

Components of premating reproductive isolation in threespine stickleback

by

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Abstract

Reproductive isolation, the reduction in gene flow between two species, is central to the study of speciation. Many so-called isolating barriers may be involved in preventing two species from successfully hybridizing, but the importance of each barrier and the presence of interactions between them have not often been measured. I investigated habitat and mating isolation in the recently diverged benthic-limnetic species pairs of threespine stickleback (*Gasterosteus aculeatus* species complex). In these species, the males of which build nests and guard territories during the breeding season, there is a strong pattern of male habitat choice, with limnetics nesting in open habitats and benthics nesting under vegetation. When males were given a choice of nesting habitats in enclosures in an artificial pond, they almost always chose according to this pattern. However, females displayed no difference in probability of spawning with conspecifics in different habitats in no-choice mating trials, making habitat isolation at best a weak barrier to hybridization in the absence of interactions with other isolating mechanisms. From an existing dataset of mate choice trials, I calculated the contributions of body size differences and male nuptial colour to mating isolation. Isolation due to body size differences was strong in both species, but stronger in benthics. Isolation due to colour preference was negligible in benthics and moderately strong in limnetics. Generalized linear models indicated that interactions with other, unspecified species-specific traits increases isolation due to body size and, in benthics, due to colour. Together, these traits provide strong but incomplete premating isolation, resulting in an expected hybridization rate higher than that observed in nature, and thus are not sufficient in themselves to maintain the species pairs.

Preface

The research in this thesis was approved by the UBC Animal Care Committee (certificate #A07-0293).

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Chapter 1

General Introduction

Speciation in sexually reproducing organisms is a process of accumulation of barriers to interbreeding (Coyne and Orr 2004). In young species that retain at least some cross-fertility, it is possible to quantify the effect of different traits on reproductive isolation (RI). Studying contemporary RI does not necessarily tell us which barriers were involved in the initiation of divergence. However, it does tell us how species are maintained and how the removal of each barrier might change interspecific gene flow (Coyne and Orr 2004).

This approach to speciation research raises questions such as how many traits contribute to current reproductive isolation, whether certain kinds of barriers are stronger than others, and whether they interact (Ramsey et al. 2003; Martin and Willis 2007). But because there are few pairs of species for which multiple isolating mechanisms are well-known, many of these questions are unanswered, or have only been addressed in one or two well-studied clades (e.g. Coyne and Orr 1989, 1997; Ramsey et al. 2003; Kitano et al. 2009). It is apparent that there often are multiple barriers, but how many are necessary to maintain a species pair is unknown (Coyne and Orr 2004). Furthermore, merely testing for the existence of barriers is only a first step towards understanding how they maintain species. Measuring their strength—by how much they reduce gene flow between two species—is often difficult, but necessary for predicting the importance of a reproductive barrier.

The existence of many barriers to hybridization that differ in their current strength raises the question of whether some kinds of barrier are typically stronger than others. Again, the lack of exhaustive studies makes this a difficult question to answer. However, conventional wisdom holds that premating isolation (any form of RI that prevents heterospecifics from mating) is often important because it prevents later-acting barriers, such as hybrid sterility or inviability, from acting at all (Coyne and Orr 1989; Schemske 2000; Coyne and Orr 2004). For example, high hybrid inviability is largely irrelevant to current RI if two species simply never hybridize. In animals, the fact that sexual signalling traits often differ between sister species further suggests a strong role for premating isolation, particularly via mate choice (Coyne and Orr 2004). The idea that the sequence of reproductive isolating mechanisms in the life cycle of an organism affects the contribution of each mechanism to total RI has been central to studies of multiple isolating mechanisms (e.g. Coyne and Orr 1989; Ramsey et al. 2003).

Studies that characterize multiple isolating mechanisms have met with criticism for several reasons, but most significantly because they ignore potential interactions between barriers (Martin and Willis 2007; Lowry et al. 2008). All would acknowledge that some barriers must interact—for example, it is easy to imagine that adaptation to different habitats in the parental species, which could cause habitat isolation or immigrant inviability, could also make intermediate hybrids less fit than parentals (i.e. extrinsic hybrid inviability). However, the search for evidence of interactions has not been a priority, perhaps because measuring barriers alone is difficult in itself. Data on the existence of interactions, their contribution to RI, and the traits involved in them are sorely needed.

Before the general questions about contemporary isolating mechanisms can be addressed, we need to quantify more barriers in a variety of organisms. To that end, I studied two premating reproductive isolating barriers in a well-studied pair of species. Threespine stickleback (*Gasterosteus aculeatus* species complex) are an emerging model system for speciation research (Hendry et al. 2009). Repeated colonizations of freshwater habitats by marine stickleback have led to multiple recent speciation events (McPhail 1994; Hendry et al. 2009). Here, I focus on species pairs that occupy different habitats in several British Columbia lakes: a ‘limnetic’ form specialized for feeding on pelagic zooplankton and a ‘benthic’ form adapted to life in the littoral zone (McPhail 1992; Schluter 1993). While many potential barriers to hybridization are known in these species, their effects have not been compared and thus their role in maintaining the species pairs is not known. Data on the strength of these barriers are all the more urgently needed, as a recent, massive increase in hybridization has caused the collapse of one species pair (Taylor et al. 2006).

My goal was to determine the contributions of nest site choice and mate choice to premating reproductive isolation in one of these species pairs. I conducted experiments to confirm the existence of male nesting habitat preferences and to determine the effect of nest habitat on the probability of spawning. I analyzed a dataset of previous stickleback mate choice trials to quantify mating isolation, further dissect mate choice to assess the roles of body size and male nuptial colour in maintaining the species, and determine whether interactions among barriers exist and how much they alter reproductive isolation. These data will lay a foundation for further studies of the causes of reproductive character displacement between benthics and limnetics, the sequence of establishment of barriers to reproduction during the course of speciation, and the mechanisms causing the collapse of one species pair into a hybrid swarm.

Chapter 2

Components of Premating Reproductive Isolation in Threespine Stickleback

2.1 Introduction

The study of speciation was fundamentally changed by Ernst Mayr's biological species concept (BSC), which defines species as groups of actually or potentially interbreeding organisms. Despite persistent controversy over the BSC's validity and applicability, it is clear that interbreeding—or rather, the lack thereof—plays a critical role in speciation in sexually reproducing organisms (Coyne and Orr 2004). Thus, since the BSC was introduced, the enumeration of barriers to reproduction between sister species has been a large component of speciation research. While this approach does not necessarily yield information on the order in which these barriers evolved, and thus their importance during speciation, it does create a snapshot of how species boundaries are currently maintained.

