were raised together but split across multiple tanks to achieve roughly equal densities (ca., 25 individuals per 100 L). Rearing tanks were randomly assigned to 1 of 4 environmental chambers. Sexes were not separated during rearing because they could not be reliably identified before maturity. For the

first few months, fish were fed live brine shrimp nauplii (*Artemia* sp.), which was later followed by a mixed diet of brine shrimp nauplii, frozen blood worms (*Chironomus* sp.), and frozen brine shrimp. In the months leading up to maturity, the fish were fed a mixture of live brine shrimp and live blackworms (*Lumbriculus* sp.).

The fish were maintained under constant "summer" conditions (16 h of light and 8 h of dark, 17 °C) until March 2005, when they were switched to constant "winter" conditions (8 h of light and 16 h of dark, 12 °C). The different environmental chambers were switched back to summer conditions at various times from September through October 2005 to allow an extended experimental period. No nesting material was provided in the rearing tanks to prevent any nesting activity prior to the mating trials.

Mating trials

The experiment involved 18 large tanks (102 L; length = 92 cm, width = 32 cm, depth = 39 cm), each filled with water to a depth of 20 cm and having their bottom covered with dark gravel. A patch of fine sand (15 cm long and 2.5 cm deep) was added at the left end of each tank to serve as a nesting site. Previous pilot experiments had shown that stickleback in the Misty system prefer dead plant material for building their nests. We therefore collected this material from the Misty system and provided it, together with some moss, in all the experimental tanks. All sides of the experimental tanks were covered with brown packing paper to prevent external disturbances.

At maturity (judged by appearance of nuptial color), individual males were placed into experimental tanks and stimulated to build a nest by exposing them to a gravid female for 15 min once or twice each day. In each case, the stimulus female was placed into the tank in a clear glass jar with a mesh lid therefore allowing chemical and visual cues but no direct contact. Care was taken to ensure that all the males saw a range of stimulus females and that each stimulus female was presented to many of the males. A total of 43 males were considered to be ready for the actual mating trials (below) when they guarded a well-structured nest. Males that did not build a nest within a week were removed and replaced with a new male.

For the actual mating trials, we simulated the photic environment of the Misty system, which is highly tannic and dominated by red light (Millar N, unpublished data). This was done by using full spectrum flourescent light passed through an appropriate filter (rust, code 777, Lee filters, Burbank, CA). The experiments were run under summer conditions (16 h light and 8 h dark, 18 °C), and each "trial" was initiated by introducing a single gravid female (judged by abdomen shape and cloacal coloration) into the tank of a male with a ready nest.

Our study design sought to control for variation among males by exposing each male to females of each ecotype: Lake (L), Inlet (I), and Outlet (O). The different females were introduced to a given male individually and sequentially, interspersed by at least 3 h (more often 1 day) without a female in the tank. This procedure was designed to mimic conditions in the wild, where males can encounter and spawn with multiple females (Foster 1994). The order of the female ecotypes was alternated so that each male ecotype was exposed to the different female ecotype sequences in roughly similar proportions. However, some exceptions occurred owing to variation in the availability of gravid females of the right ecotype at the right time. For the same reason, 6 of the 43 total males were tested with only 2 female ecotypes and 3 were tested with only 1 female ecotype. Each female was used only once, and males and females from the same family were not combined in any trial. Of the 117 total trials, the numbers per male–female

ecotype combination (male ecotype listed first) were 14 II, 15 IL, 16 IO, 15 LL, 12 LI, 12 LO, 12 OI, 11 OL, and 10 OO.

Because each male was tested with multiple females, we did not allow females to actually spawn with the male. If a female entered a nest, we induced her to leave by gently squeezing her caudal peduncle with long forceps. After a trial was completed, the female was removed and her abdomen pressed gently to confirm that her eggs were ripe (ripe eggs require only gentle pressure to extrude). Trials where the females' eggs were not ripe were considered invalid, and a male from such trials was later presented with a different female of the same ecotype. When a male had completed valid trials with all 3 female ecotypes (whenever possible), he was removed from the tank and another male was introduced for use in further trials after replacing all tank material. After the last trial for a given fish, it was killed with an overdose of MS222, photographed, dry blotted, and weighed after removal of eggs (with a digital scale to the nearest 10 mg).

