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Testing for mating isolation between ecotypes: laboratory experiments with lake, stream and hybrid stickleback

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Abstract

Mating isolation is a frequent contributor to ecological speciation – but how consistently does it evolve as a result of divergent selection? We tested for genetically based mating isolation between lake and stream threespine stickleback (*Gasterosteus aculeatus* L.) from the Misty watershed, Vancouver Island, British Columbia. We combined several design elements that are uncommon in the studies of stickleback mate choice: (i) we used second-generation laboratory-reared fish (to reduce environmental and maternal effects), (ii) we allowed for male–male competitive interactions (instead of the typical no-choice trials) and (iii) we included hybrids along with pure types. Males of different types (Lake, Inlet, hybrid) were paired in aquaria, allowed to build nests and then exposed sequentially to females of all three types. We found that Lake and Inlet males differed in behaviours thought to influence stickleback mate choice (inter- and intra-sexual aggression, display and nest activities), whereas hybrids were either intermediate or apparently 'inferior' in these behaviours. Despite these differences, Lake and Inlet fish did not mate assortatively and hybrid males did not have a mating disadvantage. Our study reinforces the noninevitability of mating isolation evolving in response to ecological differences and highlights the need to further investigate the factors promoting and constraining progress towards ecological speciation.

Introduction

Adaptation to different environments can drive the evolution of reproductive isolation and thereby cause 'ecological speciation' (Dobzhansky, 1940, 1951; Mayr, 1963; Schluter, 2000; Rundle & Nosil, 2005). Particularly, strong reproductive isolation is expected when divergent (or disruptive) selection leads to positive assortative mating (Fry, 2003; Gavrilets *et al.*, 2007; Thibert-Plante & Hendry, 2009). In fact, assortative mating in sexual organisms is thought to be critical for divergence in sympatry and to facilitate divergence in parapatry (Dieckmann & Doebeli, 1999; Thibert-Plante & Hendry, 2009). Assortative mating could arise through several different mechanisms, including habitat choice, temporal differences and mate choice (Coyne & Orr, 2004). We here concentrate on mate choice, where positive assortative mating is often seen between conspecific groups adapted to divergent environments and showing limited gene flow in nature (Funk, 1998; Rundle *et al.*, 2000; Nosil *et al.*, 2002; McKinnon *et al.*, 2004; Boughman *et al.*, 2005; Vines & Schluter, 2006; Langerhans *et al.*, 2007). Assortative mating based on mate choice is likely critical in the many instances where individuals from different environments encounter each during the

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mating period. This mate choice barrier to interbreeding could act at two levels. First, it could reduce the probability of mating between pure-type individuals that encounter each other in nature (Nagel & Schluter, 1998; Huber *et al.*, 2007). Second, it could reduce the possibility that hybrids backcross to the parental species (Hatfield & Schluter, 1996; Vamosi & Schluter, 1999; Naisbit *et al.*, 2001; Turelli *et al.*, 2001; van der Sluijs *et al.*, 2008). Strong mate choice at either level can dramatically reduce gene flow and facilitate progress towards ecological speciation.

But how inevitable are the above outcomes? Despite strong support for ecological speciation in a number of empirical systems (Rundle & Nosil, 2005), a growing number of studies find that divergent selection does not always lead to strong reproductive barriers (Räsänen & Hendry, 2008; Berner *et al.*, 2009; Hendry, 2009; Nosil *et al.*, 2009). For instance, some ecologically differentiated populations show weak or no evidence for positive assortative mate choice, despite strong adaptive divergence (Magurran, 1998; Ellers & Boggs, 2003; Rundle, 2003; Jones *et al.*, 2008; Kwan & Rundle, 2010). This variation suggests that mating isolation is not an inevitable outcome of divergent environments. Alternatively, the expression of mate choice might depend on the environmental context, such that assortative mating that occurs in nature might not be detectable using typical laboratory assays. Informing these topics requires a diversity of mate choice experiments among multiple population types in multiple natural systems. Our intention is to provide another such experiment, which is designed in a way that circumvents several previous concerns.

Threespine stickleback (*Gasterosteus aculeatus* L.) are very useful for studying ecological speciation because they exist as a complex of populations in different ecological environments (McKinnon & Rundle, 2002; Hendry *et al.*, 2009). Gene flow is often reduced among these populations even when they come into close contact (Taylor & McPhail, 2000; Hendry *et al.*, 2002; Jones *et al.*, 2006; Ólafsdóttir *et al.*, 2007), and for the reasons explained earlier, investigators have sought to determine whether assortative mating is part of the reason. Most of the studies do find assortative mating (e.g. McKinnon *et al.*, 2004; Boughman *et al.*, 2005; Ólafsdóttir *et al.*, 2006) but some do not (Jones *et al.*, 2008). Stickleback therefore present a useful system for determining the conditions under which divergent selection does or does not lead to assortative mating. Our study seeks insight into this question by focusing on parapatric lake and stream population pairs. The particular utility of this system is that different population pairs differ dramatically in their levels of adaptive divergence and gene flow (Reusch *et al.*, 2001; Hendry & Taylor, 2004; Berner *et al.*, 2009). This allows us to ask how reliably and under what conditions assortative mating evolves and how it might influence the levels of gene flow. We here begin this investigation by testing for assortative mating in a highly divergent lake–stream pair.

Our study incorporates three design elements that differ from most previous studies of assortative mate choice in stickleback. First, we present females to paired males of different types in a competitive context, as opposed to the common ‘no-choice’ design (where a single male–female pair is present in a given trial). This allows us to consider the potential effects of both female choice and male–male competition, which together could influence mating patterns in nature. Second, we compare all possible combinations of two divergent pure types and their hybrids. This allows us to simultaneously consider the two levels of potential isolation mentioned elsewhere: between pure types and against hybrids. Finally, we use the second-generation laboratory-reared offspring of individuals captured from the wild, rather than wild-caught individuals. This allows us to better isolate genetically based differences from most potential environmental and maternal effects.

Study system

Our work focuses on the Inlet and Lake ecotypes of threespine stickleback in the Misty watershed, northern Vancouver Island, British Columbia, Canada. These ecotypes differ strongly in genetically based morphology (Lavin & McPhail, 1993; Hendry *et al.*, 2002; Moore *et al.*, 2007; Sharpe *et al.*, 2008) and these differences closely parallel those seen in other paired lake–stream systems (Hendry & Taylor, 2004; Berner *et al.*, 2008, 2009). This parallelism, as well as close correlations between traits and habitat features (Berner *et al.*, 2008, 2009), implies that the lake–stream differences reflect adaptive divergence. The standard theory of ecological speciation would therefore predict, all else being equal, the evolution of ecologically based reproductive barriers between these parapatric populations. The Misty Lake and Inlet ecotypes certainly do show very low gene flow in nature (Hendry *et al.*, 2002; Moore *et al.*, 2007), but the most important reproductive barriers are still uncertain.

