The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2013, **110**, 466–476. With 1 figure

Weak habitat isolation in a threespine stickleback (*Gasterosteus* spp.) species pair

LAURA SOUTHCOTT[1]*, LAURA NAGEL[2], TODD HATFIELD[3] and DOLPH SCHLUTER[1]

[1]*Department of Zoology and Biodiversity Research Centre, University of British Columbia, Vancouver, BC, V6T 1Z4, Canada*
[2]*Biology Department, Queen's University, Kingston, ON, K7L 3N6, Canada*
[3]*Solander Ecological Research, Victoria, BC, V8S 1C7, Canada*

Received 4 February 2013; revised 19 April 2013; accepted for publication 19 April 2013

Reproductive isolation is central to the study of speciation. Multiple isolating barriers may prevent species from hybridizing, although their individual strength and the interactions between them are rarely measured. We quantified habitat isolation in a recently diverged threespine stickleback species pair (*Gasterosteus aculeatus* complex) and controlled for any such interactions. Using enclosures in an outdoor pond, we confirm that males of the two species strongly prefer different nesting habitats: limnetic males build nests in open habitats, whereas benthic males nest under vegetation. However, forcing males to nest in their nonpreferred habitat did not reduce the probability of spawning by females. As a result, habitat isolation between the species is estimated to be weak. We compared the strength of habitat isolation estimated in the present study with estimates of other behavioural barriers using previously published data. We discovered that, although total mating isolation between the species is strong, the contributions of differences in body size and male nuptial colour are similarly individually weak. Instead, interactions with other, undetermined species-specific traits were responsible for most of the isolation resulting from differences in body size and, in benthics, colour. This is one of the first attempts to estimate individual isolating barriers at the same time as controlling for interactions. *Biological Journal of the Linnean Society*, 2013, **110**, 466–476.

ADDITIONAL KEYWORDS: behaviour – gene flow – hybridization – reproductive isolation – speciation.

INTRODUCTION

The study of speciation has been shaped by Ernst Mayr's biological species concept, which defines species as groups of actually or potentially interbreeding organisms that are reproductively isolated from other such groups. Despite persistent controversy over the validity of this definition, it is clear that interbreeding (or rather the lack thereof) plays a critical role in speciation in sexually reproducing organisms (Coyne & Orr, 2004). Since the biological species concept was introduced, the enumeration of barriers to reproduction between sister species (i.e. factors that prevent hybrid formation or persistence) has been a large component of speciation research. According to the few exhaustive studies performed to date, reproductive isolation in young species is typically the result of many imperfect barriers (Ramsey, Bradshaw & Schemske, 2003; Martin & Willis, 2007; Takami, Nagata & Sasabe, 2007; Lowry *et al*., 2008; Lowry, Rockwood & Willis, 2008; Kitano *et al*., 2009; Groot *et al*., 2010). It is therefore necessary to quantify how much gene flow different barriers allow, in addition to simply identifying them, to understand which are most critical for species persistence. Although measuring individual barrier strength does not necessarily yield information on the order in which these barriers evolved, it creates a snapshot of how species boundaries are currently maintained. This approach thus gives an indication of at least some of the barriers involved in speciation, especially in young species (Coyne & Orr, 2004).

We used this approach to investigate habitat isolation in a sympatric species pair of threespine stickleback (*Gasterosteus aculeatus* species complex), a fish

*Corresponding author. E-mail: lsouthco@zoology.ubc.ca

that has provided much valuable insight into the origin and maintenance of new species. The ancestral threespine stickleback is marine (Taylor & McPhail, 2000), occurring throughout the northern hemisphere. It has repeatedly colonized freshwater lakes and streams from the ocean following the end of the last ice age. In British Columbia, five watersheds contain two stickleback species: one feeding in the open water (the 'limnetic' species), the other inhabiting the littoral zone (the 'benthic' species). Benthics are larger and deeper-bodied with fewer, smaller gill rakers, whereas limnetics are smaller and shallow-bodied with more gill rakers (McPhail, 1992), a pattern that has evolved in parallel in multiple lakes (Rundle *et al*., 2000; Boughman, Rundle & Schluter, 2005). These differences are considered to be adaptations to different foraging habitats: limnetics are zooplanktivorous, whereas benthics feed on benthic macroinvertebrates (Schluter, 1993).

The low frequency of wild hybrids (McPhail, 1992; Gow, Peichel & Taylor, 2006), combined with little apparent intrinsic hybrid inviability (McPhail, 1992; Hatfield & Schluter, 1999), implies that strong premating isolation may exist between benthics and limnetics. Indeed, mating isolation, resulting from increased mate preference for conspecifics relative to heterospecifics in the absence of habitat cues (also called sexual, behavioural, or ethological isolation), is substantial in these species pairs (Hatfield & Schluter, 1996; Nagel & Schluter, 1998; Boughman *et al*., 2005). Body size difference is known to contribute to mating isolation. Benthic stickleback are typically larger than limnetics, and interspecific spawning occurs most often between fish of similar size (Nagel & Schluter, 1998). Male nuptial colour also contributes to the preference for conspecifics, although its importance may vary among species pairs in different lakes (Nagel & Schluter, 1998; Boughman *et al*., 2005). However, the contribution of habitat preference to premating reproductive isolation has not been estimated previously.

Habitat isolation between benthic and limnetic stickleback resulting from genetically based habitat preference has been postulated in several previous studies (McPhail, 1994; Hatfield, 1995; Vamosi & Schluter, 1999). Although males of both species build and defend nests in the littoral zone, they prefer different microhabitats (hereafter 'habitats'). Limnetic males nest on open substrates, including logs, sand, and rubble, whereas benthic males build nests in patches of dense vegetation between open areas (Ridgway, 1982; McPhail, 1994; Hatfield, 1995). In an experiment in which laboratory-reared benthic and limnetic males were released singly into enclosures in Paxton Lake containing both habitat types, the two species consistently chose their different habitats (Hatfield, 1995). The collapse of the Enos Lake species pair into a hybrid swarm followed the almost complete removal of the lake's vegetation by introduced crayfish (Taylor *et al*., 2006), suggesting that habitat isolation may have been important in this pair. However, the importance of aquatic vegetation in maintaining reproductive isolation between benthic and limnetic ecotypes generally is far from clear because a nearby lake containing another stickleback species pair has very little vegetation (Ormond, 2011).