Although there are few exhaustive studies of multiple isolating mechanisms between species, it is apparent that multiple barriers is the norm (Coyne and Orr 2004). This finding implies that no individual barrier need be complete, or even very strong, in order for gene flow to be almost zero. It is therefore important to quantify how much gene flow different barriers allow, in addition to simply identifying them, in order to understand their role in species persistence. However, the practice of investigating individual barriers separately, especially in an experimental rather than observational setting, has raised the objection that it fails to account for interactions among barriers (Martin and Willis 2007; Lowry et al. 2008). An individual's ability to pass through one isolating barrier might make it more or less likely to pass through another, so that the observed gene flow due to both barriers is different from the expected gene flow based on measurements of each barrier alone. Currently, data on whether barriers interact are rare, and interactions could both increase or decrease RI, so the extent of this problem is unknown. Thus, in addition to quantifying barriers, it would be useful to have an indication of whether each barrier is independent of other species recognition cues.

The number of species in which multiple isolating mechanisms have been characterized in detail is small. This is especially the case for animals. Below I describe two components of premating reproductive isolation

in the threespine stickleback (*Gasterosteus aculeatus* species complex), a fish that has already been a major source of insight into both the origin and maintenance of species. The ancestral threespine stickleback is a marine fish that occurs 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, several lakes contain two stickleback species, one of which feeds in the open water (the ‘limnetic’ species), the other of which inhabits the littoral zone (the ‘benthic’ species). Benthics are larger and deeper-bodied with fewer, smaller gill rakers, while limnetics are smaller and thinner with more gill rakers (McPhail 1992)—a pattern that has evolved in parallel in multiple lakes (Rundle et al. 2000; Boughman et al. 2005). These differences are thought to be adaptations to different foraging habitats: limnetics are zooplanktivores, while benthics feed on benthic macroinvertebrates (Schluter 1993).

In Paxton Lake, the most often studied of the species pair lakes, F1 hybrids between benthics and limnetics make up only 1–2% of the adult population (McPhail 1992) while 3–5% may be backcrosses (Gow et al. 2006). Hybrids are much more easily generated in the lab, with, for example, 10% of no-choice inter-specific mating trials in Nagel and Schluter (1998) resulting in spawning. Hybrids are intermediate between the parental species in morphology and, in the lab, appear to suffer no intrinsic inviability or sterility except possibly in the benthic backcross (McPhail 1992; Hatfield and Schluter 1999). However, in the wild, hybrids grow more slowly than the parental types on their respective habitats (Hatfield and Schluter 1999; Rundle 2002); thus, substantial extrinsic hybrid inviability exists.

The low frequency of wild hybrids combined with the apparent lack of intrinsic hybrid inviability implies that premating isolation exists and could be strong. Premating barriers could take a variety of forms, particularly since sticklebacks are well known for their complex courtship and parental care behaviour. Previous research has noted differences in nesting habitat, male nuptial colour, and courtship behaviour between benthics and limnetics, and some of these differences are associated with mate choice. Although some of the traits underlying mate choice are known, their contributions to RI have not been quantified, and the extent to which these traits interact is unknown.

Habitat isolation— isolation due to genetically determined occurrence in or preference for different habitats— has been cited as a potential premating reproductive barrier in stickleback (McPhail 1994; Hatfield 1995; Vamosi and Schluter 1999). Males of both species build and defend nests in the littoral zone, but prefer different microhabitats within it: limnetics nest on open sand or rubble, while benthics nest in dense vegetation (Ridgway 1982; McPhail 1994; Hatfield 1995). In the wild, nest site differentiation is apparent at both large and small spatial scales (pers. obs., M. Arnegard pers. comm., McPhail 1994). For example, a large macrophyte bed will contain mostly benthic nests, but limnetic nests will be found on any open patches within it—sand, rock, or even the surface of submerged logs. Furthermore, in a nest site choice experiment on lab-reared benthic, limnetic, and F1 hybrid males in enclosures in Paxton Lake, males of the two species consistently chose different habitats in the absence of heterospecific competitors (hybrids tended to nest in the open, like the limnetics; Hatfield 1995). Interestingly, the collapse of the species pair in Enos Lake was accompanied by denudation of the lake’s macrophyte beds by introduced crayfish, so habitat isolation may have been a factor maintaining separate species (Taylor et al. 2006). Yet macrophyte abundance is not

correlated with the occurrence of a species pair; for instance, Little Quarry Lake has very little submerged vegetation compared to the other lakes containing species pairs (Ormond 2011).

Male nest site choice alone does not contribute to assortative mating unless females also have habitat preferences. Nevertheless, female habitat preference has rarely been studied. Vamosi and Schluter (1999) noted that more gravid benthic than limnetic females were caught in traps set in vegetated parts of Paxton Lake, and vice versa for open areas; however, this pattern could reflect only a tendency of females to associate with conspecifics rather than habitat preference. Furthermore, females visit multiple male territories and can assess males in different habitats. Thus, the female component of habitat isolation may depend not on their occurrence in different habitats but on their willingness to spawn in different habitats. To date, there has been no experimental demonstration of female habitat preference in stickleback species pairs, and until it has been shown it is not clear whether nest habitat choice is truly a barrier to hybridization in this system.

Mating isolation (also called sexual, behavioural, or ethological isolation), which I define as reproductive isolation due to decreased preference for heterospecifics as mates in the absence of habitat cues, is classically regarded as a major component of RI in animals. Although Coyne and Orr (2004) note that this belief rested on assumptions about female preference that were rarely tested, mate choice has been extensively tested in the stickleback species pairs, and it is clear that mating isolation is substantial (Hatfield and Schluter 1996; Nagel and Schluter 1998; Boughman et al. 2005). Several mate choice cues have been identified, including courtship behaviour (Ridgway and McPhail 1984; Boughman et al. 2005), olfactory cues (Rafferty and Boughman 2006), body size differences (Nagel and Schluter 1998; Boughman et al. 2005), and male nuptial colour (Boughman 2001; Boughman et al. 2005).