In the Misty system, intrinsic genetic incompatibilities are not present (Lavin & McPhail, 1993; Hendry *et al.*, 2002), and so the very low gene flow might reflect some combination of partial physical separation (parapatry), phenological isolation, natural selection against migrants and hybrids, and assortative mate choice. Some of these potential reproductive barriers have been investigated, but none of them appears to be particularly strong, consistent and symmetrical (see Discussion and Hendry *et al.*, 2009). We therefore here consider mate choice as a potential reproductive barrier, perhaps manifest as assortative mating among pure types (e.g. Nagel & Schluter, 1998; Boughman *et al.*, 2005; Ólafsdóttir *et al.*, 2006) or sexual selection against hybrids (e.g. Hatfield & Schluter, 1999; Vamosi & Schluter, 1999; Hay & McPhail, 2000).

We investigated these possibilities in four steps. First, we tested for divergence among Lake (L), Inlet (I) and hybrid (H) male 'types' in courtship and competitive behaviours. Divergence in these behaviours might contribute to mating isolation if they influence the ability of male migrants or hybrids to maintain a territory, build a nest or obtain mates. Second, we directly compared the levels of competitive behaviours among paired males of different types (L–I, L–H, I–H), as would occur in nature when ecotypes disperse between environments or when hybrids encounter parental types. If some male types are better competitors than others, this might influence their success in mating. Third, we identified the determinants of overall male mating success (i.e. irrespective of female type) to identify male characteristics (type, size, courtship, competitive behaviours) that might be typically preferred by females. Finally, we evaluated whether female responsiveness and mate choice is assortative when females have the opportunity to choose among male types in competition with each other.

Material and methods

Source material

In June 2004, mature threespine stickleback were collected from Misty Lake site 1 and Misty Inlet site 4 (for a map see Moore & Hendry, 2005). These sites were chosen because they contain Lake and Inlet fish that are highly divergent with respect to morphology and neutral genetic markers (Moore & Hendry, 2005; Moore *et al.*, 2007). We used standard artificial crossing methods (Hatfield & Schluter, 1996) to generate eight pure Lake families and four pure Inlet families (mature females were not common in the Inlet at the time of collection). Each family was the product of a unique male–female pair. The fertilized eggs were shipped to our laboratory at McGill University, Montréal, Canada, where the families were then raised at similar densities (25 individuals/100 L water) in separate tanks as described in more detail by Delcourt *et al.* (2008). At maturity, these fish were used for the studies of morphology (Sharpe *et al.*, 2008) and male courtship (Delcourt *et al.*, 2008). Artificial crossing methods (as earlier) were then used on these first-generation (F1) laboratory-reared fish to produce a new generation (F2) of pure Lake and pure Inlet fish (six families each), as well as Lake–Inlet hybrids (four Inlet male × Lake female families and three Lake male × Inlet female families). Each cross represented a unique male–female pair and siblings were not mated together. These new families were raised using the same protocols and reached maturity in April–June 2007, at which time they were used for our experiment. Because these fish were F2 laboratory-reared fish, any differences between types (Lake = L, Inlet = I, hybrid = H) were likely genetic, rather than environmental or maternal (e.g. plasticity, prior experience or imprinting; Albert, 2005; Kozak & Boughman, 2009). This cohort of fish has also been used to investigate variation in nest characteristics, wherein further rearing details are provided (Raeymaekers *et al.*, 2009).

The experiment

The experiment was conducted under 'summer' conditions (16L : 8D, 18 °C) in fifteen large aquaria (length = 92 cm, width = 32 cm; depth = 39 cm; volume = 102 L). The bottom of either end of each aquarium was covered with a sand layer extending 20 cm towards the centre of the tank, which was covered with dark gravel. Strips of black garbage bag tied around handfuls of gravel were used to create two plastic 'plants' per tank, one at each intersection of sand and gravel. Three types of nesting material were added in excess to each tank: 1600 pieces of 7-cm-long white sisal rope separated into individual threads, 400 pieces of 7-cm-long black sewing thread and 21 cm^3 of dead plant material originally collected from Misty Lake and Misty Inlet. The tanks were filled with water to a depth of 20 cm, and all sides were covered with brown paper so as to minimize visual disturbances.

Two male stickleback of differing types were placed into each tank, creating pairs of Lake and Inlet males (L–I), Lake and hybrid males (L–H) and Inlet and hybrid males (I–H). These males were 'stimulated' to build nests by the twice-daily presentation, in the centre of their tank, of a single gravid female (Vines & Schluter, 2006). These presentations were started on the day males were introduced into their aquaria and continued until the day before a trial. The stimulus female was contained within a clear glass jar with mesh over the top, which allowed males to see and smell the female but prevented physical interactions and movement of the female towards a nest. This manner of presentation of females was chosen to encourage males to build nests without being influenced by female responses to the nest. Stimulus females included all three female types (L, I, H), presented sequentially and in random order across days.

As soon as one of the males had constructed a nest and was guarding it, and the other male had at least started building a nest, we stopped the presentation of stimulus females and started the experimental trials. This implies that variation in nest readiness, which has the potential to influence interbreeding in the wild, could potentially affect the outcome of our experiment. Immediately prior to each trial, we placed above each tank a light filter (rust, code 777; Lee, Burbank, CA, USA) and above that a full-spectrum bulb (Vita-Lite 40 W; Duro-Test, Toronto, Canada). This filter was used because water in the Misty system is highly tannic (Lavin & McPhail, 1993) and therefore dominated by red light (confirmed with spectroradiometer measurements by Nathan Millar, unpublished data). For the trials, we sequentially (on separate days) introduced one gravid female of each type (L, I and

H in random order) into the tank of a given male pair. These females were always from a family different from the males already in the tank.

The experiment was initiated by gently introducing a gravid female into a given tank occupied by a pair of males. A given trial (i.e. a given presentation of a female to a given male pair) then lasted for 45 min or was terminated earlier if the female entered a nest. If this happened, we induced the female to leave the nest before ovipositing by gently squeezing her tail with a pair of long tweezers. The entire length of each trial was videotaped as described in the following sections. After each trial, the readiness of the female to mate was confirmed by gentle pressure on her abdomen – ripe eggs require only gentle pressure to extrude. Any trials with nonripe females were discarded, and the males were tested again with another female of the same type after a lag time of at least 3 h. The aforementioned procedures ensured that males interacted freely with a gravid female of each type, but that none of the males actually had eggs in their nest.

We designated two tank 'types' depending on the status of the males therein: in 'double' tanks, both males actively guarded completed nests, and in 'single' tanks, only one male had done so. Male pairs from double tanks were exposed to at least one female of each type and, depending on availability and family restrictions, to one to three randomly selected additional females. This was carried out to maximize the number of different female types with which a given male was filmed (both males could not be simultaneously filmed on a given trial). Male pairs from single tanks were exposed to only one to three randomly selected females – because greater effort was expended on the double tanks.

After the trials, all fish were measured (with a ruler to the nearest ± 0.1 cm) for standard length (tip of upper jaw to end of hypural plate) and body depth (anterior insertion of first dorsal spine to bottom of the pelvic girdle, perpendicular to the lateral line). Water was then drained from the tank, and the nests were removed and measured as described in Raeymaekers *et al.* (2009). By the end of the experimental period, we obtained 131 videos from double tanks: 33 videos for 9 L–I male pairs, 45 videos for 11 L–H male pairs and 53 videos for 11 I–H male pairs. In addition, we obtained 31 videos from single tanks: 20 videos for nine Inlet males, 10 videos for four inlet males and 1 video for a Lake male. Remarkably, 12 of the 14 males that failed to construct and actively guard a nest in these single tanks were Lake males.