Male behavior

Male behavior was recorded on digital video (model ZR90, Canon) from the moment a female was introduced to a tank until she was removed (after 1 h or earlier—if she entered the male's nest). We here analyze the first 15 min (starting from the first behavior a male directed toward a female) or until the female entered a nest. We chose this length of time because it encompasses the most intense interactions and because it is at least as long as in most previous studies of stickleback (Nagel and Schluter 1998; Rundle and Schluter 1998; Albert and Schluter 2004). Female behavior was not recorded because females were sometimes out of the camera's field of view during the tracking of males (males were always in the field of view).

Behaviors were scored from the digital videos by a single person (M.D.) on an event recorder (The Observer, Noldus Technologies, Leesburg, VA). Eighteen different male behaviors (see Appendix 1) were counted. We refer here to all behaviors as courtship behaviors. Note, however, that at least the "aggressive" behavioral components may also be used to chase away females rather than to attract female's attention during mating (Scotti and Foster 2007). Subsets of these behaviors were then grouped into more inclusive categories so as to increase independence among variables and to reduce type I errors that might result from multiple tests. The categories were designed to reflect functional relevance, and they were supported by principal component analysis (not shown). The categories were aggressive behaviors (bite, dorsal pricking, and chase), displays (zigzag, circle dance, and lead), and nest activities (nest fanning, material placing, nest glueing, and nest pecking). For each individual behavior and for each of the 3 behavioral categories, we calculated the frequency of occurrence (counts per minute) and then used square root transformations to improve normality.

Statistical analyses

We first used discriminant functions for comparisons of overall male behavior among the 9 male–female ecotype combinations. Specifically, we used frequencies of the 3 behavioral categories to classify trials from each of the ecotype combinations back into those combinations. This analysis takes into account the multivariate behavioral scoring to first define typical courtships for each ecotype combination and to subsequently reassign each courting male into the combination that shows the closest courtship pattern. We next used a mixed-model multivariate analysis of variance (MANOVA) to determine whether frequencies in the 3 behavioral categories were influenced

by male ecotype (fixed), female ecotype (fixed), the male–female ecotype interaction (fixed), trial order (fixed), male family (random, nested within male ecotype), and male identity (random, nested within male family). We also used similarly structured univariate analyses of variance (ANOVAs) in analyses of each behavioral category and each of the 18 specific behaviors. Pairwise comparisons were based on post hoc Tukey tests on least square (LS) means.

Our presentation will focus mostly on the 3 behavioral categories (for the reasons noted above). In addition, we explicitly discuss analyses for "bites" and "zigzags"—because these behaviors can be particularly important for mate choice and species recognition (McPhail and Hay 1983; Foster 1995). Analyses for the other individual behaviors were done but are not discussed in the text. Finally, we considered possible effects of body size—because this can strongly influence courtship and mate choice (e.g., Nagel and Schluter 1998; Rundle and Schluter 1998; McKinnon et al. 2004; Boughman et al. 2005). We did so by rerunning the analyses after including relative body size (male mass minus female mass), as well as its interactions with male and female ecotypes, as a covariate (i.e., analysis of covariance). We first ran a full model including all interactions, followed by sequential removal of nonsignificant interactions. We only present the results of the final models. Including relative body size in the analyses allowed us to test whether male courtship behavior was influenced by the relative size of the female he was encountering.