Habitat preference by males does not contribute to assortative mating unless females of the same species have the corresponding habitat preference. This situation may arise by either of two processes (Snowberg & Bolnick, 2012). In the first, females of the two species confine their general activities to different habitats and mate with the males that they predominantly encounter. This scenario occurs in many of the best-known cases of habitat isolation in animals (e.g. pea aphids: Via, 1999; *Rhagoletis* flies: Linn *et al*., 2003; stick insects: Nosil, Sandoval & Crespi, 2006; lake and stream threespine stickleback: Bolnick *et al*., 2009). In the second process, habitat isolation arises because females actively prefer males nesting in one habitat over the other. Neither of these possibilities has been tested experimentally in benthic-limnetic stickleback species pairs. In support of the first process, Vamosi & Schluter (1999) noted that more gravid benthic than limnetic females were caught in traps set in vegetated parts of Paxton Lake, whereas gravid limnetic females predominated in catches from open areas. However, this pattern might have other causes in that gravid females may search for males of their own species by cues other than habitat, putting them more often in one habitat than the other. Furthermore, the littoral zone is a patchwork of open and vegetated areas and females clearly see and encounter males nesting in both habitats. It is thus likely that any female component of habitat isolation, if present, would include active preference for males nesting in one habitat over males in the other.

To date, there has been no direct estimate of female habitat preference in benthic or limnetic stickleback. This information is required to determine the contribution of nest habitat choice to reproductive isolation and hybridization between them. In the present study, we experimentally assessed the female contribution to habitat isolation by confining nesting males to one habitat or the other and determined how this affects female willingness to spawn. We used conspecific mating trials only because this is the only way to isolate the effect of habitat independent of all other species differences. We compared our estimates of habitat isolation with estimates of mating isolation

(preference for conspecific mates in the absence of habitat cues) and to the standalone contributions of body size difference and male nuptial colour using data from previously published studies. The information obtained will contribute to our understanding of the maintenance of reproductive isolation between benthics and limnetics and is a first step toward determining whether isolating barriers interact.

MATERIAL AND METHODS

Habitat isolation

We conducted separate experiments to determine the probability of males of each species nesting in two different habitats and the probability of a female spawning with a male confined to either habitat. Both experiments were conducted in 1 × 1-m window screen enclosures with open bottoms placed in water (depth of approximately 80 cm) in two ponds at the University of British Columbia's experimental pond facility (11–12 enclosures per pond). Each pond is 15 × 25 m and ranges in depth from 0 to 6 m. The bottom edges of the enclosure sides were buried in gravel and the top edges were suspended above the water surface from nylon ropes.

Fish were captured using minnow traps between March and May 2010 and in April 2011 in Paxton Lake on Texada Island, British Columbia, and transported to the University of British Columbia, where benthics and limnetics were kept in separate 107-L aquaria. They were fed frozen bloodworms and mysis shrimp *ad libitum* daily and kept at 17 °C with 12 h of daylight initially, increased to 14 h in May. Fish collected in 2010 were used to assess male nest site choice, whereas those collected in 2011 were used in the female habitat preference experiment. As a result of a shortage of gravid wild-caught females, additional pond-reared females (15 benthic and six limnetic) were included in the latter experiment. These fish were raised in single-species ponds and are descended from wild-caught fish introduced into the ponds in 2008. Apart from the holding period in aquaria, these fish were handled in the same way as the wild-caught females.

We used 21 wild-caught males in 12 of the enclosures to assess male nest site choice (Hatfield, 1995). Half of each enclosure (chosen randomly) was covered with bladderwort (*Utricularia* sp.) collected from mature experimental ponds at the University of British Columbia, whereas the other half was left as uncovered sand and limestone gravel. Males were chosen haphazardly from the holding tanks as they came into breeding condition and transported in bags to the ponds. They were allowed to acclimate for at least 30 min by gradually adding pond water to the bags before release into enclosures (one male per enclosure). Fish in enclosures were fed frozen bloodworms two or three times per week. Each male was presented with a gravid female in a mesh-covered jar for 10 min per day to encourage nest-building. The jar containing the female was placed in the centre of the enclosure on the border between vegetated and unvegetated halves to avoid biasing the males' nest site choice. Once a male built a nest, the nesting substrate immediately surrounding the nest was classified as 'open', 'vegetated', or 'partially vegetated' (nest is within 5 cm of vegetation) *sensu* Hatfield (1995). Males were then removed by trapping or dip netting, euthanized with an overdose of buffered tricaine methanesulphonate (MS-222), measured (standard length), and preserved in 95% ethanol. The nest was destroyed after the trial so that the enclosure could be reused.

We used 23 enclosures in May to July 2011 to examine how nest habitat influences the probability that a female will spawn with a single nesting male. Enclosures were randomly assigned to vegetated or unvegetated treatments. The floor of vegetated enclosures was covered with stonewort (*Chara* sp.), whereas unvegetated enclosures had open sand and limestone gravel. Different macrophyte species were used in different years as a result of a lack of stonewort available in the ponds during the first year. Because both species are present and abundant in the vegetated parts of Paxton Lake, this difference is unlikely to affect the results of either experiment. A male of either species was randomly assigned to each enclosure and shown a captive gravid female each day, as described above. For both experiments, these captive females came from several different lakes or crosses depending on availability but were conspecific with the males whenever possible and were never used in mating trials.