Behavioural analysis

All behavioural analyses were based on digital video (model ZR90, Canon) recordings of male behaviours after the introduction of a gravid female into a tank. The video camera was used to actively follow one of the two males, the 'focal' male, in a given trial. In 'single' tanks, the focal male was always the male who had the active nest. In 'double' tanks, one of the males was selected to be the focal male in a given trial and the other male was then chosen for the next trial. Behaviours were scored from the digital videos by a single person (M. Boisjoly) using an event recorder (The Observer, Noldus Technologies). Analyses were based on the first 15 min after a male first directed behaviour towards a female, except when the female entered a nest within 15 min. In these cases, data recording ended at nest entry. The behaviours of the focal male were scored continuously. The behaviours of the 'opponent' male and the female were scored when they were in the field of view of the video frame that was centred on the focal male. As a result, we were only able to record the behaviours of the opponent male and the female when they were interacting with, or in the neighbourhood of, the focal male.

We scored each occurrence of 13 male courtship behaviours and four male competitive behaviours (see Appendix A for definitions). Subsets of these behaviours were grouped into four main behaviour categories (cfr. Delcourt *et al.*, 2008): aggression towards the female (bite, dorsal pricking and chase), displays (zigzag, circle dance and lead), nest activities (nest fanning, material collecting/placing, nest glueing and nest pecking) and male–male competition (biting the opponent male, stealing material from the opponent's nest, chasing the opponent and menacing the opponent). These categories were developed to summarize the behavioural variation of broad functional relevance. For each male behaviour and for each male behaviour category, we calculated the frequency of occurrence (counts/min) and then used square root transformations to improve normality. Female responses were scored as head up, nest inspection and nest entry (see Appendix A). Head up and nest inspection were quantified as the frequency of occurrence (counts/min) and as the relative total duration (%). Nest entry was a single, dichotomous variable (yes or no).

Statistical analyses

We tested for (i) divergence in male behaviours; (ii) competitive interactions among paired males of different types (L–I, L–H, I–H); (iii) determinants of overall male mating success (i.e. irrespective of female type); and (iv) differential responses of different female types to different male types. No differences were detected in any of these analyses between the reciprocal hybrids (I × L and L × I), and so they are considered together as a single hybrid type. All analyses were performed in STATISTICA (version 8.0, StatSoft, Tulsa, OK, USA) or SAS (version 9.0, SAS Institute, Cary, NC, USA).

Divergence in male behaviours

Using the data for all single and double tanks, we first calculated the average frequency of each of the behaviours for each focal male (i.e. the male on which the video frame was centred). For a given focal male, these averages summarize his behaviour across different females but in the presence of the same opponent male. We then used MANOVA (for all behaviours combined) and ANOVAS (for individual behaviours), followed by Tukey tests where applicable, to consider the general effect of male type on male behaviour. We also tested for an effect of opponent male type on male behaviour (single tanks excluded) by adding this variable to the aforementioned models. Finally, we performed a discriminant analysis (DA; single tanks included) to quantify how well the different male types could be discriminated from each other based on their behaviours (for an earlier example, see Delcourt *et al.*, 2008). We performed all of these analyses twice, once with and once without the hybrids. Models are presented without family effects. These effects were weak when included as a random factor, and did not affect our conclusions.

Competitive interactions

We compared the paired opponent males with respect to their reciprocal rates of biting, chasing or menacing, the rate at which they stole material from each other's nest, and their overall engagement in competitive behaviours. This was carried out in each of the male pair categories separately (L–I, L–H, I–H; single tanks excluded), applying a significance level that controls for a false discovery rate (FDR; Verhoeven *et al.*, 2005) across the three pair types. The analysis involved repeated measures ANOVAS, allowing for direct comparison between the competing males within each video trial. Male type (not the type of the 'focal' male but the type of the male performing the competitive behaviour) was included as the repeated measure accounting for the within-video trial dependence among the competing males. Female type and the male type by female type interaction were included as fixed factors. A significant male type effect would indicate that levels of competition are biased towards one of the male types (irrespective of female type). A significant female type effect would indicate that the level of competition depends on the specific female type (irrespective of male type). A significant male type by female type interaction would indicate that the level of competition depends on the combination of male and female types. A binary variable indicating whether the observed male was the focal male was included as a fixed block factor, correcting for potential observational bias (behaviours of the focal male may have had a higher chance to be scored). Male pair effects were included as a random factor. Subsequently, we added male body size to the models to test whether males of different size show different competitive behaviours. Note that body size differs among the three types in the field (I < L; Moore & Hendry, 2005) and in the laboratory (I < H < L; J.A.M. Raeymaekers and A.P. Hendry, unpublished data).

Determinants of overall male mating success

We first constructed a binary response variable indicating whether a female entered the nest of a given focal male during at least one of the experimental trials. Logistic regression models were then constructed to determine how focal male mating success depended on his type, his average behaviours (as listed earlier) and his body size (all tanks included). Additional models were constructed to evaluate whether type, average behaviours and body size of the opponent male influenced focal male mating success (single tanks excluded).

Female responses and deviations from random mating

Our three measures of female response to males were (i) head up, (ii) nest inspection and (iii) nest entry. Head up can be considered to indicate female responsiveness, nest inspection to indicate a transition between female responsiveness and female preference and nest entry to indicate female preference. Each of these variables has been used in previous work on stickleback (e.g. Hatfield & Schluter, 1996; Rundle & Schluter, 1998; Kozak *et al.*, 2009). Here, it is important to remember that these female responses might be influenced by male–male interactions. We first evaluated, for each male pair category separately (L–I, L–H, I–H; correction for multiple testing applied as elsewhere), whether head up and nest inspection were biased towards a male of a specific type. To do so, we directly compared these responses of the female towards each of the two males during a single video trial (single tanks excluded). As earlier, the within-video trial dependence among both competing males was taken into account with a repeated measures ANOVA. Male type (not the type of the focal male but the type of the male to which the female responded) was included as the repeated measure within-video trials, whereas female type and the male type by female type interaction were included as fixed factors. A significant male type effect would indicate that head up and nest inspection were biased towards one of the male types (irrespective of female type). A significant female type effect would indicate that head up and nest inspection depend on female type (irrespective of male type). A significant male type by female type effect would indicate that head up and nest inspection depend on the combination of male and female types. This last effect is the most relevant for inferring whether both responses are assortative by type. A binary variable indicating whether the behaviour of the female was directed towards the focal male was again included as a fixed block factor, correcting for potential observational bias (behaviours of the female towards the focal male may have had a higher chance to be scored). Male pair effects were included as a random factor. Subsequently, we added male body size and the four behavioural categories to the model (i.e. total display, nest activities, aggression towards the female and aggression towards the other male). Significant covariate effects would suggest that head up and nest inspection depend on these specific male traits.