In the above analyses, a significant main effect of male ecotype would suggest that male ecotypes differ "genetically" in courtship behavior (independent of female ecotype). Note that because we used full-sib families from parents caught in the wild, maternal effects cannot be fully excluded. Given the lack of maternal care and that there is no difference in egg size among these study populations (Baker JA, Räsänen K, Moore J-S, Hendry AP, unpublished data), maternal effects seem unlikely—but some caution is warranted in interpretation. A significant main effect of female ecotype would suggest that female ecotype induces plastic differences in male courtship behavior (independent of male ecotype). A significant male–female ecotype interaction would suggest that male ecotypes differ in their responses to different female ecotypes, that is, a genotype (male ecotype) by environment (female ecotype) interaction. A significant effect of relative body size would suggest that male behavior depends on own versus female body size. All statistical analyses were run in JMP (version 6.0.0).

RESULTS

Multivariate analyses

The discriminant analyses revealed that male behavior differed among the male–female ecotype combinations (Chi-square test: $\chi^2 = 122.110$, degrees of freedom [df] $= 16$, $n = 117$, $P < 0.001$; Figure 1A). In particular, Inlet males differed strongly from Lake and Outlet males, which were very similar (Figure 1). These differences remained when female ecotypes were pooled within each male ecotype (Chi-square test: $\chi^2 = 47.508$, df $= 4$, $n = 117$, $P < 0.001$; Figure 1B), but not when male ecotypes were pooled within female ecotypes (Chi-square test: $\chi^2 = 1.163$, df $= 4$, $n = 117$, $P = 0.884$). We therefore conclude that the main effect of male ecotype (genetic) is considerably stronger than the main effect of female ecotype (plastic).

The MANOVA confirmed that male behavior differed strongly among male ecotypes (Wilk's lambda $= 0.33$, $\eta^2 = 0.306$, $P < 0.001$) and revealed a significant, although weaker, effect of female ecotype (Wilk's lambda $= 0.73$, $\eta^2 = 0.1$, $P = 0.002$). There was no significant interaction between male
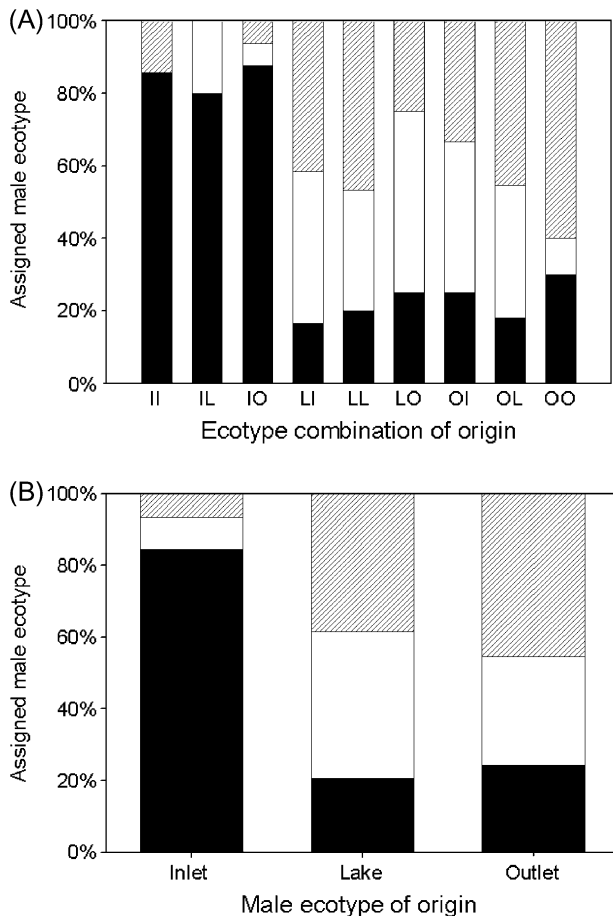
Figure 1
Variation in male courtship behavior among ecotypes as revealed by the discriminant function constructed from the 3 behavioral categories (see Materials and methods). Shown are the proportions of trials in a given ecotype combination (*x* axis) that were assigned to a given male ecotype (*y* axis). In Panel A, female ecotypes are pooled within male ecotypes for the ecotype categories to which trials were assigned (*y* axis). In Panel B, female ecotypes are pooled within male ecotypes for the categories of origin (*x* axis) and for the categories to which trials were assigned (*y* axis). Filled areas correspond to the proportion of assignment to Inlet ecotype, open areas to Lake ecotype, and hatched areas to Outlet ecotype.