I used data from some of these studies to estimate mating isolation and to further explore the contributions of two of these mate choice cues, size and colour. Benthic stickleback are typically larger than limnetics, and interspecific spawning occurs most often between fish of similar size, in both directions of hybridization (Nagel and Schluter 1998). Male nuptial colour—in particular, the male's red throat—is a classic sexual signalling trait that could feasibly contribute to preference for conspecifics. However, results in this area have been ambivalent and may vary among the different species pairs (Nagel and Schluter 1998; Boughman et al. 2005). Furthermore, the proportion of mating isolation that is caused by body size- or colour-related preferences has never been determined.

In this study, I quantified habitat and mating isolation between benthic and limnetic sticklebacks from Paxton Lake in order to compare their roles in maintaining the species pair. I experimentally assessed the male and female contributions to habitat isolation. I then used a dataset of previous mate choice trials to assess mating isolation, quantify the contributions of body size and nuptial colour to RI, and look for evidence of non-independence of components of mating isolation. These results contribute to our understanding of the maintenance of reproductive isolation between benthics and limnetics and suggest an initial approach to determining whether barriers interact.

2.2 Methods

2.2.1 Habitat isolation

Experimental design

To measure habitat isolation, I conducted two experiments: one to determine the probability of a male of each species nesting on two different habitats, and another to determine the probability of a female spawning with a male in either habitat. The second experiment placed females in enclosures only with males of the same species in order to measure only the component of habitat isolation that is independent of other species differences—in other words, habitat isolation independent of other premating reproductive barriers. Moreover, using only conspecific mate choice trials to determine spawning rates on different habitats allowed greater statistical power, given the limited number of fish that permits allowed us to remove from Paxton Lake, by both reducing the number of treatments and increasing the number of trials per treatment.

As in previous studies of stickleback mate preference (e.g. Nagel and Schluter 1998; Boughman 2001), I employed a no-choice design, in which a single male-female pair “chooses” whether to spawn in a defined time period. This design avoids aggressive interactions among males, which may interfere with spawning and confound female habitat preference with preference for other factors such as male size, dominance, or colour. No-choice experiments are also conservative with respect to detecting a preference; fish are more likely to show a preference if they have a choice of mates.

Fish

Benthic and limnetic stickleback were captured using minnow traps between March and May 2010 and in April 2011 in Paxton Lake, on Texada Island, British Columbia and transported back to UBC, where they were kept in 107 L aquaria separated by species. They were fed frozen bloodworms and mysis shrimp *ad libitum* daily and kept at 17°C with 12 hours of daylight initially, increased to 14 hours in May. Fish collected in 2010 were used for assessing male nest site choice and for a pilot project on female habitat preference (results not shown). Fish collected in 2011 were used for the female habitat preference experiment. Due to a lack of gravid wild-caught females, additional pond-reared females were included in the latter experiment. These fish were raised in single-species ponds and descend from wild-caught fish introduced into the ponds in 2008. Apart from the holding period in aquaria, these fish were handled identically to the wild-caught females.

Experimental enclosures

I constructed 23 1 m x 1 m window screen enclosures and put them in two ponds at UBC’s experimental pond facility in approximately 80 cm deep water. The bottom edges of the enclosure sides were buried in gravel and the top edges were suspended from nylon ropes across the ponds. As they came into breeding condition and whenever enclosures were available, males were chosen haphazardly, bagged, and transported

to the ponds. They were acclimated for at least 30 min by gradually adding pond water to the bags and released into enclosures. Starting in May, fish in enclosures were fed frozen bloodworms 2-3 times per week.

Male nest site choice

I reproduced Hatfield's (1995) experiment using wild-caught males in 12 of the enclosures. Half of each enclosure (chosen randomly) was covered with bladderwort (*Utricularia* sp.) collected from mature experimental ponds at UBC while the other half was left uncovered (sand and limestone gravel). Males were used in the pilot female preference experiment before being randomly reassigned to habitat choice enclosures, which were located in a different pond. 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. When a male built a nest, the nesting substrate was classified as "open", "vegetated", or "partially vegetated" (within 5 cm of the vegetation) according to Hatfield's (1995) methods. Males were then removed by trapping or dipnetting, 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 the enclosure could be reused.

Nest habitat and female mate preference

I used 23 enclosures in 2011 to examine how nest habitat influences the probability that a female will spawn. Enclosures were randomly assigned to vegetated or unvegetated treatments. The floor of vegetated enclosures was covered with stonewort (*Chara* sp.), while unvegetated enclosures had open sand and limestone gravel. A male of either species was randomly assigned to each enclosure and shown a captive gravid female each day, as above. These females came from several different populations or crosses depending on availability, but were conspecifics whenever possible and were never re-used for mating trials.

When a male had nested, a gravid conspecific female was acclimated to the pond in the same manner as the males for at least 30 min before being introduced into the enclosure. A pilot study that used both conspecific and heterospecific trials indicated that spawning never took place in the first 30 min after introduction of the female but was almost certain after 24 hours, even in hybrid pairings; thus, I ended trials after 2 hours. Each trial was filmed using Active WebCam recording software (Py Software) and a waterproofed Gearhead WC7351 webcam anchored in the enclosure about 50 cm from the nest. At the end of each trial, I removed the fish by dipnetting or trapping and checked the nest for eggs. If spawning did not occur, I checked if the female was ready to spawn by gently pressing on her abdomen and looking for eggs in the oviduct. As in the previous experiment, fish were euthanized, measured, and preserved after their removal from the enclosure.

Data analysis

I used Fisher’s exact test to test whether males of the two species chose different nest habitats. To look for differences in spawning probability in different habitats, I used a generalized linear model (GLM) of spawning versus female species and habitat. I also conducted another GLM in which I pooled results for the two species and reclassified the habitats as “own habitat” (the habitat in which conspecific males would normally nest, i.e. open for limnetics and vegetated for benthics) or “foreign habitat” (the habitat in which heterospecific males would normally nest). These and all subsequent analyses were conducted in the R statistical environment (version 2.11.1).

