Strong assortative mating between allopatric sticklebacks as a by-product of adaptation to different environments

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Speciation involves the evolution of reproductive isolation between populations. One potentially important mechanism is the evolution of pre- or postzygotic isolation between populations as a by-product of adaptation to different environments. In this paper, we tested for assortative mating between allopatric stickleback populations adapted to different ecological niches. Our experimental design controlled for interpopulation interactions and non-adaptive explanations for assortative mating. We found that prezygotic isolation was surprisingly strong: when given a choice, the majority of matings occurred between individuals from similar environments. Our results indicate that the by-product mechanism is a potent source of reproductive isolation, and likely contributed to the origin of sympatric species of sticklebacks.

Keywords: ecological speciation; *Gasterosteus aculeatus*; by-product mechanism; adaptation; prezygotic isolation

1. INTRODUCTION

Reproductive isolation between populations may evolve as an incidental consequence of adaptation to different environments (Mayr 1942; Dobzhansky 1951). The idea behind this 'by-product mechanism' is that the evolution of reproductive isolation is not directly favoured, but arises as a side effect of divergent selection on other traits (Funk 1998; Schluter 2001; Coyne & Orr 2004). For example, prezygotic isolation may evolve because mate choice happens to be based on traits that are the target of divergent natural selection (Ratcliffe & Grant 1983; Podos 2001; Servedio 2004), or because divergent selection may favour shifts in mate choice criteria onto traits that are most conspicuous in each environment (Endler 1992; Schluter & Price 1993; Boughman 2001; Leal & Fleishman 2004). Alternatively, the different alleles favoured in different environments may reduce survival or fecundity when brought together in a hybrid individual (Dobzhansky 1951).

The by-product mechanism is certainly plausible, but evidence from evolution experiments in the laboratory give mixed results about how often it occurs. For example, Kilias *et al.* (1980) raised *Drosophila melanogaster* in either cold-dry-dark or warm-wet-light conditions, and Dodd (1989) kept lines of *D. pseudobscura* on starch- or maltose-based media. Both studies found that some prezygotic isolation developed between flies from different environments, whereas almost no isolation evolved between different lines living in the same conditions. In contrast, Mooers *et al.* (1999) and Rundle (2003) found no effect of divergent selection on assortative mating between replicate *Drosophila* lines.

There are also two types of examples of the by-product mechanism from natural populations. First, several studies have demonstrated that traits under divergent

natural selection are also involved in reproductive isolation. For example, beak shape in Galapagos finches determines diet (Schluter & Grant 1984), but it is also involved in visual (Ratcliffe & Grant 1983) and auditory (Podos 2001) mate recognition. Similarly, adaptation by *Agrostis tenuis* and *Anthoxanthum odoratum* to lead and zinc contaminated mine tailings has also changed their flowering times, reducing gene flow between tolerant plants and nearby populations on normal soils (McNeilly & Antonovics 1968). Second, a few researchers have shown that stronger assortative mating exists between allopatric populations from different environments compared to allopatric populations in similar environments (Funk 1998; Jiggins *et al.* 2001; Nosil *et al.* 2002; McKinnon *et al.* 2004). These studies used allopatric populations because assortative mating can arise between sympatric populations in response to the production of unfit hybrids (i.e. reinforcement: Dobzhansky 1940; Servedio & Noor 2003). However, the by-product mechanism is still implicated when differentially adapted populations produce fully functional hybrids, as there is then no incentive to avoid hybridization. For example, hybrids between the lower and upper shore morphs of the marine snail *Littorina saxatilis* are fitter than either parent in the midtidal zone, yet there is still some assortative mating between the parental morphs (Rolan-Alvarez *et al.* 1997; Cruz *et al.* 2004).

This paper tests the efficacy of the by-product mechanism in generating prezygotic isolation between stickleback populations adapted to different environments. Threespine sticklebacks inhabit many lakes and streams in the Northern Hemisphere, and freshwater populations typically range between small, slim, plankton-feeding fishes that live mostly in open water and large, deep-bodied fishes that feed on littoral and lakebed invertebrates (Lavin & McPhail 1986; Schluter & McPhail 1992). Interestingly, sticklebacks at the extremes of this

axis coexist as reproductively isolated populations in several lakes in British Columbia; the plankton feeder is termed the limnetic species and the lakebed feeder is the benthic species.