ecotype and female ecotype (Wilk's lambda = 0.86, $\eta^2 = 0.049$, $P = 0.613$). MANOVA further revealed that male behavior was influenced by family (Wilk's lambda = 0.21, $\eta^2 = 0.405$, $P < 0.001$), suggesting potential genetic/maternal effect variation within populations, and by male identity (Wilk's lambda = 0.17, $\eta^2 = 0.447$, $P < 0.001$). A significant trial-order effect (Wilk's lambda = 0.72, $\eta^2 = 0.104$, $P = 0.002$) indicated that males were more active at their nest in the second and third trials than in the first trial (data not shown).

Univariate analyses

The ANOVAs indicated that the effects of male and female ecotypes on male behavior differed among the 3 behavioral categories (Table 1). For aggression, male ecotype effects were strong, with Inlet males being less aggressive than Lake/Outlet males (Figure 2A). In contrast, overall aggression did not depend on female ecotype or the interaction between male and female ecotypes (Table 1). A marginally significant male ecotype × body size effect (Table 2) arose because Inlet males

were more aggressive toward relatively small females (slope = 0.442 ± 0.209, $t = 2.12$, $P = 0.039$), whereas Lake and Outlet males showed no significant relationship with relative body size (both $P > 0.28$). After inclusion of body size, the male ecotype effect remained significant. Similar patterns were evident when individually analyzing bites, the most overt aggressive behavior (Tables 1 and 2): Inlet males bit females much less frequently than Lake/Outlet males did (Figure 2B). However, a significant male ecotype × body size effect (Table 2) arose because Inlet males bite smaller females more (slope = 0.496 ± 0.154, $t = 3.22$, $P = 0.002$), whereas Lake males bite larger females more (slope = -0.430 ± 0.181, $t = -2.38$, $P = 0.021$), and there was no body size effect on Outlet males ($P = 0.82$). Taken together, these results suggest genetic divergence between Inlet and Lake/Outlet males in aggressive behaviors toward females but that the extent of aggression is influenced by differences in male and female body sizes.

In contrast to aggressive behaviors, frequency of displays and nest activities did not differ among male ecotypes but instead depended on female ecotype (Table 1). In particular, all male ecotypes displayed least in the presence of Lake/Outlet females and were most attentive to their nest in the presence of Inlet females (Figure 2C,E). Again, no interaction was evident between male and female ecotypes (Table 1). Similar patterns were evident when analyzing zigzags, the most overt display behavior (Table 1). The only difference was that the male ecotype effect was marginally significant: Inlet males zigzag more than do Lake/Outlet males (Figure 2D). Including relative body size as a covariate revealed that males displayed more toward relatively small females (slope = 0.413 ± 0.188, $t = 2.19$, $P = 0.033$) but did not reveal a direct influence of relative body size on nest activities (Table 2). For both behavioral categories, however, the addition of relative body size eliminated the female ecotype effect. This suggests that males may react differently to Lake/Outlet females simply because these are larger (Table 3).

Note that we included male identity and male family in all ANOVAs but for the sake of simplicity do not present the result details. In short, male identity often had significant effects (in absence of body size in the models, aggressive behaviors, display, nesting activities, and bites had all $P < 0.03$; with body size in the model, $P < 0.04$ for display and nesting behaviors), whereas male family effects were never significant (all $P > 0.06–0.40$)—suggesting little within-population variation in genetic/maternal effects.