Comparing the strengths of multiple isolating barriers requires an equivalent formula for each barrier (Ramsey et al. 2003). Several methods have been proposed; I used equation 2.1 (J. Sobel, pers. comm.), which I modify in subsequent sections to apply it to different barriers.

$$RI = 1 - 2 \frac{H}{H + C} \quad (2.1)$$

In general, H represents the expected number of hybrids and C the expected number of pure-species individuals after a particular barrier has acted, assuming equal opportunities for each type of mating. Thus, $\frac{H}{H+C}$ is the proportion of the total population (in the case of premating isolation, of spawning events) that is hybrid; the proportion that has “passed through” a barrier. Equation 2.1 can be applied to any isolating barrier and measures the decrease in hybrids relative to the total population, unlike other methods of calculating RI which compare hybrids/heterospecific matings to pure species/conspecific matings (J. Sobel, pers. comm.). Its values can range from -1 for disassortative mating to 1 for perfect assortative mating, with RI = 0 indicating random mating. By convention, in animals, RI for premating barriers is calculated separately for females of each species (Coyne and Orr 2004).

To calculate habitat isolation (hereafter RI(habitat)), I used the following formula:

$$RI(\text{habitat}) = 1 - 2 \frac{s_{\text{veg}} p_{\text{het,veg}} + s_{\text{open}} p_{\text{het,open}}}{s_{\text{veg}} p_{\text{het,veg}} + s_{\text{open}} p_{\text{het,open}} + s_{\text{veg}} p_{\text{con,veg}} + s_{\text{open}} p_{\text{con,open}}} \quad (2.2)$$

The frequency of spawning on a given habitat, from the female habitat choice experiment, is given by s_{veg} and s_{open} . The values of p denote the proportion of conspecific or heterospecific males nesting on each habitat (i.e. $p_{\text{het,veg}}$ is the proportion of males nesting in vegetation that were heterospecifics and $p_{\text{het,veg}} + p_{\text{con,veg}} = 1$), as determined in the male nest site choice experiment.

2.2.2 Mating isolation

Dataset

To quantify mating isolation, I used existing data from no-choice mating trials within and between the benthic and limnetic species. Trials were conducted by Laura Nagel on wild-caught fish from Paxton and Priest lakes and by Todd Hatfield on lab-reared fish from Paxton Lake. Additional details of the experimental setup

can be found in Nagel and Schluter (1998) and Hatfield and Schluter (1996). Analyses were conducted on the following subsets of the data: Paxton fish only (Nagel data), Priest fish only (Nagel data), Paxton limnetics only (Hatfield data). No benthic females attempted to spawn in any of Hatfield's trials so reproductive isolation could only be calculated for limnetics. Additionally, the range of size differences in the Hatfield limnetic data was more restricted than in the Nagel dataset. Estimates of RI for Priest fish are included here as a comparison among independently-derived species pairs. However, Priest benthic males did not acclimate well to activity and had a lower spawning rate, even with conspecific females (Nagel and Schluter 1998, so interpretation of the Priest Lake data is difficult.

Mating isolation was calculated for females of each species as follows:

$$\text{RI(mating)} = 1 - 2 \frac{H}{H + C} \quad (2.3)$$

where H and C are the proportion of heterospecific and conspecific trials respectively that resulted in spawning (or nest entry in the case of Hatfield's data; females were prevented from spawning after nest entry). I resampled the data 1000 times and took the 2.5% and 97.5% quantiles of RI(mating) to estimate the bootstrap 95% confidence intervals.

Isolation due to body size

To calculate reproductive isolation due to body size differences, I modified equation 2.1 as follows:

$$\text{RI(body size)} = 1 - 2 \frac{\sum_i \frac{m_{i,\text{het}}}{m_{\text{het}}} d_{i,\text{het}}}{\sum_i \frac{m_{i,\text{het}}}{m_{\text{het}}} d_{i,\text{het}} + \sum_i \frac{m_{i,\text{con}}}{m_{\text{con}}} d_{i,\text{con}}} \quad (2.4)$$

Here, $d_{i,\text{het}}$ is the probability of a pair with size difference i being heterospecific, $m_{i,\text{het}}$ is the probability of a heterospecific pair spawning given size difference i , $d_{i,\text{con}}$ and $m_{i,\text{con}}$ are the equivalent values for conspecific pairs, and i ranges from the smallest to the largest size difference in the dataset (among both con- and heterospecific pairs). The two values m_{het} and m_{con} are the maximum spawning rates for heterospecific and conspecific pairs respectively; dividing by these values corrects for differences in spawning rates that are not due to body size. Simulations (unpubl. data) indicate that this method returns RI = 0 when there is no effect of size on mating isolation.

To estimate the contribution of size differences alone to RI, I used generalized linear models (GLM) to estimate $d_{i,\text{het}}$, $m_{i,\text{het}}$, $d_{i,\text{con}}$, and $m_{i,\text{con}}$ as functions of size difference. For females of each species, I first determined the relationship between body size difference and probability of spawning with con- or heterospecific males ($m_{i,\text{con}}$ and $m_{i,\text{het}}$). I used a GLM with binomial error and body size difference (absolute value of (male standard length - female standard length)) and male species as fixed effects without an interaction term to define $m_{i,\text{het}}$ and $m_{i,\text{con}}$ as functions of body size difference.

To determine whether other, unknown factors interact with body size in mate choice (i.e. whether RI(body size) is independent of other traits causing mating isolation), I compared these GLMs to GLMs

with the interaction between size difference and male species included. A significant interaction indicates that the relationship between size difference and probability of spawning, the slope of the linear predictor in a logistic regression, differs when a female is paired with a conspecific or heterospecific male. This difference implies that fish detect other differences between species and change how they react to size difference (change their “preference function”) in response to these cues. (The no-choice design makes it impossible to determine whether it is females responding differently to males, or males responding differently to females and causing further changes in female response.) Thus, they react differently to the combination of size difference and other, unspecified traits than they would to either alone.

I estimated $d_{i,\text{con}}$ by calculating the means and standard deviations of the lengths of males and females of each species from a larger dataset that included the trials studied here (Hatfield and Schluter 1996; Nagel and Schluter 1998; Rundle et al. 2000; Boughman et al. 2005; Albert and Schluter 2004). These values defined normal distributions from which I generated 1000 random pairs of sizes for each male species-female species combination. From these I fitted another GLM to derive the relationship between body size difference and the probability of a pair being conspecific; note that $d_{i,\text{het}} = 1 - d_{i,\text{con}}$.

These GLMs yielded logistic equations which I substituted for $d_{i,\text{het}}$, $m_{i,\text{het}}$, $d_{i,\text{con}}$, and $m_{i,\text{con}}$. I used the local maxima of the $m_{i,\text{het}}$ and $m_{i,\text{con}}$ functions (in the observed size range) as m_{het} and m_{con} . I calculated RI(body size) by summing the values of these functions for the range of body size differences in the dataset in intervals of 0.1 mm. I calculated RI(body size) using first the equations derived from GLMs without the size difference by male species interaction and then using the equations that included an interaction.