As in previous studies (Nagel & Schluter, 1998; Boughman, 2001), we employed a no-choice design in which a single male–female pair is given a defined time period in which to spawn. This design avoids aggressive interactions among males, which may interfere with spawning and confound female habitat preference. It also had the advantage of confining males to a specific habitat by providing no alternative (males switch to the preferred habitat if available). No-choice experiments may provide a conservative estimate of habitat isolation because females presented with two males nesting in contrasting habitats might be more likely to show a habitat preference than in the present design. The present study used only conspecific pairs to control for differences in all other phenotypic traits known to differentiate benthics and limnetics. Our goal was to investigate the contribution of habitat isolation independent of

other behavioural and morphological mechanisms of reproductive isolation.

Once a male had nested, a gravid conspecific female was acclimated to the pond (as described for males) for at least 30 min before being introduced into the enclosure. An earlier pilot study indicated that spawning rarely took place in the first 30 min after introduction of the female but had almost always occurred after 24 h; thus, we ended trials after 2 h (Hatfield & Schluter, 1996). At the end of each trial, fish were removed by dip netting or trapping and the nest was checked for eggs. If spawning did not occur, the female was examined for readiness to spawn by gently pressing on her abdomen and looking for eggs in the oviduct. Trials in which the female was not ready to spawn were excluded from analyses, leaving 51 trials for our analyses. As in the previous experiment, fish were euthanized, measured, and preserved after their removal from the enclosure.

We used Fisher's exact test to test whether males of the two species chose different nest habitats. To compare spawning probability in different habitats for each species, we used a generalized linear model (GLM) with binomial error and species and habitat as fixed effects. We also conducted another GLM in which we pooled results for the two species and reclassified the habitats as 'preferred' (the habitat in which conspecific males would normally nest; i.e. open for limnetics and vegetated for benthics) or 'nonpreferred' (i.e. the habitat in which heterospecific males would normally nest). These and all subsequent analyses were conducted in R, version 2.11.1 (R Development Core Team, 2011).

Comparing the strength of habitat isolation to other isolating barriers requires an equivalent formula (Ramsey *et al*., 2003). Our analysis was based on the general formula (Sobel, 2010):

$$RI = 1 - 2\frac{H}{H + C} \tag{1}$$

where H represents the expected number of hybridization events and C is the expected number of pure-species mating events after a particular barrier has acted, assuming equal opportunities for conspecific and heterospecific mating. Thus, $\frac{H}{H+C}$ is the proportion of all mating events that are hybridizations. RI can range from −1, when all matings are hybridizations, to 1 when all matings are intraspecific, with $RI = 0$ indicating random mating. Reproductive isolation for premating barriers is calculated separately for females of each species (Coyne & Orr, 2004). We modified this general formula to calculate habitat isolation:

$$RI\,(habitat) = 1 - 2\frac{s_{veg}p_{het,veg} + s_{open}p_{het,open}}{s_{veg}p_{het,veg} + s_{open}p_{het,open} + s_{veg}p_{con,veg} + s_{open}p_{con,open}} \tag{2}$$

The observed frequency of spawning in each habitat is given by s_{veg} and s_{open}. The values of p denote the proportion of conspecific or heterospecific males nesting in each habitat (i.e. $p_{het,veg}$ is the proportion of all males nesting in vegetation that were heterospecifics, $p_{con,veg}$ is the proportion of all males nesting in vegetation that were conspecifics, and $p_{het,veg} + p_{con,veg} = 1$), as observed in the male nest habitat choice experiment. Thus, H and C in Eqn (1) are replaced by the sum of the spawning rates in each habitat weighted by the probability of finding a heterospecific or conspecific mate respectively in each habitat. Data were resampled 1000 times and the 2.5% and 97.5% quantiles of *RI(habitat)* were used to estimate the bootstrap 95% confidence intervals (Efron, 1987).

Mating isolation

We compared our estimates of habitat isolation with the strength of other known premating barriers in Paxton Lake stickleback: mating isolation and two components of mating isolation (body size difference and male nuptial colour). We used data from previous no-choice mating experiments within and between the benthic and limnetic species. These trials were conducted in aquaria using wild-caught (Nagel & Schluter, 1998) and laboratory-reared fish (Hatfield & Schluter, 1996) from Paxton Lake, the same lake studied in our habitat isolation experiments. Analyses were conducted on each dataset, hereafter referred to as wild-caught and laboratory-reared datasets, separately. No benthic females spawned in any of Hatfield & Schluter's (1996) trials, and so reproductive isolation could only be calculated for laboratory-raised limnetics. The range of size differences in the laboratory-reared limnetics was more restricted than in the wild-caught fish, although this dataset is still useful for investigating overall mating isolation.

Mating isolation, or *RI(mating)*, was calculated for females of each species using Eqn (1), where H and C are the proportions of heterospecific and conspecific trials, respectively, that resulted in spawning (or nest entry in the case of the laboratory-reared fish; females were prevented from spawning after nest entry). Data were resampled 2000 times and the 2.5% and 97.5% quantiles of *RI(mating)* were used to estimate the bootstrap 95% confidence intervals. Sample sizes for these trials are presented in Table 1.

Isolation by body size

Again, we used only data from conspecific trials in the previously studied wild-caught and laboratory-raised

Table 1. Sample sizes for trials used to calculate *RI(mating)*, *RI(body size)*, and *RI(colour)*

Source	Treatment	*N*
Wild	Benthic × Benthic	24
	Limnetic × Limnetic	9
	Benthic × Limnetic	19
	Limnetic × Benthic	19
Lab	Limnetic × Limnetic	20
	Limnetic × Benthic	15

datasets in the measurements of isolation by body size and by male nuptial colour. As for habitat isolation, this method separates the effects of these two traits from isolation arising from interactions between them and other, unknown differences between the two species. In general, to calculate reproductive isolation as a result of body size differences, we modify Eqn (1):

$$RI\,(\text{body size}) = 1 - 2\frac{\sum_{\text{i}} \frac{m_{\text{i,het}}}{m_{\text{het}}} d_{\text{i,het}}}{\sum_{\text{i}} \frac{m_{\text{i,het}}}{m_{\text{het}}} d_{\text{i,het}} + \sum_{\text{i}} \frac{m_{\text{i,con}}}{m_{\text{con}}} d_{\text{i,con}}} \quad (3)$$