Finally, we studied nest entry patterns as an indicator of likely spawning to assess deviations from random mating. First, nest entries that would have generated Lake or Inlet pure-type crosses, F1 and F2 hybrid crosses, and Lake or Inlet backcrosses were counted. A chi-square test was then used to compare the observed number of crosses with the number of crosses expected if mate choice would be random (i.e. number of trials leading to a specific cross type multiplied by overall nest entry success). This test does not account for the complexity of the design (different male pair categories having different possible outcomes were lumped) and has low statistical power (only 14 of 146 trials in double tanks ended in a nest entry). As will be seen, however, the results are clear.

Results

Divergence in male behaviours

MANOVA revealed significant behaviour differences among the three male types (Wilks' lambda = 0.38, $F_{34,112} = 2.05$, $P = 0.0027$). Discriminant analysis (DA) showed that Lake, Inlet and hybrid males were reasonably well classified based on their behaviours (per cent misclassified: L = 29%; I = 25%; H = 31%; Fig. S1). Loadings of behaviours on the discriminant functions pointed to important contributions of stealing, menacing, zigzagging and biting the female (Fig. 1). Hybrid male behaviours generally showed multivariate intermediacy between pure-type males (Fig. 1) but were sometimes more similar to Inlet males than to Lake males (none of the hybrids were misclassified to the Lake type). When hybrids were excluded from the DA, classification success improved, and the misclassifications included more Inlet-like Lake males (19%) than Lake-like Inlet males (11%). The discriminant functions from this last analysis again classified most of the hybrid males as Inlet-like (65%).

Univariate ANOVAS (Table 1) revealed that the differences between the three types were driven mainly by stealing (L > H = I; Tukey tests: L vs. I, $P = 0.0017$; L vs. H, $P = 0.0023$; Fig. 2d). Additional, albeit marginally nonsignificant, differences seem likely for menacing the opponent (L > H > I; $P = 0.0544$; Fig. 2b) and biting the female (L > H > I; $P = 0.0793$; Fig. 2a). The same behaviours were important when hybrid males were excluded, and an additional effect was seen for the rate of zigzag displays (L < I; Fig. 2c; Table 1). Opponent type did not explain a significant amount of the variation in male courtship behaviours when added to the aforementioned models. In short, Lake, Inlet and hybrid males showed genetically based differences in some elements of courtship and competitive behaviours.

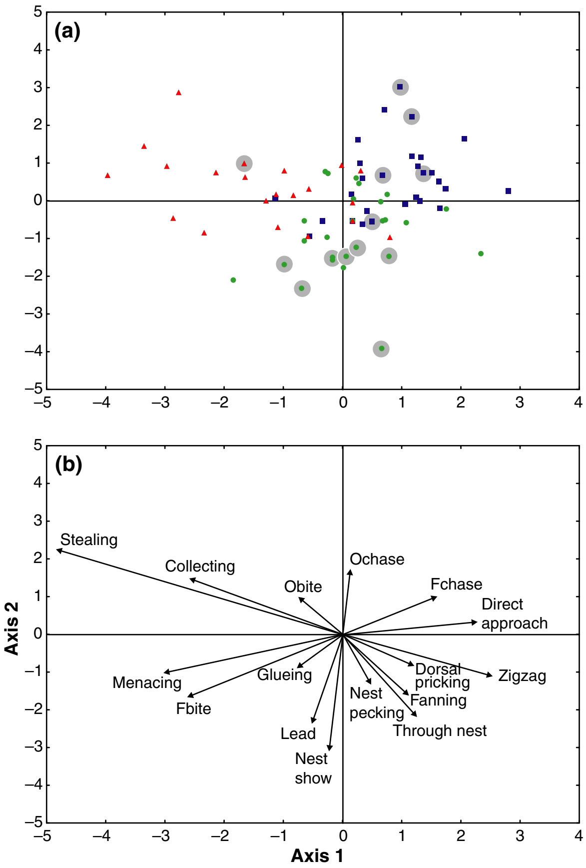

Fig. 1 Discriminant analysis plot showing threespine stickleback males as differentiated by their behaviours. (a) Behavioural divergence between Lake males (triangles), hybrid males (dots) and Inlet males (squares). Shaded symbols represent males that were preferred by females as indicated by at least one female nest entry. (b) Loadings of the behaviours with respect to the discriminant functions are represented as vectors. Circle dance did not point in either dimension and was left unlabelled. Labels for the behaviours are clarified in Appendix A.

Competitive interactions

Total aggressive behaviours were more frequent for Inlet males than for Lake males when they were paired as opponents ($F_{1,50} = 7.03$; $P = 0.0107$; Fig. 3e; Table 2). This difference could be attributed to a strong effect of biting ($F_{1,50} = 8.47$; $P = 0.0054$; Fig. 3a; Table S1) and a smaller effect of chasing ($F_{1,50} = 4.74$; $P = 0.0342$; Fig. 3d; Table S1). In contrast, total aggressive behaviours were more frequent for Lake males than for hybrid males when they were paired as opponents ($F_{1,72} = 6.25$; $P = 0.0147$; Fig. 3e; Table 2). This difference was mainly because of effects of biting ($F_{1,72} = 4.5$; $P = 0.0374$; Fig. 3a; Table S1) and stealing ($F_{1,72} = 5.27$; $P = 0.0246$; Fig. 3b; Table S1). As expected from the aforementioned, total aggressive behaviours were more frequent for Inlet

Table 1 (A) Mean courtship and competitive behaviours by male type (Lake, hybrid, Inlet; standard errors in parentheses). (B) and (C) show analyses of variance results (B) with and (C) without hybrid males included in the analyses.