DISCUSSION

We here report likely genetically based divergence in intersexual behavior between Inlet and Lake/Outlet males in the Misty system. In particular, Inlet males appear to be less aggressive toward females than are Lake/Outlet males. However, male behavior is also plastically influenced by female phenotype. In particular, males of all ecotypes displayed less toward Lake/Outlet females, which are big, and were more attentive to their nest in the presence of Inlet females, which are small. Moreover, Inlet males appeared to be more aggressive toward relatively smaller females. In light of these main findings, we now discuss 1) genetic divergence between Inlet and Lake males, 2) the lack of divergence between Lake and Outlet males, and 3) the environmental (plastic) effect of female ecotype. We close with a discussion of implications of genetic versus plastic courtship behaviors for ecologically driven reproductive isolation.

Divergence between Inlet and Lake males

Stickleback in the Misty Inlet and Lake experience strongly divergent environments, with different patterns of water
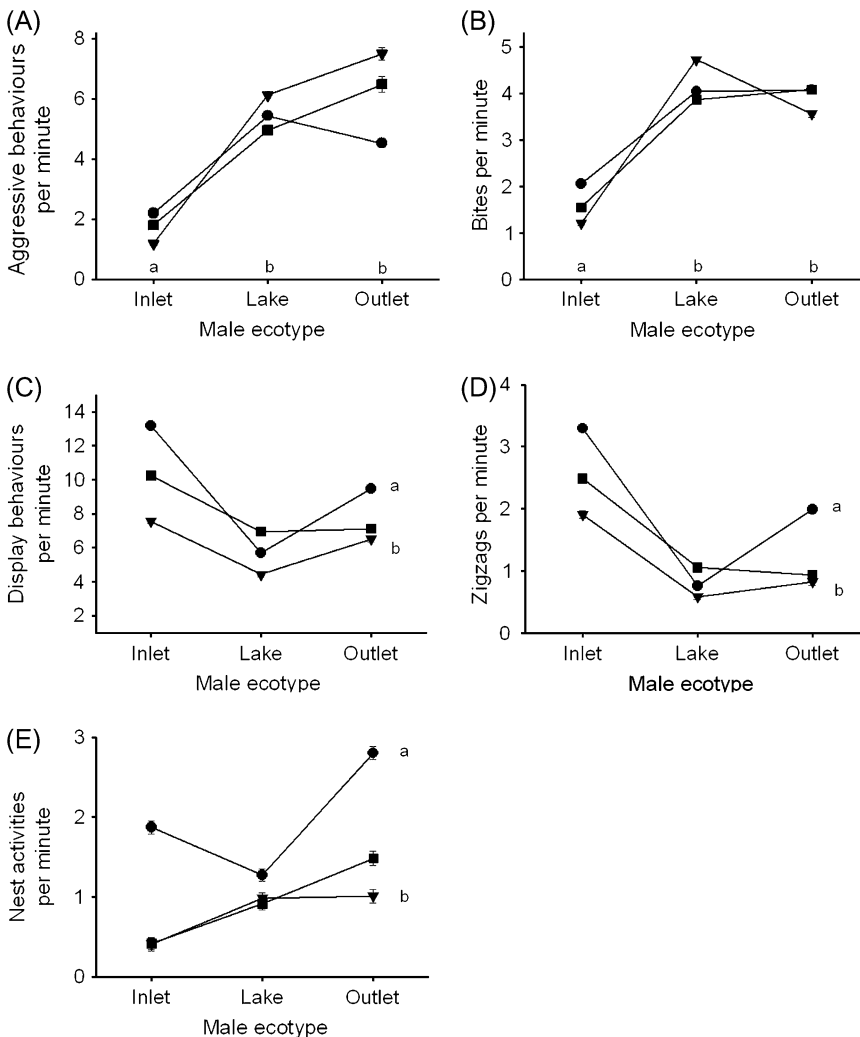
Table 1

Mixed-model ANOVA for the effects of male and female ecotypes on 3 behavioral categories and 2 selected behavioral components (bite and zigzag) in Misty lake and stream stickleback