Each pair probably formed when a limnetic-like population invaded a lake containing an intermediate or benthic-like solitary population, after which competition for resources caused further divergence in morphology (Schluter & McPhail 1992; Schluter 1994, 2003). Assortative mating between sympatric stickleback species is well-documented (Ridgway & McPhail 1984; Nagel & Schluter 1998) and has evolved in parallel in independent species pairs (Rundle *et al.* 2000). Comparative studies indicate that assortative mating became strengthened after the two populations came together in a lake, but the amount of reproductive isolation that predated sympatry, arising as a by-product of prior divergent adaptations, has not been estimated.

We tested the by-product mechanism by examining the level of assortative mating between allopatric (i.e. solitary) limnetic-like and benthic-like stickleback populations. If premating reproductive isolation can indeed be generated as a by-product of adaptation of populations to contrasting ecological niches, then the majority of matings should occur between sticklebacks adapted to similar environments. Our experiment is an important step towards establishing the importance of the by-product mechanism to speciation in the wild.

2. MATERIAL AND METHODS

(a) *Fish populations*

We collected males from a benthic-like population in Brannen Lake (49°12′ N, 124°3′ W) and from a limnetic-like population in Sproat Lake (49°14′ N, 124°54′ W) on Vancouver Island. Benthic-like females were collected from Dougan Lake (48°42′ N, 123°36′ W) on Vancouver Island and limnetic-like females came from North Lake (49°44′ N, 123°57′ W) on the Sechelt peninsula. The populations used in the experiment are summarized in table 1. Fish were collected between April and June 2004 using either minnow traps or beach seines. The fish were kept in separate aquaria in an environment chamber, before they were introduced into the pond enclosures; the room temperature was varied to match the water temperature in the ponds.

(b) *Morphological analysis*

We quantified size and shape of the four experimental populations to determine whether they really were similar to the sympatric benthics and limnetics. Size was measured using standard length. The body size of both benthic-like populations was larger than that of the two limnetic-like populations, as is the case between true benthics and limnetics (figure 1).

To measure shape we placed 19 landmarks onto digital photographs of the fish with tpsDig (Rohlf 2001*a*). We included 19 fish from Brannen Lake, 17 each from Dougan, Sproat and North Lakes, and 20 limnetics and 15 benthics from Priest Lake on Texada Island, British Columbia (McPhail 1992). These landmarks are similar to the 14 used by Walker (1997), with an additional five that further describe the eye and jaw (see also Albert & Schluter 2004).

Landmarks were analysed using the program tpsRelw (Rohlf 2001*b*), which creates a consensus body shape that is

Table 1. Summary of the stickleback populations used in the experiment.

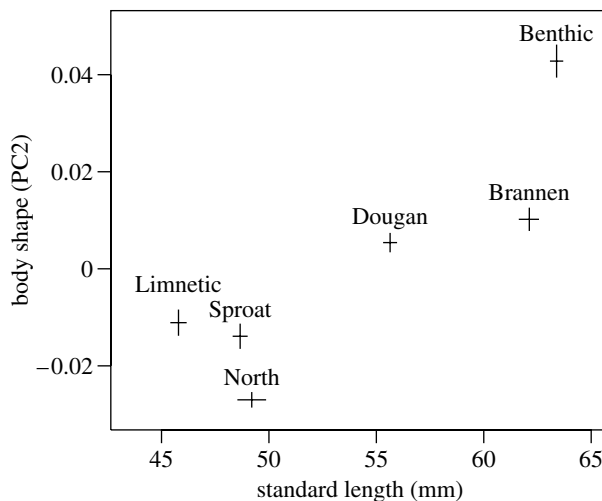|	benthic-like	limnetic-like
male	Brannen lake	Sproat lake
female	Dougan lake	North lake

Figure 1. Population means of body size (standard length) and the second principal component of body shape (PC2) for the Priest Lake species pair (Limnetic and Benthic) and the four study populations, see text for details. Error bars indicate ±1 s.e.

standardized for both geometry and rotation. The program then calculates partial warps scores that describe the amount of energy required to 'bend' the consensus configuration to fit the landmarks of an individual fish (Rohlf & Marcus 1993). The matrix of bending energies is then reduced with principal component analysis to a set of relative warps. The first principal component axis explained 29% of the total variation, and the second explained 18%. The first axis was dominated by variation in the size of the pelvic girdle, and it separated Brannen fish from the other five populations. The second axis separated the limnetic and benthic body shapes, and is the axis plotted in figure 1. The shape differences between the four experimental populations paralleled those of the limnetic and benthic, but were smaller. The two limnetic-like populations resembled the true limnetic in body shape. The two benthic-like populations had body shapes about half-way between those of the limnetic and benthic (figure 1).