Here, $d_{\text{i,het}}$ is the predicted probability of a randomly-paired male and female with size difference i being heterospecific, $m_{\text{i,het}}$ is the predicted probability of a heterospecific pair spawning given size difference i, $d_{\text{i,con}}$ and $m_{\text{i,con}}$ are the equivalent values for conspecific pairs, and i ranges from the smallest to the largest size difference in the dataset (among both conspecific and heterospecific pairs). m_{het} and m_{con} are the maximum spawning rates for heterospecific and conspecific pairs, respectively; dividing by these values corrects for differences in mean spawning rates between cross-types not a result of body size. This method returns $RI = 0$ when body size has no effect on mating.

To calculate *RI(body size)* independent of other species-specific differences, as for *RI(habitat)*, we used only spawning rates from conspecific trials, thus setting $m_{\text{i,het}} = m_{\text{i,con}}$ and paralleling Eqn (2). This simplifies Eqn (3):

$$RI\,(\text{body size}) = 1 - 2\frac{\sum_{\text{i}} m_{\text{i,con}} d_{\text{i,het}}}{\sum_{\text{i}} m_{\text{i,con}}} \quad (4)$$

GLMs were used to estimate $m_{\text{i,con}}$ as a function of size difference. We used models with binomial errors (logistic regression) and body size difference measured as the absolute value of the difference between male and female standard length. For females of each species, predicted probabilities from the model for each size (in increments of 0.1 mm) were used to calculate *RI(body size)* using Eqn (4).

Other phenotypic differences between species may interact with body size to influence mating, producing stronger (or weaker) isolation for a given body size difference between species than within species. Because data from heterospecific trials were also available, we could try to determine whether this occurs and calculate reproductive isolation as a result of these traits together with such interactions. Using data from both conspecific and heterospecific trials, we compared GLMs of spawning versus body size difference and male species with and without an interaction term between size and species. A significant interaction indicates that the effect of body size difference on spawning probability differs when a female is paired with a conspecific or heterospecific male, implying the influence of other traits. In addition to testing for the significance of the interaction terms, we considered the change in the corrected Akaike information criterion (ΔAICc) (Burnham & Anderson, 2004) between models with and without interaction terms as another measure of the contribution of interactions. To calculate *RI(body size)* with these interactions, we applied Eqn (3) using the estimates of $m_{\text{i,con}}$ and $m_{\text{i,het}}$ from the models with an interaction term.

We estimated $d_{\text{i,con}}$ for both scenarios (conspecific only and both conspecific and heterospecific including interactions) by calculating the means and SDs of the lengths of males and females of each species in samples of wild fish used in the trials analyzed in the present study (Hatfield & Schluter, 1996; Nagel & Schluter, 1998) and in other mate choice studies of the Paxton Lake species pair (Rundle *et al.*, 2000; Albert & Schluter, 2004; Boughman *et al.*, 2005). Size-frequency distributions were fitted with normal distributions from which we generated 1000 random pairs of sizes for each male species–female species combination. From these, we fitted another GLM to derive the relationship between body size difference and the probability of a pair being conspecific ($d_{\text{i,con}}$); note that $d_{\text{i,het}} = 1 - d_{\text{i,con}}$.

Isolation by male nuptial colour

Eqn (3) is generalizable to other categorical or continuous traits. The dataset for wild-caught fish contained subjective scores for the intensity of the red throat patch of males just prior to mating trials (1 = almost no colour, 5 = highest intensity red; in increments of 0.5). Using the same approach as the *RI(body size)* calculation, we estimated *RI(colour)* from only conspecific data by fitting GLMs of female spawning on male colour score. In this case, $m_{\text{i,con}}$

Table 2. Male nest site frequencies

	Open	Partial	Vegetated
Benthic	0	1	10
Limnetic	7	3	0

represents the predicted probability of spawning with a conspecific of colour score *i*. We also added male species as a fixed effect in the models to calculate *RI(colour)* combined with interactions with other species-specific traits. We compared GLMs with and without an interaction term between colour score and male species to investigate whether colour interacts with other species differences to affect mate choice. Because colour scores were not normally distributed, we calculated the probability of a male being conspecific for each possible colour score ($d_{i,con}$) directly from the data, correcting for differences in sample size between species.

Finally, we calculated mating isolation as a result of body size and colour combined as:

$$RI\,(combined) = 1 - 2\frac{H_{size}H_{colour}}{H_{size}H_{colour} + C_{size}C_{colour}} \quad (5)$$

Here, $H_{size} = \sum_i m_{i,con}d_{i,het}$ and $C_{size} = \sum_i m_{i,con}d_{i,con}$ are as calculated in Eqn (4). H_{colour} and C_{colour} are the corresponding values for male nuptial colour. This method of calculating the combined effect of two barriers assumes that they are independent; hence, we used only the conspecific data for this estimate. This calculation allows us to compare the combined effects of size and colour to *RI(mating)*, which may include contributions from other, untested traits.

RESULTS

Habitat isolation

Data from these experiments, as well as from the mating isolation datasets, are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.95qc4. Males of the two species differed significantly in nest site choice (Fisher's exact test, $P < 0.0001$), with benthics strongly preferring vegetation and limnetics favouring open sites (Table 2). These results match those of Hatfield (1995), who used laboratory-raised males in enclosures in Paxton Lake and natural vegetation. Our replication of these results suggests that stickleback perceived the substrates in the enclosures much as they would a natural lakebed.

By contrast, neither benthic, nor limnetic females showed a lower probability of spawning in their 'nonpreferred' habitat (Table 3). This was true both when the species were considered separately ($P = 0.71$) and when they were pooled ($P = 0.69$). Limnetic females were overall less likely to spawn than benthics, although this result was not significant ($P = 0.92$). There was no overall difference in spawning rate between habitats ($P = 0.86$).