Source	Male ecotype		Female ecotype		Male ecotype × female ecotype		Trial order	
Trait	$F_{2,14}$	P	$F_{2,66}$	P	$F_{4,66}$	P	$F_{2,66}$	P
Aggressive behaviors	9	**<0.001**	0.35	0.703	1	0.415	1.1	0.325
Display behaviors	1.47	0.262	3.76	**0.029**	0.7	0.595	2.23	0.116
Nest activities	0.77	0.480	6.13	**<0.004**	0.89	0.475	12.15	**<0.001**
Bite	6.67	**0.009**	0.21	0.812	0.5	0.735	2.1	0.131
Zigzag	3.01	0.082	3.88	**0.026**	0.96	0.435	0.23	0.794

Random effects of male identity and family (see text) not shown.

movement, water depth, aquatic vegetation, food types, and (probably) predation (Lavin and McPhail 1993; Moore and Hendry 2005; Moore et al. 2007). These ecological differences have caused adaptive divergence in a broad suite of morphological traits (Lavin and McPhail 1993; Hendry et al. 2002; Hendry and Taylor 2004; Moore and Hendry 2005). We here found strong divergence also in the frequency of aggressive behavioral components that males direct toward females (Table 1 and Figure 2A), and we now consider possible evolutionary explanations.

One possible explanation for divergence in aggressive behaviors relates to egg predation (cannibalism) by females. Nesting stickleback males from populations that show high levels of egg predation usually also show higher aggression toward females (Foster 1994; Rundle and Schluter 1998; Albert and Schluter 2004). Several lines of indirect evidence suggest that a similar pattern is likely also in the Misty system. First, high egg predation rates appear to be a property of lakes rather than streams (Ridgway and McPhail 1988; Foster 1994; Foster and Baker 1995), although this has yet to be confirmed for the

Figure 2
Aggressive behaviors (A), bites (B), display behaviors (C), zigzags (D), and nest activities (E) per minute shown as LS means ± standard errors of frequencies. The 3 lines represent the 3 female ecotypes (Inlet: circles, Lake: triangles, Outlet: squares). Different letters indicate a significant difference among male ecotypes (horizontal comparison) and among female ecotypes (vertical comparison) in Tukey tests on LS means of transformed data.

Table 2

Mixed-model analysis of covariance for male and female ecotypes and relative body size (male mass − female mass) effects on 3 behavioral categories and 2 selected behavioral components (bite and zigzag) in Misty lake and stream stickleback

Source	Male ecotype		Female ecotype		Male ecotype × female ecotype		Trial order		Relative body size		Male ecotype × relative body size	
Trait	$F_{2,53}$	P	$F_{2,53}$	P	$F_{4,53}$	P	$F_{2,53}$	P	$F_{1,53}$	P	$F_{2,53}$	P
Aggressive behaviors	7.25	0.007	1.04	0.360	0.57	0.688	0.92	0.404	0.12	0.729	2.63	0.081
Display behaviors	0.85	0.447	0.68	0.512	0.68	0.610	2.01	0.144	4.8	0.033	—	—
Nest activities	0.38	0.692	2.45	0.096	0.81	0.524	7.64	0.001	0.29	0.590	—	—
Bite	5.43	0.018	0.04	0.959	0.49	0.745	1.49	0.235	0.01	0.931	5.76	0.005
Zigzag	3.2	0.09	0.98	0.381	0.92	0.46	0.39	0.679	1	0.322	—	—

Misty system. Second, large females are more likely to prey on eggs (Foster and Baker 1995) as well as to elicit aggression from males (Albert and Schluter 2004), and Misty Lake/Outlet females are considerably larger than are Inlet females (Table 3, Lavin and McPhail 1993; Hendry et al. 2002). Note that this may hold especially in the Lake population because Lake males appear to bite more when females are relatively larger. Third, "zigzagging" is more common in noncannibalistic populations (Foster 1995) and was here most common in Inlet males (Figure 2). Fourth, all male types performed fewer nest activities (which reveal the location of the nest) in the presence of the Lake females than in the presence of Inlet females.