Our test assumes that freshwater populations similar in phenotype are nevertheless phylogenetically independent. Globally, separate stickleback populations in freshwater are related to one another by a shared marine ancestor that colonized lakes and streams many times independently (Orti *et al.* 1994; Taylor & McPhail 1999, 2000; McKinnon *et al.* 2004). Previous phylogenetic studies of several populations in British Columbia (Taylor & McPhail 1999, 2000) give little indication that phenotypically similar populations inhabiting separate watersheds are each others' closest relative.

To investigate this further in the four experimental populations, we sequenced 729 bp of the mitochondrial control region (Takahashi & Goto 2001; Mattern 2004) for five individuals from Brannen, Dougan and North Lake, and four from Sproat Lake. We also sequenced fish from two marine populations: six from the Nanaimo River
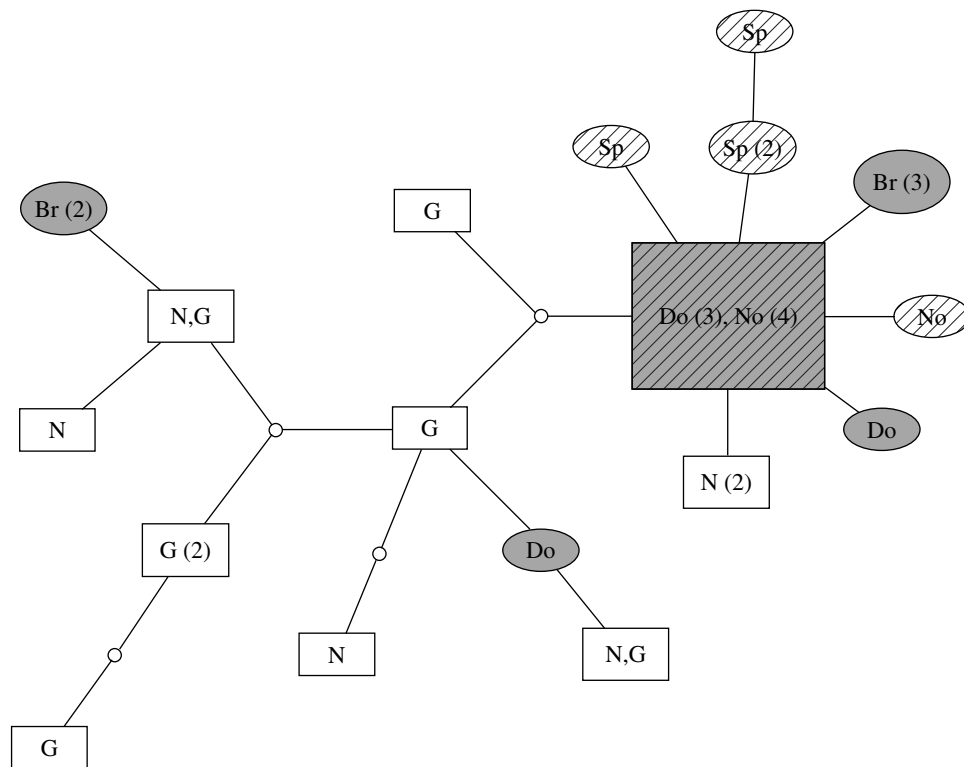
Figure 2. Statistical parsimony network indicating interrelationships among haplotypes for control region mitochondrial DNA. Letters represent the population of origin: 'G', Grappler Inlet; 'N', Nanaimo River; 'Br', Brannen Lake; 'Do', Dougan Lake; 'No', North Lake and 'Sp', Sproat Lake. Each haplotype is represented by a rectangle (marine) or an oval (freshwater); those from benthic-like freshwater populations are shaded, and haplotypes from limnetic-like populations are hatched. The numbers in parentheses are the number of individuals possessing that haplotype. Each line connecting haplotypes represents a single mutational step. Haplotypes that were not found but are necessary intermediates are shown as small circles.

(on the Strait of Georgia) and seven from Grappler Inlet (near Bamfield on the west coast of Vancouver Island).