	(A) Male type			(B) With hybrids		(C) Without hybrids	
Behaviour	Lake (N = 21)	Hybrid (N = 26)	Inlet (N = 28)	$F_{2,72}$	P	$F_{1,47}$	P
All display behaviours	0.21 (0.09)	0.34 (0.08)	0.32 (0.06)	0.77	0.4689	1.02	0.3187
Zigzag	0.10 (0.05)	0.24 (0.05)	0.25 (0.05)	2.24	0.1143	3.73	0.0594
Lead	0.10 (0.06)	0.14 (0.05)	0.06 (0.03)	0.95	0.3912	0.46	0.5019
Circle dance	0.12 (0.06)	0.12 (0.04)	0.13 (0.04)	0.01	0.9944	0.01	0.9439
Direct approach	0.31 (0.05)	0.40 (0.04)	0.44 (0.05)	1.67	0.1962	3.03	0.0883
All nest activities	0.55 (0.08)	0.63 (0.09)	0.59 (0.09)	0.21	0.8112	0.07	0.7916
Nest pecking	0.25 (0.06)	0.31 (0.07)	0.27 (0.05)	0.24	0.7868	0.09	0.7668
Fanning	0.32 (0.07)	0.46 (0.06)	0.40 (0.08)	0.78	0.4642	0.46	0.4999
Collecting	0.20 (0.06)	0.10 (0.03)	0.10 (0.03)	2.20	0.1187	2.85	0.0977
Glueing	0.07 (0.03)	0.07 (0.03)	0.04 (0.02)	0.32	0.7280	0.45	0.5073
Showing the nest	0.12 (0.06)	0.21 (0.06)	0.08 (0.04)	1.57	0.2145	0.27	0.6041
Creeping through	0.03 (0.02)	0.08 (0.03)	0.07 (0.02)	1.18	0.3125	1.15	0.2882
Total aggression towards female	0.73 (0.12)	0.64 (0.08)	0.46 (0.07)	2.33	0.1048	4.18	**0.0466**
Dorsal pricking	0.00 (0.00)	0.01 (0.01)	0.01 (0.01)	0.54	0.5866	0.75	0.3921
Bite	0.72 (0.12)	0.63 (0.08)	0.45 (0.07)	2.63	0.0793	4.74	**0.0345**
Chase	0.02 (0.01)	0.04 (0.02)	0.06 (0.03)	0.96	0.3876	1.76	0.1908
Total aggression towards opponent	0.58 (0.08)	0.41 (0.07)	0.4249 (0.0863)	1.27	0.2882	1.54	0.2213
Menacing	0.20 (0.03)	0.14 (0.04)	0.0772 (0.0291)	3.03	0.0544	7.10	**0.0105**
Stealing	0.18 (0.05)	0.04 (0.02)	0.0417 (0.0165)	8.14	**0.0007**	10.29	**0.0024**
Bite	0.37 (0.08)	0.28 (0.06)	0.3157 (0.0833)	0.32	0.7278	0.19	0.6685
Chase	0.15 (0.04)	0.12 (0.04)	0.1641 (0.0332)	0.49	0.6155	0.06	0.8033

Variables were square-rooted to improve normality. Significant *P*-values are in bold.

males than for hybrid males when they were paired as opponents ($F_{1,88}$ = 4.8; P = 0.0311; Fig. 3e; Table 2). In particular, bite rates were markedly higher for Inlet males than for their hybrid opponents ($F_{1,88}$ = 14.05; P = 0.0003; Fig. 3a; Table S1). Female type influenced the level of male–male competition in only one instance: Inlet and Lake males chased each other most frequently in the presence of Lake females ($F_{2,50}$ = 5.51; P = 0.0069; Table S1). Male body size was never associated with the level of competition (results not shown). In short, engagement in competitive interactions with opponents was most frequent for Inlet males, intermediate for Lake males and least frequent for hybrid males.

Determinants of male mating success

All of the successful males (i.e. those attaining at least one female nest entry) are highlighted in Fig. 1. Logistic regression models revealed the following effects (effects not mentioned here were not significant): the mating success of focal males increased with increasing rates of display (total: χ^2 = 6.47, d.f. = 1, P = 0.0109; zigzag: χ^2 = 4.56, d.f. = 1, P = 0.0328), nest activities (total: χ^2 = 5.4, d.f. = 1, P = 0.0201; nest pecking: χ^2 = 5.52, d.f. = 1, P = 0.0188), aggression towards females (total: χ^2 = 8.38, d.f. = 1, P = 0.0038; bite: χ^2 = 8.17, d.f. = 1, P = 0.0043) and showing the nest (χ^2 = 5.24, d.f. = 1, P = 0.022). Focal male type was also a statistical predictor of mating success, and this was highest for hybrid males – although only significantly so when single tanks were included (χ^2 = 6.42, d.f. = 2, P = 0.0404). Furthermore, focal male mating success was not influenced by opponent type but decreased with increasing rates of aggression of the opponent male towards females (total: χ^2 = 4.95, d.f. = 1, P = 0.0261; bite: χ^2 = 4.59, d.f. = 1, P = 0.0322) and direct approaches of the opponent towards females (χ^2 = 8.5, d.f. = 1, P = 0.0035). Remarkably, none of the aggressive behaviours between males, nor their body sizes, significantly influenced mating success. In short, interactions of the two males with the female, but not with each other, influenced focal male mating success, and hybrid males did not show lower mating success than pure-type males.

Female responses and deviations from random mating

Rates and total durations of 'head up' and nest inspection did not depend on the interaction between male type and female type in any of the male pair categories (L–I, L–H, I–H; Table S2). This indicates that specific female types did not show differential responsiveness towards specific male types, implying that females did not respond assortatively (Fig. S2). Head up and nest inspection were usually positively correlated with male display, aggression towards the female and nest building and negatively correlated with aggression towards the opponent male (Table S2). Male body size did not influence these

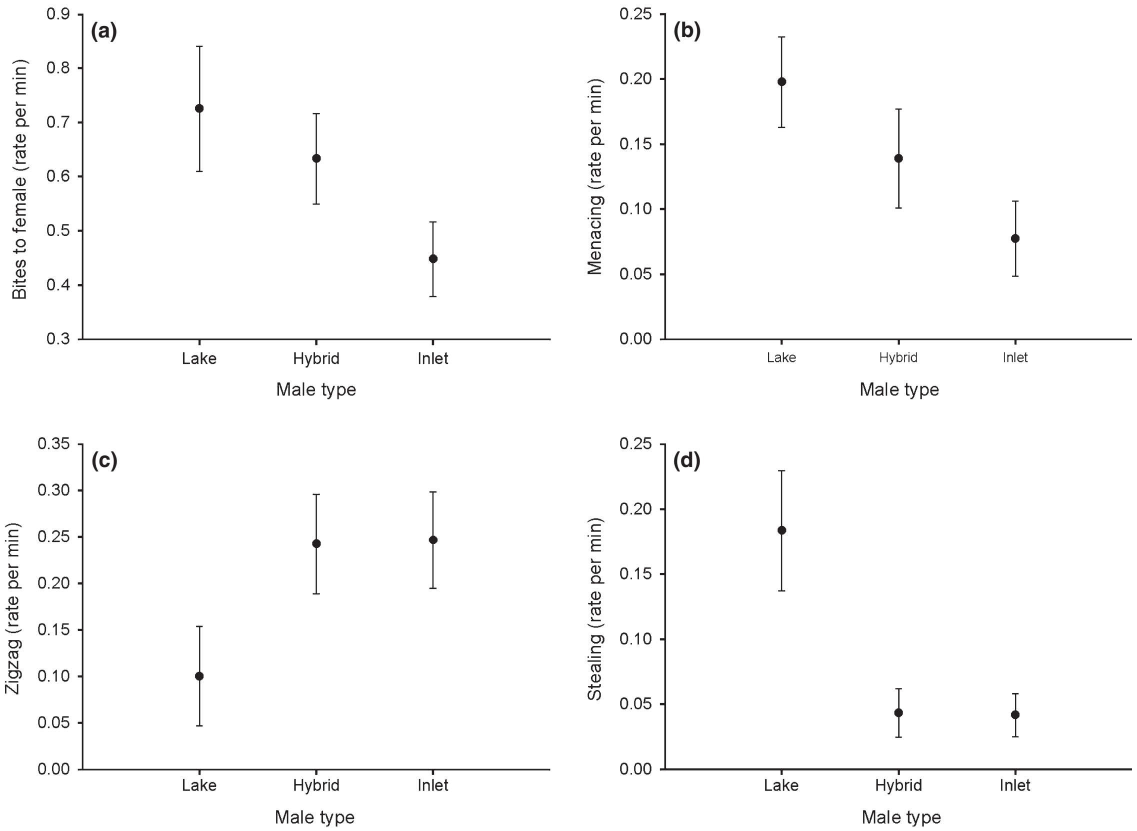

Fig. 2 Male courtship and competitive behaviours by male type (Lake, hybrid, Inlet). (a) Bites to the female per minute; (b) menacing per minute; (c) number of zigzags per minute; and (d) stealing attempts per minute. Vertical bars denote standard errors. Variables were square-root transformed so as to improve normality.