Isolation due to male nuptial colour

Equation 2.4 is generalizable to other categorical or continuous traits. The Nagel dataset contained subjective scores for the intensity of the red throat patch of males (1 = almost no colour, 5 = highest intensity red, in increments of 0.5). Using the same approach as the RI(body size) calculation, I estimated RI(colour) by conducting GLMs of female spawning versus male colour score and male species. In this case, $m_{i,\text{het}}$ represents the probability of spawning with a heterospecific of colour score i . Again, I compared models with and without interaction terms to investigate whether colour interacts with other species recognition cues during mate choice. Because colour scores were not normally distributed, I calculated the probability of a male being conspecific for each possible colour score ($d_{i,\text{con}}$) directly from the data, correcting for differences in sample size between species.

Finally, I calculated mating isolation due to body size and colour combined, to compare to the original measurement of RI(mating):

$$\text{RI(combined)} = 1 - 2 \frac{H_{\text{size}} H_{\text{colour}}}{H_{\text{size}} H_{\text{colour}} + C_{\text{size}} C_{\text{colour}}} \quad (2.5)$$

Here, $H_{\text{size}} = \sum_i \frac{m_{i,\text{het}}}{m_{\text{het}}} d_{i,\text{het}}$ and $C_{\text{size}} = \sum_i \frac{m_{i,\text{con}}}{m_{\text{con}}} d_{i,\text{con}}$ as calculated in equation 2.4. 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 I used only the functions without interaction terms for size and colour isolation for this estimate. The size difference by male species and colour by male species interaction terms may represent interactions with the same traits—thus, the interaction terms of the two components of mating isolation may not themselves be independent.

2.3 Results

2.3.1 Habitat isolation

Male nest site choice

Males of the two species differed significantly in nest site choice (Fisher’s exact test, $p < 0.0001$), with benthics preferring vegetation and limnetics favouring open sites (Table 2.1). These results closely resemble those of Hatfield (1995), who used lab-raised males in enclosures in Paxton Lake with natural vegetation. My replication of these results suggests that stickleback perceived the enclosures much as they would a natural lakebed.

Table 2.1: Male nest site frequencies.

	Open	Partial	Vegetated
Benthic	0	1	10
Limnetic	7	3	0

Nest habitat and female mate preference

Neither benthic nor limnetic females showed a lower probability of spawning on their “non-preferred” habitat (Table 2.2, Table 2.3). This was true both when the species were considered separately and when they were pooled. Limnetic females were overall less likely to spawn than benthics, but this result was not significant.

Table 2.2: 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
	Non-preferred	9	17

Table 2.3: Analyses of deviance results for GLMs of spawning versus species and nest habitat (benthics and limnetics considered separately) and of spawning versus nest habitat (both species pooled; habitat classified as “preferred” or “non-preferred”.)

Analysis	Factor	Df	Deviance	Residual Df	Residual Deviance	$P < \chi^2 $
Species separate	(null)			50	67.35	
	Habitat	1	0.033	49	67.32	0.86
	Species	1	0.01	48	67.31	0.92
	Habitat*Species	1	0.14	47	67.17	0.71
Species pooled	(null)			50	67.35	
	Habitat	1	0.16	49	67.19	0.69

Reproductive isolation due to habitat choice

Because several males nested in “partially vegetated” habitat but spawning rate in partial vegetation was not measured, I calculated RI(habitat) in three different ways: including “partial” with the preferred habitat (i.e. vegetated for benthics, open for limnetics), including “partial” with the non-preferred habitat, and splitting the “partial” nests equally between preferred and non-preferred habitats (Table 2.4). Regardless of the calculation method, RI(habitat) is close to zero (ranging from -0.15 to 0.17), and there is no consistent difference between benthic and limnetic females.

Table 2.4: Reproductive isolation due to nest habitat choice. RI(habitat) is calculated in three different ways: first, counting partially vegetated nests as nests in vegetation; second, counting partial as open; third, counting half the partial nests as vegetated and half as open. Bootstrapped 95% confidence intervals, where calculated, are given in parentheses.

Species	RI(habitat)		
	Partial = Vegetated	Partial = Open	Partial = Split
Benthic	-0.18 (-0.74,0.44)	0.13 (-0.53,0.74)	-0.03
Limnetic	0.29 (-0.20,0.73)	0.01(-0.50,0.56)	0.14

2.3.2 Mating isolation

Mating isolation, the reduction of gene flow due to preference for conspecific mates, was high in fish from both Paxton and Priest lakes (Table 2.5). It was also asymmetric, but in a different direction in the two lakes: isolation was higher in benthics in Paxton but higher in limnetics in Priest. Mating isolation was lower in the lab-raised Hatfield limnetic dataset than in the wild-caught Nagel limnetics.

Body size

Probability of spawning decreased with absolute body size difference for both benthic and limnetic females (Figure 2.1; only the results for Paxton Lake fish are shown). The probability of a pair being conspecific for a given size difference is shown in Figure 2.2 for Paxton fish. RI(body size) without a size difference by

Table 2.5: Mating isolation for all subsets of the dataset. Bootstrapped 95% confidence intervals are shown in parentheses where calculated. Fish were either wild (Nagel data) or lab-raised (Hatfield data).

Source	Lake	Species	RI(mating)
Nagel	Paxton	Benthic	0.86 (0.59,1)
		Limnetic	0.58 (0.23,0.89)
	Priest	Benthic	0.61 (-0.4,1)
		Limnetic	0.73 (0.4,1)
Hatfield	Paxton	Limnetic	0.33 (0.086,1)

male species interaction is substantial, but lower than RI(mating) in all cases but the Paxton limnetics (Table 2.5), and is in fact negative (implying disassortative mating by size difference) in Priest benthics (Table 2.7). Models with a size difference by male species interaction term were as good as or better than the models without an interaction (Table 2.6), implying that size difference is treated differently as a mate choice cue in conspecific and heterospecific trials. RI(body size) calculated from models with an interaction term was higher in all cases than when the interaction was not included. Although RI(body size) with an interaction was also higher than RI(mating), it fell within the 95% confidence intervals (Table 2.5).