The phylogenetic relationships among the haplotypes were inferred using statistical parsimony (Templeton *et al.* 1992) as implemented in TCS (Clement *et al.* 2000); the resulting network is shown in figure 2. All lake haplotypes detected were closely related to haplotypes present in the marine populations ($psd \leq 0.41\%$) indicating a recent origin of all four lake populations, consistent with the retreat of the glaciers from the Strait of Georgia around 12 000 years ago. The haplotypes are also consistent with the assumption of phylogenetic independence, as similar phenotypes in different lakes shared no haplotypes. Each lake contained several unique sequences, and the only overlap observed was one haplotype shared between four individuals from North Lake (a limnetic-like population) and three from Dougan Lake (benthic-like). Other unique haplotypes were either closely related to this shared haplotype or to haplotypes found in the marine populations.

(c) *Experimental protocol*
Mating experiments were carried out in enclosures in two artificial ponds on the University of British Columbia campus. These ponds are 23 m across and consist of a polyurethane liner overlaid with limestone sand (see also Hatfield & Schluter 1999; Schluter 2003). The enclosures were constructed from 1 mm door screen mesh attached to a wooden frame and buried in the sand, and each enclosure was 3.6 m wide and between 2 and 4 m from the shore to the back wall. The maximum depth at the back wall ranged from 0.5 to 1 m. As stickleback populations may differ in preferred nesting substrate (Ridgway & McPhail 1984; Vamosi &

Schluter 1999), a randomly chosen side of each enclosure was planted with bladderwort (*Utricularia vulgaris*) and artificial plants made from shredded green plastic bags, and the other side was left open.

A mating trial consisted of placing a female from either Sproat Lake (limnetic-like) or Dougan Lake (benthic-lake) into an enclosure containing four males, two of them benthic-like (from Brannen Lake) and two of them limnetic-like (from Sproat Lake). We used males from different populations than the females to avoid same-lake effects deciding the outcome of assortative mating trials. Males were in good condition and in full breeding coloration. We stimulated nesting behaviour by suspending a jar containing a gravid benthic-like female in the enclosure for 10–60 min each day prior to mating trials. Males that died or became sick were replaced with another individual from the same population.

When an enclosure had at least one nest for each ecotype, a gravid limnetic-like (from North Lake) or benthic-like female (from Dougan Lake) was then selected from the aquaria and left to acclimatize in a floating jar for 30 min. After this period, the female was released. Females were typically recaptured the following day. If she had laid her eggs, we killed her with an overdose of 0.1% clove oil anaesthetic, measured her body length and preserved her in 95% ethanol. Females that were still gravid were released back into the enclosure and re-examined each day until they had laid their eggs. At this time, we also recorded the substrate (vegetation or bare sand) and depth of each nest, and caught the males to measure their standard length.

We identified where the female had laid her eggs by examining male nests. The eggs were then gently removed. The enclosure was then left alone for a day so that the males

Table 2. Results of the 31 mating trials for the nine separate enclosures.

	enclosure								
	1	2	3	4	5	6	7	8	9
no. of within-ecotype matings	4	2	3	4	5	5	2	0	2
no. of between-ecotype matings	0	2	1	0	0	0	0	1	0
proportion matings within ecotype	1.0	0.5	0.75	1.0	1.0	1.0	1.0	0	1.0

could repair their nests before another female was added. Up to five females were added sequentially to an enclosure, after which the males were removed, anaesthetized in a 0.1% clove oil solution and preserved in ethanol.

3. RESULTS

(a) Mate choice

There was strong assortative mating between the two ecotypes: benthic-like females mated almost exclusively with benthic-like males, while limnetic-like females preferred limnetic-like males (table 2). Of the 31 trials, only four involved a mating between different ecotypes. This is significantly different from the random mating expectation of 50 : 50 (binomial test, $p < 0.0001$). Males were used repeatedly, in which case the choices of different females might not be independent. The 31 trials involved only nine separate sets of males, and it is more conservative to treat male set as the unit of replication. We fitted the regression model $\text{logit}[p_i] = \mu + \varepsilon$, where p_i is the proportion of within-ecotype matings in enclosure i, ε is an error term and μ is the rate of assortative mating. $\mu = 0$ corresponds to random mating or to a situation in which both benthic-like and limnetic-like females preferred the same male ecotype. The one-sided alternative hypothesis is that $\mu > 0$, which indicates preferential mating within ecotypes. Results gave $\mu = 1.42$, $\chi_8^2 = 3.609$, $p = 0.0287$, which supports the pattern of strong assortative mating shown above.