responses after controlling the false discovery rate (Table S2). Taking nest entry as an indicator of spawning, none of the apparent deviations from randomness (Fig. 4) were statistically significant ($\chi^2 = 5.49$, d.f. = 5, $P = 0.36$), and the trends were not even in the *direction* of assortative mating between pure types. For instance, and ignoring statistical significance, hybrid males obtained the highest mating success (nine of 106 trials; 8.5%), followed by Inlet males (four of 96 trials; 4.2%) and Lake males (one of 90 trials; 1.1%). In short, female responses (head up and nest inspection) were not assortative when females had the opportunity to choose among competing male types, and nest entry patterns did not deviate from randomness.

Discussion

We draw three main inferences. First, the Lake and Inlet pure male types from the Misty system differ genetically in a number of traits thought to influence stickleback mate choice. These differences could reduce the mating success of migrants between the environments. Second, hybrids were either intermediate or ‘inferior’ in these behavioural traits, suggesting they could be disadvantaged in both the Lake and the Inlet. Third, despite the aforementioned differences, pure types in the laboratory did not show assortative mating and hybrid males did not show a mating disadvantage. In the following four paragraphs, we outline the results of the four analysis steps (see Introduction) and explain how they led us to the aforementioned inferences. We then close with a consideration of the implications for ecological speciation and its detection.

Differences in male courtship behaviour are common among stickleback populations (e.g. McPhail & Hay, 1983; Ridgway & McPhail, 1984; McKinnon *et al.*, 2004; Boughman *et al.*, 2005; Ólafsdóttir *et al.*, 2006; Delcourt *et al.*, 2008). Using no-choice mating trials for Misty stickleback, Delcourt *et al.* (2008) showed that F1 laboratory-reared Lake males court more aggressively, whereas Inlet males have a more display-oriented courtship. Our present results confirm these findings with F2 laboratory-reared males in competition with each other. Additionally, we found that Lake males engage in more aggression towards opponent males (stealing nesting material and menacing). The evolutionary reasons for

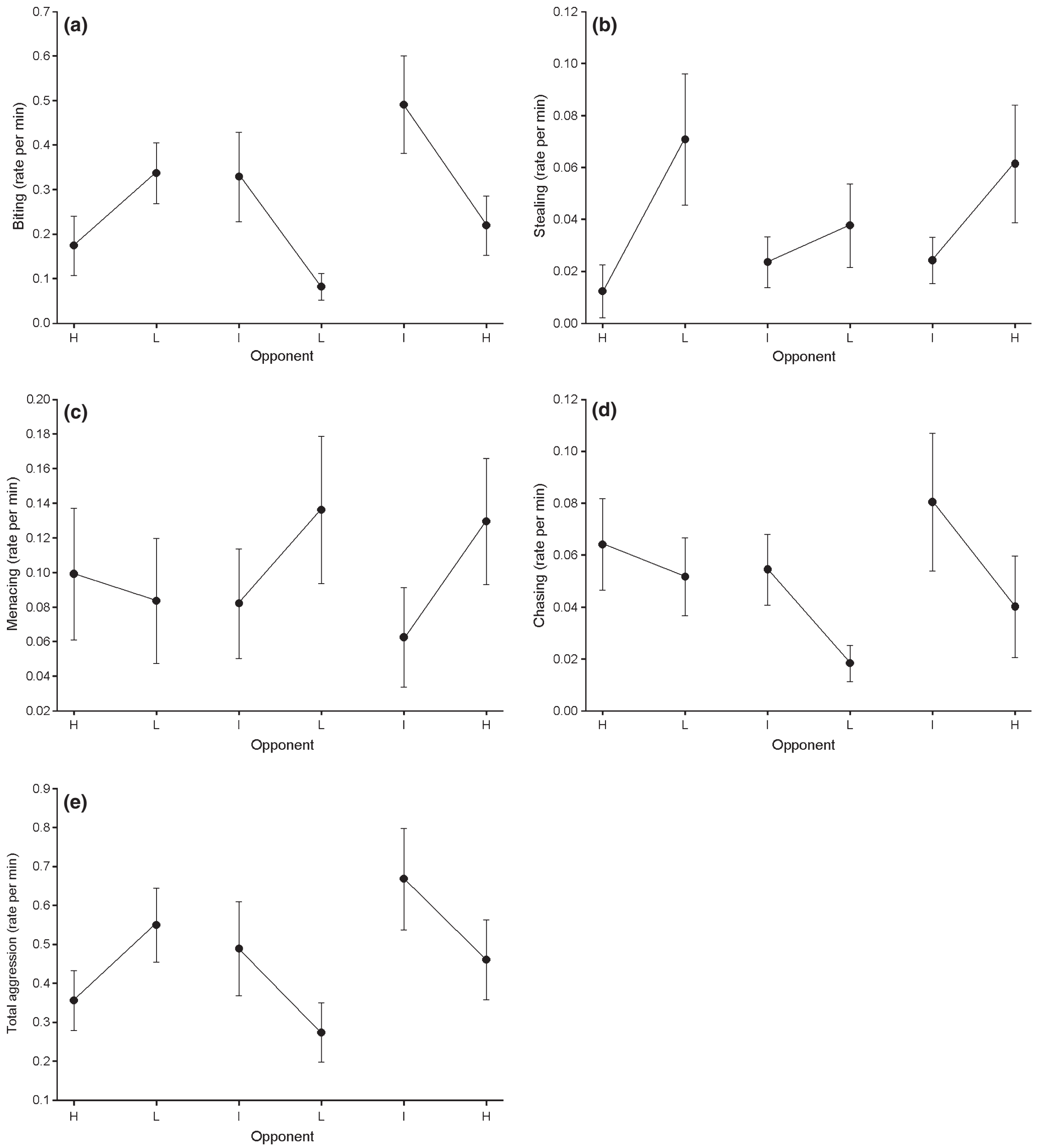

Fig. 3 Competitive interactions between threespine stickleback males of different types. The dots connected by lines indicate reciprocal mean levels of competition between H–L, I–L, and I–H male pairs. (a) Bites per minute; (b) stealing attempts per minute; (c) menacing per minute; (d) chases per minute; (e) total number of aggressive behaviours per minute. Vertical bars denote standard errors.

these genetic differences are currently unknown. Another new finding is that hybrid male behaviour was generally intermediate between that of the two pure types, although it was sometimes more Inlet-like than Lake-like. Perhaps hybrid males are thus maladapted for either parental environment (especially the Lake) and might suffer reduced mating success in those environments (i.e. an ecologically dependent disadvantage). In addition, the intermediate hybrid males could be disfavoured by pure females of each type irrespective of the actual mating environment (ecologically independent disadvantage) – an hypothesis to which we later return.