Other adaptive explanations are also possible, however. For example, higher levels of predation may favor less conspicuous behaviors, whereas intense competition for breeding sites may favor more aggressive behaviors (Candolin 1997; Bell 2005). Both predation and competition intensity have yet to be quantified for the Misty system, but the Lake population does have a much higher density (Moore JS, Hendry AP, unpublished data). It is therefore possible that the lake environment is more competitive and favors higher rates of aggression, which then simply may carry over to male–female interactions. Such "behavioral syndromes" (reviewed by Sih et al. 2004) have recently been argued for stickleback populations, albeit in a different context (Bell 2005; Scotti and Foster 2007). More data are clearly needed on variation in competition and predation, as well as their influence on behavioral evolution.

Although adaptive divergence does seem most likely, nonadaptive explanations, including arbitrary "runaway" (Lande 1981) or "chase-away" (Holland and Rice 1998) sexual selection, genetic drift, and different ancestries, cannot be excluded. Genetic drift may play a role especially in the Inlet given the small population sizes at some Inlet sites (Moore JS, Hendry AP, unpublished data) and the lack of gene flow from the Lake (Hendry et al. 2002; Moore et al. 2007). Ancestry effects are also possible given that different ancestral lineages of mtDNA predominate in the Inlet and Lake populations (Thompson and Taylor 1997; Hendry et al. 2002). The contributions of these effects to male behavior cannot be assessed at present but would be interesting to examine in future work.

Table 3

Mean (±standard error) body mass (in grams) of males and females in Misty lake and stream stickleback

	Inlet	Lake	Outlet
Males	2.80 ± 0.06	3.41 ± 0.06	3.53 ± 0.09
Females	3.11 ± 0.07	3.92 ± 0.10	3.80 ± 0.09

Lack of divergence between Lake and Outlet males

Outlet and Lake males did not differ in their behavior (Figure 1), consistent with previous results for morphology (Hendry et al. 2002; Moore and Hendry 2005; Moore et al. 2007). At least 4 nonexclusive explanations may account for this pattern. First, the Lake and Outlet populations may have similar ancestries given that the same mtDNA clade predominates in both (Thompson and Taylor 1997; Hendry et al. 2002). Second, the lake and outlet habitats are much more similar than are the lake and inlet habitats (Moore and Hendry 2005; Moore et al. 2007), which may have selected for similar behaviors in Lake and Outlet males. Third, high gene flow from the Lake population to the Outlet population may constrain adaptive divergence in male behavior, just as it does for morphology (Hendry et al. 2002; Moore et al. 2007). Finally, the similar body sizes of Lake and Outlet females (Hendry et al. 2002, Table 3) may impose similar selection on male behaviors (see above). In accordance with our work on morphology (Moore et al. 2007), we suggest that the lack of divergence in male behavior between Lake and Outlet populations reflects a combination of weak divergent selection and high gene flow.

Plastic responses to female phenotype

Male stickleback in the Misty system responded in part plastically to female ecotype: all male ecotypes directed fewer displays toward Lake females and conducted more nest activities in the presence of Inlet females. The effect of female ecotype on male display behaviors appeared to be primarily driven by variation in the relative body size of males and females: males displayed less toward larger (Lake and Outlet) than toward smaller (Inlet) females and adding relative body size to the statistical models generally eliminated any significance of the female ecotype effect. Intriguingly, also aggressive responses of the different ecotypes were in part determined by relative body size of the sexes: Inlet males bit females more when these were relatively small, whereas the opposite was true for Lake males. In short, stickleback in the Misty system appear to respond differently to the different female ecotypes in part because of differences in body size.