(b) Body size and mate choice

Our data are consistent with assortative mating by body length at the between-ecotype level. Females chose the male ecotype with an average size closest to her own in 23 out of the 31 trials (binomial test: $p = 0.01$). This pattern is not significant when male set is the unit of replication: as above, we fitted the regression model $\text{logit}[p_i] = \mu + \varepsilon$, where p_i is the proportion of females that mated with the ecotype with mean size closest to her own in enclosure i, ε is an error term and μ is the rate of size-assortative mating. Results gave $\mu = 1.12$, $\chi_8^2 = 2.46$, $p = 0.11$, implying some size assortative mating (μ is positive), but not enough for statistical significance.

There was a marked difference between the two types of females in the correlation between body length and mate choice. The limnetic-like North females chose the ecotype closest in length to herself in 11 out of 12 trials, including a trial where a large North female chose the larger Brannen ecotype. By contrast, Dougan females chose the longer Brannen males (12 of 14 trials), even though they were often closer in length to the Sproat males. However, neither female type showed size assortative mating within ecotype: in trials where both males of the chosen ecotype

had nests, she chose the male closest to herself in length in only 9 of 19 trials.

(c) Nest locations

The benthic-like males typically nested in deeper water than limnetic-like males: a benthic-like male had the deepest nest in all nine sets of males (binomial test: $p = 0.003$). By contrast, neither male type showed any preference for the vegetated or bare side of the enclosure: 9 of 14 benthic-like males and 3 of 14 limnetic-like males nested on the vegetated side (binomial test: $p = 0.42$ and 0.057, respectively).

4. DISCUSSION

Premating reproductive isolation between populations may evolve as a by-product of adaptation to different environments (Rice & Hostert 1993; Coyne & Orr 2004). For example, adaptation may alter mate preferences, and these can drive changes in the secondary sexual traits used in mate recognition. Alternatively, existing display traits may be hard to detect in a new environment, and therefore mate preferences might evolve to prefer more conspicuous traits (Endler 1992; Schluter & Price 1993). Here, we tested whether premating isolation between stickleback populations might evolve as a by-product of adaptation to different environments. We found that, given a choice, allopatric benthic-like females prefer benthic-like males, and allopatric limnetic-like females prefer limnetic-like males. This result suggests that mate preferences change readily as a consequence of ecological adaptation.

Our result clarifies the contribution of the by-product mechanism to the evolution of complete reproductive isolation between sympatric species of limnetic and benthic sticklebacks. Previous evidence indicated that reinforcement has contributed to the evolution of reproductive isolation between these species (Rundle & Schluter 1998), but this gave little indication of the amount of reproductive isolation that had built between the populations prior to their coming together in a single lake. We presumed that some premating reproductive isolation was there initially, otherwise it is difficult to understand how they would have persisted in the early stages of sympatry (Hatfield & Schluter 1999; Rundle 2002). However, this contribution has not been estimated. The present paper demonstrates that ecologically differentiated populations can show surprisingly strong premating isolation when they first come into contact.

Our finding of partial premating isolation between allopatric, ecologically differentiated forms was derived from field-based experiments in which females had a choice of males, in contrast to our usual 'no-choice' laboratory studies (Rundle & Schluter 1998; Albert & Schluter 2004). Furthermore, males were allowed to

choose their own nest location, and benthic-like males consistently had the deepest nests in all enclosures. These nest location differences may strengthen assortative mating if benthic- and limnetic-like females tend to search for mates at different depths, or if males build better nests at their preferred depth. These differences in design may explain why previous laboratory studies of assortative mating found weaker assortative mating between allopatric populations than that detected here.

Finally, divergence in size is a common feature of ecological divergence in sticklebacks (including those used in this experiment), and several other studies have found size-assortative mating between other ecologically differentiated stickleback populations (Nagel & Schluter 1998; Ishikawa & Mori 2000; McKinnon *et al.* 2004). The observation of assortative mating between the large benthic-like and small limnetic-like fishes in our study is therefore unlikely to be a unique feature of these two pairs of populations. However, it is possible that strong assortative mating does not exist between all benthic- or limnetic-like stickleback populations, but this does not undermine the potentially key role of the by-product mechanism in the formation of stickleback species pairs. There are many more suitable lakes than species pairs (Schluter & McPhail 1992; Vamosi 2003), and this may be because sufficiently isolated benthic- and limnetic-like populations did not exist in all locations. It would thus be valuable to test the generality of our results with other populations.

In summary, our experiment demonstrated that premating isolation appears as a by-product of adaptation to different environments. This by-product mechanism probably generated most of the initial reproductive isolation between the sympatric limnetic–benthic species pairs found in other lakes in British Columbia. We hope that our results stimulate further research into the impact of ecological adaptation on mate preferences and the formation of new species.

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