Although male dominance is known to facilitate mate acquisition in stickleback (Kraak *et al.*, 1999; Östlund-Nilsson & Nilsson, 2000; Candolin & Voigt, 2001; Candolin & Salesto, 2009), the contribution of male competition to reproductive isolation has not been explicitly tested (see Seehausen & Schluter (2004) for

Table 2 Fixed effects of a repeated measures ANOVA on the total rate of aggression among paired males of different types.

Male pair category	Effect	Num DF	Den DF	*F*	*P*
H–L	Focal	1	72	0.69	0.4073
	Female	2	72	0.75	0.4758
	Male	1	72	6.25	***0.0147***
	Focal*male	1	72	3.96	0.0504
	Female*male	2	72	0.24	0.7902
I–L	Focal	1	50	1.86	0.1788
	Female	2	50	0.3	0.7456
	Male	1	50	7.03	***0.0107***
	Focal*male	1	50	0	0.9755
	Female*male	2	50	2.66	0.0797
I–H	Focal	1	88	0.16	0.6915
	Female	2	88	2.45	0.0917
	Male	1	88	4.8	***0.0311***
	Focal*male	1	88	0.54	0.4632
	Female*male	2	88	0.28	0.7562

Fixed effects included the type of the female ('female'), the type of the male showing the behaviours ('male'), a binary variable indicating whether the male was the focal male ('focal'), and interactions. The within-video trial dependence among both competing males was taken into account as a repeated measure. Male pair effects were included as random (not shown). *P*-values in bold indicate significance at $\alpha = 0.05$ and italics *P*-values are significant after controlling the false discovery rate across the three implicit comparisons (H–L; I–L; I–H).

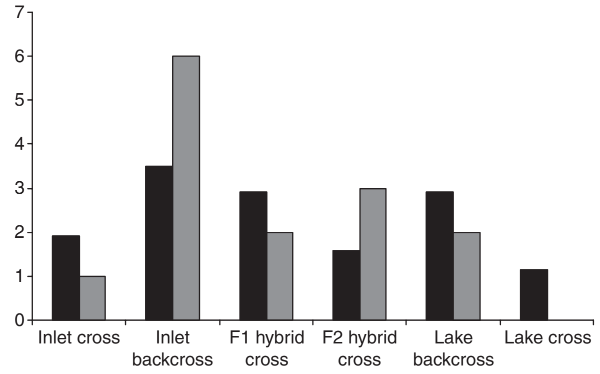

Fig. 4 Numbers of crosses resulting in female nest entry as an indicator of spawning (grey bars), and the number of crosses expected if mating was random with respect to type (black bars).

an example in cichlids). In the present study, even though Inlet males were smaller and less aggressive overall than were Lake males, they were dominant over Lake males in some respects (biting and chasing) and they constructed their nests much more quickly (J.A.M. Raeymaekers, personal observation). Moreover, Lake males were the least likely to construct nests when in competition with other male types. Perhaps Lake fish moving into the inlet therefore might be at a competitive disadvantage – particularly if Inlet male behaviours are adapted for their local environment. Hybrid males, by contrast, appeared generally inferior to both pure types, engaging in less biting than Lake and Inlet males and less stealing than Lake males. Perhaps hybrid males would have difficulties in establishing territories and building nests in both environments in nature. However, as we will explain in the following paragraphs, it did not seem to limit their mating success in the laboratory.

Work on other stickleback systems has shown that mate choice can depend on a variety of factors, including body size, male colour, male behaviour, olfactory cues, immune genes and nest characteristics (e.g. Barber *et al.*, 2001; Boughman, 2001; McKinnon *et al.*, 2004; Ólafsdóttir *et al.*, 2006; Eizaguirre *et al.*, 2009). Here, we found that male mating success generally increased with rates of display and aggression towards the female. Hybrid males, however, did not have a mating disadvantage (even with pure-type females), despite their low engagement in competitive interactions. Perhaps this was because hybrid males were larger and more aggressive courters than Inlet males and faster nest builders and more display-oriented courters than Lake males.

Assortative mating between stickleback ecotypes has been found in a number of previous studies (e.g. McKinnon *et al.*, 2004; Boughman *et al.*, 2005; Ólafsdóttir *et al.*, 2006), although not all of them (Jones *et al.*, 2008). Our study adds to the negative examples in that Lake and Inlet stickleback did not show any evidence of assortative mating: responses (head up, nest inspection and nest entry) of pure Lake and Inlet females were not greater towards their own ecotype.

Ecological speciation and its detection

It is now increasingly clear that divergent ecological environments do not inevitably drive the evolution of particular reproductive barriers (Räsänen & Hendry, 2008; Berner *et al.*, 2009; Hendry, 2009; Nosil *et al.*, 2009), including mate choice (Magurran, 1998; Ellers & Boggs, 2003; Rundle, 2003; Jones *et al.*, 2008; Kwan & Rundle, 2010). It is therefore important to explicitly study the factors that promote or constrain progress towards ecological speciation in any given situation (Hendry, 2009). The great diversity of independent instances of threespine stickleback adapting to different environments provides an outstanding opportunity for such analyses (McKinnon & Rundle, 2002; Hendry *et al.*, 2009). We therefore now contrast our results with those of other studies of threespine stickleback, allowing us to consider factors that might influence progress towards ecological speciation in a diverse radiation, as well as the experimental detection of those factors. Although our discussion focuses on comparisons within the stickleback radiation, the points are general and should apply to many systems.

Work on Misty stickleback has provided mixed evidence for progress towards ecological speciation. On the one hand, all studies suggest strong adaptive divergence and very low gene flow between the Lake and Inlet stickleback (Lavin & McPhail, 1993; Thompson *et al.*, 1997; Hendry *et al.*, 2002; Hendry & Taylor, 2004; Moore *et al.*, 2007; Delcourt *et al.*, 2008; Sharpe *et al.*, 2008; Raeymaekers *et al.*, 2009; Hendry *et al.*, in press; present study). On the other hand, the usual tests for typical reproductive barriers in stickleback have not revealed strong barriers in the Misty system: selection against migrants is weak (Hendry *et al.*, 2002; K. Räsänen and A.P. Hendry, unpublished data), breeding times overlap (J.S. Moore and A.P. Hendry, unpublished data), assortative mating is absent in the laboratory (present study), and hybrids show no mating disadvantage in the laboratory (present study). By way of contrast, many of these barriers are quite strong in the benthic/limnetic stickleback pairs (Ridgway & McPhail, 1984; McPhail, 1994; Nagel & Schluter, 1998; Vamosi & Schluter, 1999; Rundle *et al.*, 2000; Boughman, 2001; Boughman *et al.*, 2005; Vines & Schluter, 2006; Kozak *et al.*, 2009) and also in at least some of the anadromous/freshwater pairs (Hay & McPhail, 1975; McKinnon *et al.*, 2004). We can see several possible explanations for why reproductive barriers appear weaker in the Misty system than in other stickleback systems.