Table 2.6: Comparison of GLMs of spawning probability versus absolute value of size difference and male species with and without an interaction term. A difference between AIC values of <2 indicates substantial support for both models (in both cases of $\Delta AIC < 2$, the without-interaction model had higher support). Asterisks indicate significant interaction terms at the $p = 0.05$ level. A significant interaction term indicates that females assess body size differently when courted by conspecific compared to heterospecific males.

Source	Lake	Species	ΔAIC	p-value of interaction term
Nagel	Paxton	Benthic	2.98	0.026*
		Limnetic	3.85	0.016*
	Priest	Benthic	0.77	0.096
		Limnetic	8.78	0.001*
Hatfield	Paxton	Limnetic	1.49	0.47

Table 2.7: Reproductive isolation due to body size calculated from models with and without a size difference by male species interaction term.

Source	Lake	Species	RI(body size), without interaction	RI(body size), with interaction
Nagel	Paxton	Benthic	0.45	0.87
		Limnetic	0.68	0.75
	Priest	Benthic	-0.22	0.92
		Limnetic	0.37	0.93
Hatfield	Paxton	Limnetic	-0.35/0.26*	0.68/0.64*

*Hatfield's trials using lab-raised limnetics spanned a much smaller range of size differences than is found in nature (0-10 mm vs. 0-31 mm). The first value is calculated for the range size differences expected in nature; the second, for the range used in the experiment.

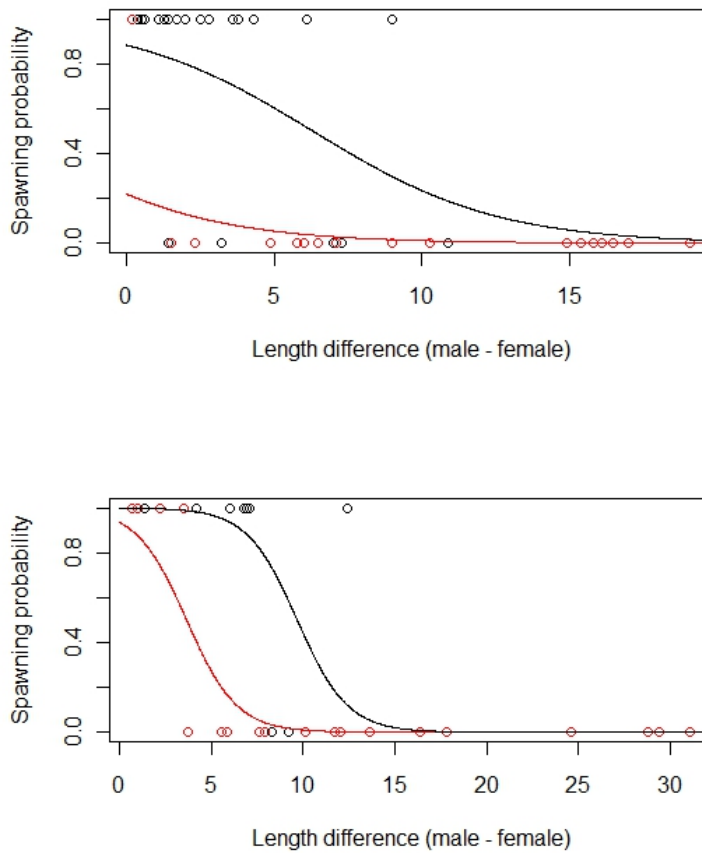


Figure 2.1: Absolute value of body size difference versus probability of spawning for benthic (top) and limnetic (bottom) females. Red circles represent heterospecific trials; black represents conspecific trials. Curves (red and black for hetero- and conspecifics respectively) are the probability of spawning for a given size, or $m_{i,het}$ and $m_{i,con}$. Curves were generated from logistic regressions without including a size difference by male species interaction term.

Male nuptial colour

Among Paxton Lake fish, male limnetics had higher colour scores than benthics (Figure 2.4). Benthic females showed little colour preference while limnetics spawned only with the males with the highest red intensity (Figure 2.3). Accordingly, isolation due to nuptial colour without a male colour by male species interaction was weak in Paxton benthics and fairly strong in limnetics (Table 2.9). A similar pattern was found in Priest fish, in which $RI(\text{colour})$ was slightly negative for benthics and positive for limnetics. Models with a colour by male species interaction had substantial support compared to those without one (Table 2.8) and greatly increased $RI(\text{colour})$ for benthics in both lakes (Table 2.9). However, the interaction did not change $RI(\text{colour})$ for Paxton limnetics and slightly decreased it for Priest limnetics.