Table 3. Frequency of spawning with conspecific males in open or vegetated habitats

Species	Habitat	Spawn	No spawn
Benthic	Vegetated	4	6
	Open	4	7
Limnetic	Vegetated	5	10
	Open	6	9
Combined	Preferred	10	15
	Nonpreferred	9	17

We calculated *RI(habitat)* in two ways: by grouping 'partial' vegetation with the preferred habitat (i.e. vegetated for benthics, open for limnetics) and by including 'partial' with the nonpreferred habitat instead. Grouping was necessary because 'partially vegetated' habitat was not used in the female mating trials. Regardless, *RI(habitat)* was close to zero, and there is no consistent difference between benthics and limnetics (Fig. 1). However, 95% confidence intervals for *RI(habitat)* range up to 0.74, and so we cannot rule out the possibility that there is some relatively weak habitat isolation.

Other barriers

Mating isolation was strong in both directions, although stronger in benthics than limnetics (Fig. 1). This was in part a result of male limnetics being less likely to court benthic females than male benthics were to court limnetic females, possibly because benthic females sometimes consume eggs already in the male's nest, whereas limnetic females rarely do (Albert & Schluter, 2004).

RI(body size) without an interaction was as weak as habitat isolation (Fig. 1). The negative value for laboratory-reared limnetics is probably an artefact of the limited size range tested in that study. *RI(body size)* calculated from models with an interaction term was higher in all cases than when only conspecific trials were considered. Models with a size difference by male species interaction term were as good as or better than the models without an interaction based on both *P*-values and AICc scores except for laboratory-reared limnetics (benthics, $\Delta AICc = 2.66$ and $P = 0.026$; wild limnetics, $\Delta AICc = 3.33$ and $P = 0.016$; laboratory limnetics, $\Delta AICc = 1.49$ and $P = 0.47$), implying that

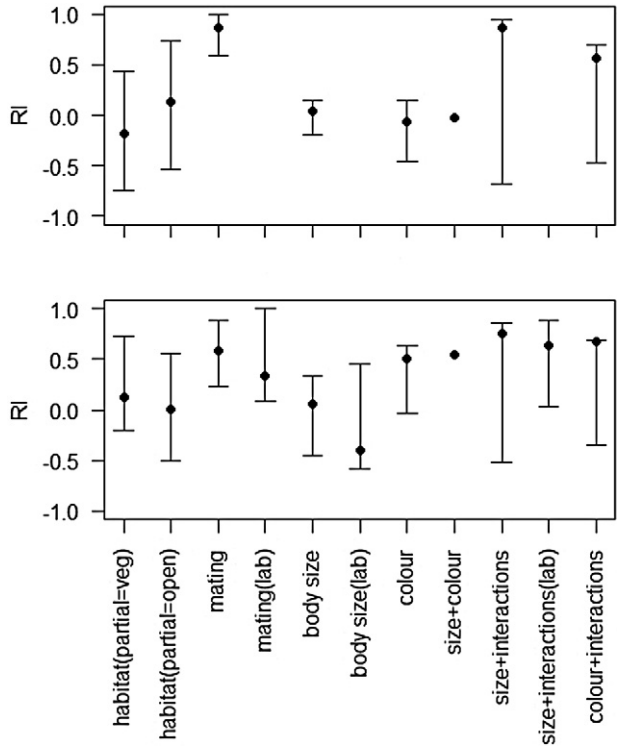

Figure 1. Reproductive isolation (*RI*) as a result of various premating barriers in benthic (top) and limnetic (bottom) threespine stickleback from Paxton Lake. Habitat isolation is calculated from the experimental results obtained in the present study. Barriers indicated by 'lab' (limnetic only) are calculated from data reported in Hatfield and Schluter (1996). All other barriers are calculated from data reported in Nagel and Schluter (1998). Error bars show bootstrap 95% confidence intervals, where calculated.

size difference was treated differently as a mate choice cue in conspecific and heterospecific trials.

Isolation as a result of nuptial colour alone was weak in benthics and fairly strong in limnetics (Fig. 1). Models with a male colour by male species interaction had substantial support compared to those without one based on ΔAICc, although the interaction terms themselves were not significant (benthics, $\Delta AICc = 0.81$ and $P = 0.200$; limnetics, $\Delta AICc = 2.50$ and $P = 0.999$). Including the interaction greatly increased *RI(colour)* for benthics but increased it only slightly for limnetics (Fig. 1).

There was no relationship between male length (or size difference) and colour score (results not shown). We therefore treated size and colour as independent components of mating isolation and calculated their combined strength from the without-interaction estimates of each barrier. *RI(combined)* was near zero for benthic females (Fig. 1). However, *RI(combined)* for limnetics was slightly larger than but within the 95% confidence intervals of the observed mating isolation.

Comparison

Habitat isolation was much lower than mating isolation, which was high in both species but higher in benthics than in limnetics (Fig. 1). The two components of mating isolation that we quantified (i.e. body size difference and male nuptial colour) were no stronger than habitat isolation when only conspecific trials were considered (i.e. when interactions with other species-specific differences were controlled for) (Fig. 1). The exception was isolation resulting from preference for male colour in limnetic females, which, at 0.51, is substantial but by no means complete. When the effects of size and colour were combined, the resulting isolation was still negligible for benthic females but stronger for limnetics. However, when interactions were included using data from conspecific and heterospecific mate choice trials, isolation by body size and by colour increased in both species (Fig. 1).