Our results concur with previous work highlighting the importance of body size to sexual selection in stickleback. Stickleback of different ecotypes often mate assortatively by size (e.g., McKinnon et al. 2004; Vines and Schluter 2006), but males may also prefer bigger females due to larger clutches that these provide (Kraak and Bakker 1998) or avoid larger females due to higher risk of nest destruction and egg cannibalism by these (Foster and Baker 1995; Ishikawa and Mori 2006). We cannot currently exclude the possibility that male behaviors were also influenced by behavior of females if, for

example, small females were also more responsive. Nevertheless, our data suggest that genetic divergence in body size between lake and stream ecotypes may influence plastic responses in courtship behavior. These results warrant more detailed investigations on the interplay between body size and ecotype in the evolution of courtship behaviors.

Implications for mating isolation

Stickleback populations in the highly divergent environments of Misty Lake and Misty Inlet seemingly show genetic divergence in courtship behaviors, particularly aggression. We next discuss the potential implications of these laboratory-based patterns for mating isolation in nature. Some caution is warranted, however, due to potential unknown genotype-by-environment interactions that may occur between the laboratory and the natural environment. In general, the implications will depend on covariation between divergence in the male traits and female responses to those traits (Schwartz and Hendry 2006). In the case of tight covariation, lower (higher) aggression in Inlet (Lake) males may be associated with a preference in Inlet (Lake) females for less (more) aggressive males. In this case, Inlet and Lake males and females are expected to mate assortatively, and symmetric mating isolation might result. In the case of no covariation (e.g., female preferences have not diverged), females of both Lake and Inlet types may prefer a single male type, and asymmetric mating isolation might result. Our future work will examine these and other possibilities.

In contrast to the above comparison of Lake and Inlet males, Lake and Outlet males did not differ in courtship behavior. This result, combined with the lack of noteworthy morphological differences, makes mating isolation seem increasingly unlikely. We suggest that high gene flow between these 2 "populations" might be both a cause and a consequence of their minimal phenotypic divergence. That is, low phenotypic divergence might prevent the pleiotropic evolution of mating isolation, and low mating isolation might allow high gene flow and thereby cause low phenotypic divergence. Moreover, plasticity would probably not contribute to mating isolation if, for example, Lake and Outlet females elicit similar responses in both Lake and Outlet males when coming into contact.

In general, the implications of genetic and environmental effects for mating isolation is an underdeveloped area of research. Our study has made some initial inroads into an improved understanding of these effects within 1 study system. Our future work will examine the effects and underlying selection of variation in male courtship behavior (and other traits) for mating isolation. We encourage similar work in other study systems because the genetic and environmental effects on mating signals and preferences for those signals will likely have dramatic consequences for progress toward speciation.

APPENDIX A

Description of male courtship behaviors following van Iersel (1953), McPhail and Hay (1983), Rowland (1989, 1994), and Kraak and Bakker (1998). Behavioral categories are indicated by A (aggressive), D (display), and N (nest activities), and behaviors used in behavioral categories as well as the 2 chosen individual behaviors (bite and zigzag) are underlined.

Bite (A): male bites the female.
Dorsal pricking (A): male erects his dorsal spine and pricks the female's belly in a backward movement.
Chase (A): male swims rapidly toward the female and ends the bout with a physical contact.
Zigzag (D): male swims toward the female in a series of horizontal dashes.

Lead (D): male leads the female to his nest in a direct movement.
Circle dance (D): male swims around the female on a circular trajectory.
Direct approach (D): male swims toward the female in 1 bout.
Material placing (N): male places an element to his nest.
Nest pecking (N): male pushes parts of the nest with his snout.
Fanning (N): male fans the nest entrance with his pectoral fins.
Glueing (N): male spreads kidney secretion on the nest.
Showing the nest (N): male swims to his nest and pokes the entrance with his snout.
Creeping through (N): male enters his nest through the entrance and swims out on other end.
Male showing his belly (N): male rolls over partially presenting his belly to the female.
Duration of material placing (N): the proportion of time during which the male is placing materials.
Duration of fanning (N): the proportion of time during which the male is fanning his nest.

Other:
Male feeding: male feeds on materials found in the aquarium.
Duration of feeding: the proportion of time during which the male is feeding.

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