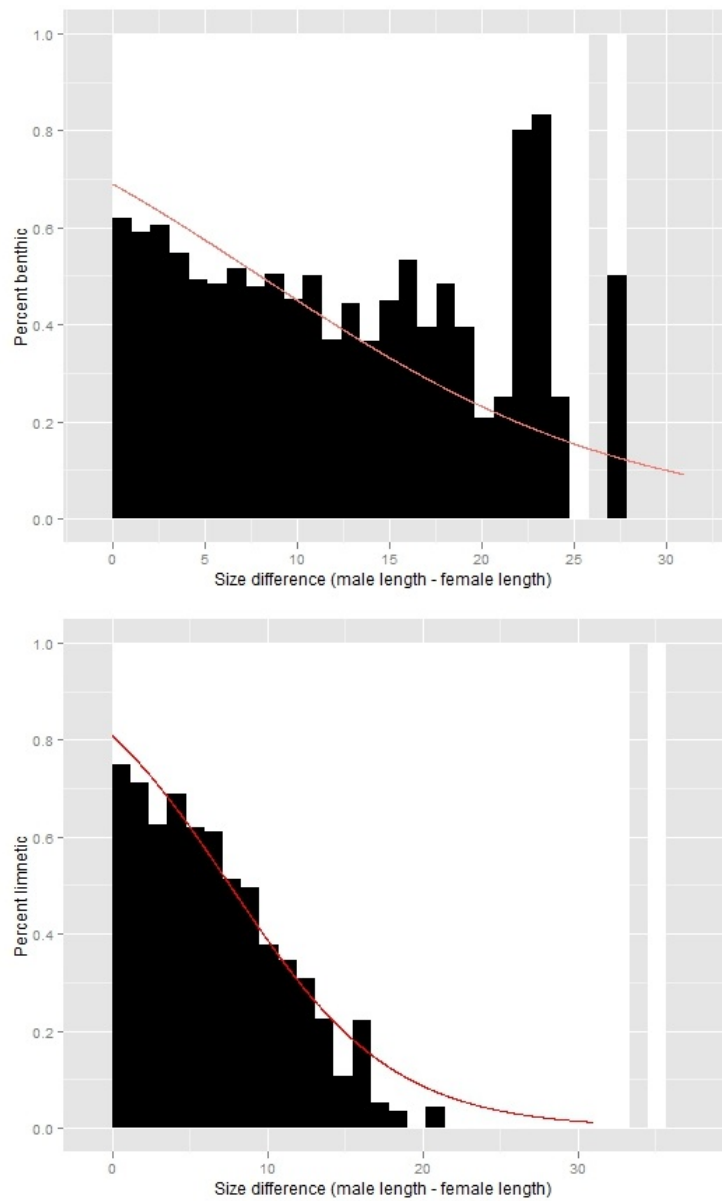


Figure 2.2: Proportion of males that are conspecific in relation to the absolute value of body size difference from benthic (top) and limnetic (bottom) females. Lines represent the probability of being conspecific for a given size difference, or $d_{i,\text{con}}$. Curves were generated from logistic regressions of cross type versus size difference.

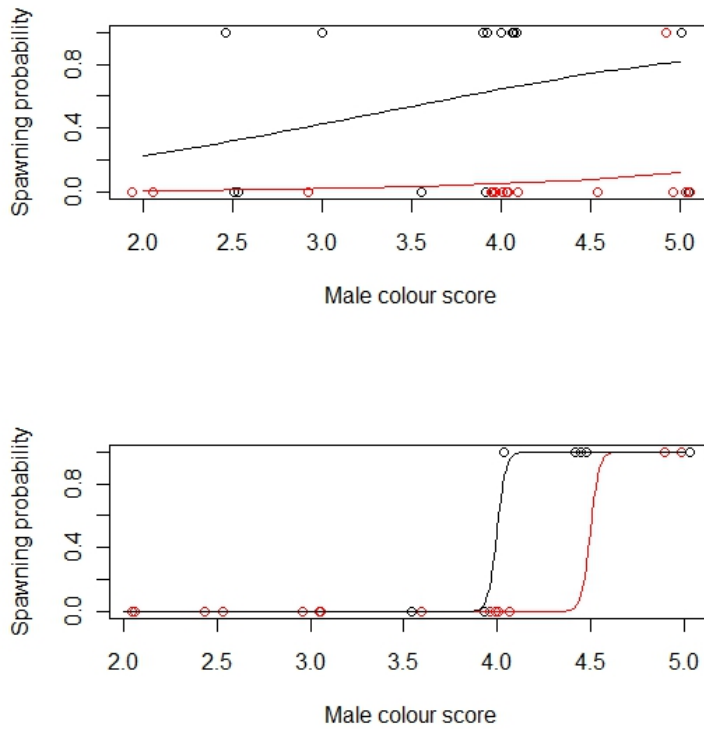


Figure 2.3: Probability of spawning versus male colour score for Paxton benthic (top) and limnetic (bottom) females. Red circles represent heterospecific trials; black represents conspecific trials. Curves (red and black for hetero- and conspecifics respectively) are the probability of spawning for a given colour, or $m_{i,het}$ and $m_{i,con}$. Curves were generated from logistic regressions without a colour by male species interaction term.

Table 2.8: Comparison of GLMs of spawning probability versus male colour score and male species with and without an interaction term. A difference between AIC values of <2 indicates substantial support for both models. Asterisks indicate significant interaction terms at the $p = 0.05$ level. A significant interaction term indicates that females assess male colour differently when courted by conspecific compared to heterospecific males. All analyses were performed on fish in the Nagel dataset only.

Lake	Species	ΔAIC	p-value of interaction term
Paxton	Benthic	0.35	0.200
	Limnetic	2.00	.999
Priest	Benthic	2.04	0.045*
	Limnetic	1.96	0.841

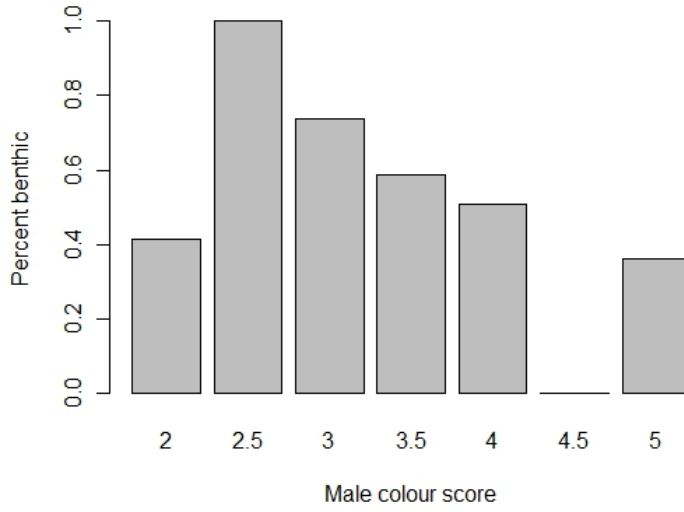


Figure 2.4: Proportion of Paxton males of a given colour score that were benthic, or $d_{i,con/het}$, corrected for differences in numbers of trials involving benthic and limnetic males.

Table 2.9: Reproductive isolation due to male nuptial colour calculated from models with and without a male colour by male species interaction term.

Lake	Species	RI(colour), without interaction	RI(colour), with interaction
Paxton	Benthic	0.08	0.57
	Limnetic	0.68	0.68
Priest	Benthic	-0.11	0.15
	Limnetic	0.48	0.38

Total premating isolation

There was no relationship between male length (or size difference) and colour score (results not shown). I therefore treated size and colour as independent components of mating isolation and calculated their combined strength from the without-interaction estimates of each barrier. Although RI(combined) is smaller than the observed RI(mating) for benthic females and larger for limnetic females (Table 2.10), it falls within the confidence intervals of RI(mating) (Table 2.5).

Table 2.10: Reproductive isolation due to both body size differences and male nuptial colour.

Lake	Species	RI(combined)
Paxton	Benthic	0.52
	Limnetic	0.93
Priest	Benthic	-0.32
	Limnetic	0.72

2.4 Discussion

2.4.1 Habitat isolation

Despite strong habitat preferences in males of both species, females did not show a lower probability of spawning on the non-preferred habitat. The small sample size of this study limited its power to detect subtle habitat effects; however, an overlooked weak habitat preference would still create only moderate reproductive isolation.