DISCUSSION

Habitat isolation

We present the first estimates of habitat isolation in the Paxton Lake stickleback species pair. The presence of habitat isolation has been postulated previously because nesting males are known to prefer different habitats (McPhail, 1994; Hatfield, 1995). By using 'no-choice' intraspecific trials, we measured this isolating mechanism's strength independent of other species-specific trait differences. Contrary to expectations, we found weak isolation by male nesting habitat. Despite strong habitat preferences in males of both species, females were just as likely to spawn with a male in either habitat. Our sample sizes limited our power to completely rule out subtle habitat effects, and the 95% confidence interval for habitat isolation was broad, ranging from −0.7 to 0.7 (Fig. 1). Yet our data do indicate that, if any habitat isolation exists, it is moderate at best, and certainly weaker than the direct preference of females for conspecific males (mating isolation; Fig. 1). It is conceivable that offering females a choice of similar males in different habitats would have detected moderate habitat isolation; however, if such isolation exists, its impact on the maintenance of benthics and limnetics is much weaker than that of mating isolation. Additionally, our no-choice study design had the advantage of eliminating male–male interactions that could confound such results.

Female preference of males in different nest habitats represents one of two possible mechanisms of habitat isolation in stickleback. In the wild, habitat isolation could also arise from broadscale habitat segregation, leading females to encounter males of their

own species more often. This appears to be the case in parapatric lake and stream stickleback, which preferentially return to their native habitat when translocated (Bolnick *et al*., 2009). Although this source of isolation remains to be examined experimentally in benthics and limnetics, there are many areas in Paxton Lake in which male nesting habitats are closely interspersed (McPhail, 1994; M. Arnegard, unpubl. data; L. Southcott, pers. observ.). Thus, habitat isolation by female preference of nesting males appears to be the most likely scenario.

Male habitat choice in the present study parallels previous observations from several benthic-limnetic species pairs that benthics tend to nest under dense vegetation and limnetics nest in the open (Ridgway, 1982; McPhail, 1994; Hatfield, 1995). Notably, male stickleback in other systems, namely the divergent lake and stream stickleback and sympatric Icelandic lake stickleback, also demonstrate nest site preferences: sand versus gravel in the former (Raeymaekers, Delaire & Hendry, 2009) and near versus far from shelter in the latter (Ólafsdóttir, Ritchie & Snorrason, 2006). The present study used primarily wild-caught fish, and thus habitat preferences could be genetically based or learned, although Hatfield's (1995) similar results for laboratory-reared fish strongly suggest that the preference is genetic. Because males were introduced singly to enclosures in the present study and that of Hatfield (1995), their choices in this experiment represent true habitat choice rather than the outcome of male–male competition.

These results then lead to a mystery: why should males of the two species evolve strong differences in habitat preference in the absence of strong female habitat preference? One possibility is that weak habitat choice by females is nevertheless sufficiently strong to drive the evolution of preferences in males. Second, male habitat preferences might be the result of a genetic correlation (linkage disequilibrium or pleiotropy) with other differences between the species favoured by selection, such as foraging niche. A lack of male habitat choice in benthic- and limnetic-like solitary stickleback populations suggests that this is not the case (Vines & Schluter, 2006). In another solitary population within which there was variation and assorative mating by trophic niche, limnetic-like traits were correlated with a tendency to nest in vegetation rather than open nest sites, although the effect was weak (Snowberg & Bolnick, 2012). In addition, if a genetic correlation explained nesting habitat preference, we might expect the effect to occur in females as well as males. The lack of such a finding raises the possibility that nest habitat choice is sex-linked. Finally, differences in nesting habitat may be an evolved response to past male–male competition between benthics and limnetics (i.e. character displacement). Our results do not allow us to distinguish between these possibilities.

The present study employed intraspecific trials so that the effect of habitat on mating could be evaluated without confounding effects from other differences between males of the two species. However, it is possible that habitat preference might play a role in an interaction with these other species differences, leading to stronger reproductive isolation than that resulting from habitat alone. This idea is made plausible by our analysis of data from previous studies, which found that two of the best-known contributors to reproductive isolation, differences in body size and male colour, have their greatest effects only by their interaction with other, unknown trait differences between the species. Furthermore, a study of assortative mating by feeding habitat between benthic-like and limnetic-like phenotypes within a solitary lake population concluded that other, unknown differences between phenotypes contributed to assortative mating beyond the effects of habitat (Snowberg & Bolnick, 2012). Future mate choice experiments in which both conspecifics and heterospecifics are tested in both habitats could be used to identify such an interaction. Interactions between habitat choice and other traits contributing to reproductive isolation might also be investigated by looking at the interactions within species between traits such as size difference, male nuptial colour, and nesting habitat.

The collapse of the species pair in Enos Lake by hybridization has drawn attention to the possibility of reproductive isolation by habitat choice between limnetics and benthics because the collapse happened simultaneously with the virtually complete loss of lake vegetation following the invasion of the lake by signal crayfish (Taylor *et al*., 2006). As in Paxton Lake, male Enos Lake benthics usually nested under vegetation, whereas limnetics nested in open areas (Ridgway, 1982). If habitat choice was a strong isolating barrier in Enos Lake, homogenization of the available nesting habitat could have led to greater gene flow between benthics and limnetics. Our results suggest a limited role for habitat isolation in the Paxton Lake species pair but, conceivably, its role in the former Enos Lake pair was greater, although no evidence of this has been obtained.

Mating isolation

We also compared our measures of habitat isolation with mating isolation (i.e. the reduction in hybridization resulting from mate choice) using previously published data. Unlike habitat isolation, mating isolation was strong in both directions but stronger in benthics than limnetics. Surprisingly, the two components of

mating isolation that we evaluated, *RI(body size)* and *RI(colour)*, were as weak on their own as habitat isolation. The exception was isolation by male nuptial colour in limnetics: female limnetics prefer more brightly-coloured males regardless of their species, leading to one-sided isolation because benthic males have reduced coloration compared to limnetic males.