First, strong reproductive barriers might be present between Lake and Inlet stickleback, but we have thus far failed to find the smoking gun. For instance, different parasite communities in lakes vs. streams could cause strong selection against migrants and assortative mate choice based on MHC genotypes (Eizaguirre *et al.*, 2009). Or perhaps adaptive preferences for local habitats could limit movement between lakes and streams (Hendry *et al.*, 2002; Bolnick *et al.*, 2009). Such habitat preferences are certainly a major contributor to ecologically driven reproductive barriers in other taxa (e.g. Via, 1999; Tonnis *et al.*, 2005; Nosil *et al.*, 2006). Future work will therefore consider these and other potential barriers.

Second, perhaps a smoking gun does not exist and reproductive isolation is instead achieved by the combination of many small reproductive barriers. In this case, any given barrier might seem weak, perhaps even statistically unconfirmable, but overall ecologically driven reproductive isolation might still be strong. A situation like this appears to be the case in a pair of phytophagous ladybird beetles (Matsubayashi & Katakura, 2009). Also, a barrier that is asymmetric in one direction could be complemented by a different barrier that is asymmetric in the other direction, as has been shown for Japan Sea vs. Pacific Ocean threespine stickleback (Kitano *et al.*, 2009). Candidates for such small barriers are numerous and include all of those mentioned earlier. Again, this point reinforces the need to consider multiple potential reproductive barriers when examining progress towards ecological speciation (e.g. Schluter, 2000; Nosil, 2007).

Third, perhaps our experimental conditions were not appropriate for revealing strong reproductive barriers. With respect to mate choice, perhaps we have not provided the right conditions for assortative mating and hybrid disadvantages to be manifest. As one possibility, traits or preferences might diverge in nature owing to prior experience and plasticity rather than genetic differences. For example, mate choice in stickleback can depend on the social environment in which individuals are raised (Kozak & Boughman, 2009). The environment for the expression of mate choice is also likely to matter. For example, Hatfield & Schluter (1996) did not find a benthic/limnetic hybrid mating disadvantage in laboratory mating trials, whereas Vamosi & Schluter (1999) did so in experimental enclosures in nature. In our case, Inlet females might prefer Inlet males only in Inlet environments (and the opposite for Lake females) – if, for example, males cannot appropriately court females or adequately build nests in alternative environments, or because females do not perceive or care about male differences outside of a natural environmental context. We suggest that more studies of ecological speciation consider plastic contributions to mate choice and variation in the environmental context for the expression of mate choice.

Finally, adaptive divergence actually might not have driven the evolution of reproductive barriers between Lake and Inlet stickleback in the Misty system, with the low gene flow instead simply reflecting partial spatial separation. If so, what factors have constrained the progress towards ecological speciation that would be expected from the strong adaptive divergence? Insufficient time for divergence is one possibility, but this seems unlikely as divergence times (about 10 000 years) are likely similar to other stickleback pairs (Taylor & McPhail, 2000; Reusch *et al.*, 2001; Raeymaekers *et al.*, 2005). Another possibility is the lack of interactions between different ecotypes that could lead to character displacement and the reinforcement of mating preferences (Hendry *et al.*, 2009). In both the benthic/limnetic and freshwater/anadromous stickleback pairs, the different ecotypes are in frequent contact and so selection to avoid maladaptive hybridization, or direct selection to avoid the other type, might strengthen assortative mating (Rundle *et al.*, 2000; Albert & Schluter, 2004). These opportunities will occur less frequently and over a much smaller spatial range for Misty Lake vs. Inlet stickleback. Moreover, our specific collection sites were far enough apart that stickleback dispersal between them is probably very low. However, it is also true that assortative mating can be evident between different ecotypes (benthic and limnetic) even if they come from solitary allopatric populations (Vines & Schluter, 2006), and this is true of other taxa as well (e.g. Langerhans *et al.*, 2007). Studies of ecological speciation would thus benefit from examining mating isolation in multiple contexts and among multiple populations. Our next step will be to determine whether assortative mating is present in some of the many other lake–stream pairs found in the same geographical region.

Conclusion

Our study adds to the growing list of examples (Magurran, 1998; Ellers & Boggs, 2003; Rundle, 2003; Jones *et al.*, 2008; Kwan & Rundle, 2010) where divergent selection seemingly does not inevitably lead to strong mating isolation. This highlights the need to study more systems in which ecological speciation is not evident despite divergent environments (Hendry, 2009; Nosil *et al.*, 2009). Only by doing so can we better understand the factors that promote and constrain progress towards ecological speciation.

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Appendix A

Male courtship and competitive behaviours were scored for both the focal male and the opponent male. Courtship behaviours included the same behaviours as in Delcourt *et al.* (2008). Description of these behaviours follows McPhail & Hay (1983), Rowland (1989, 1994), van Iersel (1953) and Kraak & Bakker (1998). Male competitive behaviours included direct and aggressive behaviours towards the other male. Description of female behaviours follows Hatfield & Schluter (1996), Rundle & Schluter (1998) and Kozak *et al.* (2009), with one modification for nest inspection. Behavioural categories are indicated by D (display), N (nest activities), AF (aggression towards the female) and AO (aggression towards the opponent male). Behaviours included in behavioural categories are underlined.

Courtship behaviours

Bite (AF; Fbite in Fig. 1): male bites the female.

Dorsal pricking (AF): male erects his dorsal spine and pricks the female's belly in a backward movement.

Chase (AF; Fchase in Fig. 1): male swims rapidly towards the female and ends the bout with a physical contact.

Zigzag (D): male swims towards 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 towards the female in 1 bout.

Material collecting and placing (N; Collecting in Fig. 1): 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; Nest show in Fig. 1): male swims to his nest and pokes the entrance with his snout.

Creeping through (N; through nest in Fig. 1): male enters his nest through the entrance and swims out on other end.

Competitive behaviours

Bite (AO; Obite in Fig. 1): male bites the other male.

Chase (AO; Ochase in Fig. 1): male swims aggressively towards the other male, but does not end by physical contact.

Menacing (AO): interference by repeated slow movements towards the other male, but without physical contact.

Stealing (AO): stealing material from the other male's nest.

Female behaviours

Head up: female has her head upwards in an approximately 45° angle.
Nest inspection: female places her snout in the nest opening, or directs its orientation towards the nest while swimming very close (< 5 cm).
Nest entry: female enters the nest within the first 45 min of the trial.

Supporting information

Additional Supporting Information may be found in the online version of this article:
Figure S1 Variation in 17 male courtship and competitive behaviours among Lake, hybrid and Inlet types as revealed by discriminant function analysis.
Figure S2 Female responsiveness towards paired males of different types (H–L; I–L; I–H) scored as (a) the rate of 'head up' and (b) the rate of nest inspection.
Table S1 Fixed effects of a repeated measures ANOVA on competitive behaviours among paired males of different types.
Table S2 Fixed effects of a repeated measures ANOVA on female responsiveness towards paired males of different types.

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