Our analyses suggest that the previously demonstrated effect of body size on reproductive isolation (Nagel & Schluter, 1998; Boughman *et al*., 2005; Conte & Schluter, 2013) occurs via the interaction between body size and other differences between males of the two species. In other words, females are more sensitive to body size differences when the potential mate is heterospecific than when conspecific. Manipulation of benthic and limnetic sizes *sensu* McKinnon *et al*. (2004) to attain values typically found in heterospecific pairings is required to confirm our result (Conte & Schluter, 2013). Similarly, including the interaction increased *RI(colour)* for benthic females; they showed no colour preference within their own species but only spawned with the brightest red limnetic male. However, this finding is based on only a single heterospecific spawning event, and more trials are needed to confirm this effect.

Our conclusion that interactions between traits may play a substantial role in previously documented sources of isolation between benthics and limnetics sheds new light on other stickleback research. Body size divergence occurs in other stickleback systems (McKinnon & Rundle, 2002), and it has been suggested that size-based mating isolation facilitated the radiation of stickleback throughout the northern hemisphere (Conte & Schluter, 2013). Tests using fish from different populations but within ecotype have found that size influences mate choice in anadromous versus stream resident (McKinnon *et al*., 2004) and Japan Sea versus Pacific Ocean stickleback (Kitano *et al*., 2009). On the other hand, size-assortative mate choice does not appear to be a source of premating isolation between lake and stream stickleback (Räsänen *et al*., 2012). However, because these are still between-population comparisons, we cannot say for sure whether this is size-based isolation without interactions.

One explanation for the lack of isolation as a result of body size alone is that another size-related trait, and not standard length, is the actual cue used in mate choice. For example, Baube (2008) used the lateral projection area, which incorporates both length and body depth (both of which differ in the species pairs), to explain reproductive isolation between Atlantic threespine stickleback and the blackspotted stickleback *Gasterosteus wheatlandi*. Because a benthic of a given standard length has a deeper body and therefore larger lateral projection area than a limnetic of the same length, the probability of a female spawning with a similarly-sized heterospecific would be even lower than expected based on the pair's length difference.

In combination, the quantities estimated in the present study do not fully account for the amount of reproductive isolation observed in nature between sympatric limnetics and benthics. We can estimate the hybridization rate arising from mating isolation alone using $\frac{H}{H+C}$ in Eqn (1), making the generous assumption that equal opportunities for each cross-type exist. For the wild-caught fish, this value is 0.07 for benthics and 0.21 for limnetics. If we assume equal population sizes and clutch sizes of the two species (admittedly an unlikely scenario), the next generation would contain 14% F_1 hybrids. This is substantially higher than the at most 5% hybrids (including the F_2 generation and backcrosses) observed in nature (McPhail, 1992; Gow *et al*., 2006). Gow *et al*. (2006) estimated that approximately 5% of Paxton fish are of hybrid descent, although no F_1 hybrids were found, suggesting that they are rare. Keeping in mind that no-choice mating trials are conservative and thus assortative mating by species could be stronger when females are given a choice of males (as is the case in nature), it is likely that other isolating mechanisms, including ecological selection against hybrids, are important for maintaining benthics and limnetics as distinct species. Based on the analyses conducted in the present study, it would be worthwhile examining further whether interactions among the known isolating mechanisms in stickleback contribute to the maintenance of the benthic–limnetic species pairs.

ACKNOWLEDGEMENTS

We thank members of the Schluter laboratory and especially G. Conte, A. Smith, T. Ingram, A. Dalziel, D. Toews, S. Kabir, T. Tai, J. Best, K. Heilbron, T. Lam, C. Kingwell, A. R. Lackey, and P. Tamkee for their help with field work and fish rearing. A. R. Lackey, S. Otto, and several anonymous reviewers greatly improved earlier versions of this manuscript, and R. Fitzjohn, L. M'Gonigle, and K. Samuk provided helpful statistical advice. This research was funded by a Natural Sciences and Engineering Research Council (NSERC) Canada Graduate Scholarship to L.S.

REFERENCES

Albert AYK, Schluter D. 2004. Reproductive character displacement of male stickleback mate preference: reinforcement or direct selection? *Evolution* **58:** 1099–1107.

Baube CL. 2008. Body size and the maintenance of reproductive isolation in stickleback, genus *Gasterosteus*. *Ethology* **114:** 1122–1134.

Bolnick DI, Snowberg LK, Patenia C, Stutz WE, Ingram T, Lau OL. 2009. Phenotype-dependent native habitat preference facilitates divergence between parapatric lake and stream stickleback. *Evolution* **63:** 2004–2016.

Boughman JW. 2001. Divergent sexual selection enhances reproductive isolation in sticklebacks. *Nature* **411:** 944–948.

Boughman JW, Rundle HD, Schluter D. 2005. Parallel evolution of sexual isolation in sticklebacks. *Evolution* **59:** 361–373.

Burnham KP, Anderson DR. 2004. Multimodal inference: understanding AIC and BIC in model selection. *Sociological Methods and Research* **33:** 261–304.

Conte GL, Schluter D. 2013. Experimental confirmation that body size determines mate preference via phenotype matching in a stickleback species pair. *Evolution* **67:** 1477–1484.

Coyne JA, Orr HA. 2004. *Speciation*. Sunderland, MA: Sinauer Associates.

Efron B. 1987. Better bootstrap confidence intervals. *Journal of the American Statistical Association* **82:** 171–185.

Gow JL, Peichel CL, Taylor EB. 2006. Contrasting hybridization rates between sympatric three-spined sticklebacks highlight the fragility of reproductive barriers between evolutionarily young species. *Molecular Ecology* **15:** 739–752.

Groot AT, Marr M, Heckel DG, Schofl G. 2010. The roles and interactions of reproductive isolation mechanisms in fall armyworm (Lepidoptera: Noctuidae) host strains. *Ecological Entomology* **35:** 105–118.

Hatfield T. 1995. Speciation in sympatric sticklebacks: hybridization, reproductive isolation and the maintenance of diversity. PhD Thesis, University of British Columbia.

Hatfield T, Schluter D. 1996. A test for sexual selection on hybrids of two sympatric sticklebacks. *Evolution* **50:** 2429–2434.

Hatfield T, Schluter D. 1999. Ecological speciation in sticklebacks: environment-dependent hybrid fitness. *Evolution* **53:** 866–873.

Kitano J, Ross JA, Mori S, Kume M, Jones FC, Chan YF, Absher DM, Grimwood J, Schmutz J, Myers RM, Kingsley DM, Peichel CL. 2009. A role for a neo-sex chromosome in stickleback speciation. *Nature* **461:** 1079–1083.

Linn C Jr, Feder JL, Nojima S, Dambroski HR, Berlocher SH, Roelofs W. 2003. Fruit odor discrimination and sympatric host race formation in *Rhagoletis*. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 11490–11493.

Lowry DB, Modliszewski JL, Wright KM, Wu CA, Willis JH. 2008. The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philosophical transactions of the Royal Society of London Series B, Biological Sciences* **363:** 3009–3021.

Lowry DB, Rockwood RC, Willis JH. 2008. Ecological reproductive isolation of coast and inland races of *Mimulus guttatus*. *Evolution* **62:** 2196–2214.

Martin NH, Willis JH. 2007. Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution* **61:** 68–82.

McKinnon JS, Mori S, Blackman BK, David L, Kingsley DM, Jamieson L, Chou J, Schluter D. 2004. Evidence for ecology's role in speciation. *Nature* **429:** 294–298.

McKinnon JS, Rundle HD. 2002. Speciation in nature: the threespine stickleback model systems. *Trends in Ecology and Evolution* **17:** 480–488.

McPhail JD. 1992. Ecology and evolution of sympatric sticklebacks (*Gasterosteus*): evidence for a species-pair in Paxton Lake, Texada Island, British Columbia. *Canadian Journal of Zoology* **70:** 361–369.

McPhail JD. 1994. Speciation and the evolution of reproductive isolation in the sticklebacks (*Gasterosteus*) of southwestern British Columbia. In: Bell MA, Foster SA, eds. *The evolutionary biology of the threespine stickleback*. Oxford: Oxford University Press, 399–437.

Nagel L, Schluter D. 1998. Body size, natural selection, and speciation in sticklebacks. *Evolution* **52:** 209–218.

Nosil P, Sandoval CP, Crespi BJ. 2006. The evolution of host preference in allopatric vs. parapatric populations of *Timema cristinae* walking-sticks. *Journal of Evolutionary Biology* **19:** 929–942.

Ólafsdóttir GA, Ritchie MG, Snorrason SS. 2006. Positive assortative mating between recently described sympatric morphs of Icelandic sticklebacks. *Biology Letters* **2:** 250–252.

Ormond CI. 2011. Environmental determinants of threespine stickleback species pair evolution and persistence. MSc Thesis, University of British Columbia.

R Development Core Team. 2011. *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing. Available at: http://www.R-project.org.

Raeymaekers JAM, Delaire L, Hendry AP. 2009. Genetically based differences in nest characteristics between lake, inlet, and hybrid threespine stickleback from the Misty system, British Columbia, Canada. *Evolutionary Ecology Research* **11:** 905–919.

Ramsey J, Bradshaw HD, Schemske DW. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution* **57:** 1520–1534.

Räsänen K, Delcourt M, Chapman LJ, Hendry AP. 2012. Divergent selection and then what not: the conundrum of missing reproductive isolation in Misty Lake and stream stickleback. *International Journal of Ecology* **2012:** Article ID 902438. doi:10.1155/2012/902438.

Ridgway MS. 1982. Reproductive isolation between two co-existing populations of stickleback (*Gasterosteus*) in Enos Lake, Vancouver Island. MSc Thesis, University of British Columbia.

Rundle HD, Nagel L, Boughman JW, Schluter D. 2000. Natural selection and parallel speciation in sympatric sticklebacks. *Science* **287:** 306–308.

Schluter D. 1993. Adaptive radiation in sticklebacks: size, shape, and habitat use efficiency. *Ecology* **74:** 699–709.

Snowberg LK, Bolnick DI. 2012. Partitioning the effects of spatial isolation, nest habitat, and individual diet in causing assortative mating within a population of threespine stickleback. *Evolution* **66:** 3582–3594.

Sobel JM. 2010. Speciation in the western North American wildflower genus *Mimulus*. PhD Thesis, Michigan State University.

Southcott L, Nagel L, Hatfield T, Schluter D. 2013. Data from: Weak habitat isolation in a threespine stickleback (*Gasterosteus* spp.) species pair. *Dryad Digital Repository*. doi:10.5061/dryad.95qc4

Takami Y, Nagata N, Sasabe M. 2007. Asymmetry in reproductive isolation and its effect on directional mitochondrial introgression in the parapatric ground beetles *Carabus yamato* and *C. albrechti*. *Population Ecology* **49:** 337–346.

Taylor EB, Boughman JW, Groenenboom M, Sniatynski M, Schluter D, Gow JL. 2006. Speciation in reverse: morphological and genetic evidence of the collapse of a three-spined stickleback (*Gasterosteus aculeatus*) species pair. *Molecular Ecology* **15:** 343–355.

Taylor EB, McPhail JD. 2000. Historical contingency and ecological determinism interact to prime speciation in sticklebacks, *Gasterosteus*. *Proceedings of the Royal Society of London Series B, Biological Sciences* **267:** 2375–2384.

Vamosi SM, Schluter D. 1999. Sexual selection against hybrids between sympatric stickleback species: evidence from a field experiment. *Evolution* **53:** 874–879.

Via S. 1999. Reproducitve isolation between sympatric races of pea aphids. I. Gene flow restriction and habitat choice. *Evolution* **53:** 1446–1457.

Vines TH, Schluter D. 2006. Strong assortative mating between allopatric sticklebacks as a by-product of adaptation to different environments. *Proceedings of the Royal Society of London Series B, Biological Sciences* **273:** 911–916.

ARCHIVED DATA

Data deposited at Dryad (Southcott *et al*., 2013).