Male habitat choice in my experiment parallels previous results for both Paxton and Enos Lake fish (Ridgway 1982; McPhail 1994; Hatfield 1995). Since males were not competing with heterospecifics in my trials, their choices in this experiment represent actual habitat choice and not exclusion from better (e.g. more sheltered) nest sites. This study used primarily wild-caught fish, and thus habitat preferences could be genetically based or learned, but Hatfield's (1995) study using lab-reared fish strongly suggests the preference is genetically based.

Male habitat choice is not a direct outcome of competition between benthic and limnetic males for nest sites, because males were tested in isolation. Why the strong habitat preference evolved in males without female habitat choice is unknown, but it may be the result of a genetic correlation with other differences between the species favoured by selection, such as trophic niche. Male habitat choice could also be an evolved response to past competition between benthic and limnetic males—i.e. character displacement. However, the results of the female habitat preference experiment appear to rule out habitat-based mate choice by females, whether due to predation risk to females in different habitats or preference for conspicuous versus inconspicuous courtship displays, as a major selective force driving nest habitat divergence.

My experiment did not look for an interaction between nest habitat and male species, and thus it is possible that habitat combined with other species differences between males causes reproductive isolation. In other words, nest habitat alone is not a species recognition cue—females did not reject males in the non-preferred habitat—but preference for other trait(s) that differ between benthics and limnetics could make females even less likely to spawn with a heterospecific in the non-preferred habitat than would be expected based on the effects of habitat preference and the other trait(s) alone. Mate choice trials using heterospecifics in both habitats, when compared to conspecific trials like those I conducted, could be used to identify such an interaction. Another way to look for interactions between habitat choice and other barriers is to investigate correlations between traits that are known to contribute to RI, such as size difference or male nuptial colour, and probability of spawning on different habitats within species. (No such relationship exists for size difference in my data, although this result must be considered preliminary because the range of sizes tested is much smaller than the possible range for heterospecific trials.)

Habitat choice has attracted attention as a potentially important barrier in part because the collapse of the species pair in Enos Lake happened simultaneously with a loss of vegetation due to introduced crayfish (Taylor et al. 2006). If habitat choice was a strong isolating barrier in Enos, homogenization of the available nesting habitat could have enhanced gene flow between benthics and limnetics. Although my results

suggest a limited role at best for habitat isolation in the Paxton Lake species pair, there may be reason to believe that its role in the former Enos Lake pair was different. Male Enos benthics had black rather than red and blue nuptial colouration (Ridgway 1982), a trait associated with light environment in stickleback (Reimchen 1989; Boughman 2001). If the littoral zone in Enos was spectrally different from that in Paxton, an interaction between habitat and nuptial colour could have caused reproductive isolation. However, the disappearance of black nuptial colour from the Enos hybrid swarm (A. Lackey, pers. comm.), and the collapse itself, makes this suggestion almost impossible to test.

2.4.2 Mating isolation

I used previously published stickleback mate choice data to determine mating isolation—the reduction in hybridization due to mate choice—and the components of it that result from preferences for body size differences and male nuptial colour. Although mating isolation is well-known in stickleback species pairs, its strength has not previously been estimated. Mating isolation is strong in both directions in both lakes, but in Paxton Lake it is stronger in benthics than limnetics. This is expected because male limnetics are less likely to court benthic females, which are known to consume eggs already in the male's nest (Albert and Schluter 2004), than male benthics are to court limnetic females. The asymmetry between female species lies in the opposite direction in Priest Lake fish; however, the overall reluctance of Priest benthic males to spawn in the lab (even with conspecifics) makes this pattern likely an artifact of captivity (Nagel and Schluter 1998) and may also explain the wider confidence intervals on this estimate.

Isolation due to body size differences alone is strongest in Paxton limnetics, but when the interaction of size and species is considered, it is stronger in benthics. In all cases, the interaction adds considerable isolation, meaning that fish are more sensitive to body size differences when the potential mate is a heterospecific than when conspecific. One explanation is that another size-related trait, not standard length, is the actual cue used in mate choice. For example, Baube (2008) used 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*; lateral projection area had previously been shown to correlate more strongly with male behaviour than did standard length. Since 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 spawning with a similarly-sized heterospecific would be even lower if area is the “true” body size cue than if we assume standard length is.

RI(body size) in Priest benthic females is the only case of (weak) disassortative mating by size. This finding is surprising, as the only spawning event between a Priest benthic female and limnetic male occurred between two fish of similar size. Again, this result may be an artifact of the low overall spawning rate of Priest benthics (Nagel and Schluter 1998). Only four out of 20 conspecific trials resulted in spawning, one of which had a size difference of 24.5 mm, the largest value in the Priest benthic dataset.

Isolation due to male nuptial colour is stronger in limnetics than in benthics in both lakes, and in fact RI(colour) in benthics is nearly negligible. This finding is similar to that of Boughman et al. (2005), who

noted than limnetic females strongly prefer brightly coloured males but benthic males tend to have duller throats than limnetics. Benthic females do not have a strong preference for brighter throats in conspecific males, explaining the low RI(colour) without a colour by male species interaction term, but, when they do spawn with limnetic males, tend to choose the brightest of them (Boughman et al. 2005), in keeping with the noticeable increase in RI(colour) when an interaction term is considered. Although the relationship between spawning and male nuptial colour has been studied previously, this is the first time the strength of reproductive isolation resulting from it has been quantified. This measurement allows us to predict interspecific gene flow based on differences in this trait alone.

In limnetics, body size and male colour make similar contributions to mating isolation, while RI(body size) is much stronger than RI(colour) in benthics from both lakes. It is interesting to consider whether size and colour combined explain most of the observed mating isolation. In limnetics from both lakes, the two traits combined produce as strong RI as the observed RI(mating) alone. However, the combination is weaker than RI(mating) for benthics, and therefore other mate choice cues—perhaps behaviour or olfaction—also cause mating isolation in benthic females. Additionally, it is not clear whether the negative RI estimated for Priest benthic females actually represents disassortative mating by size and colour or is due to the compounding of two non-significant but negative effects of the individual barriers; the previously noted low spawning rate of Priest benthic males with conspecifics may obscure the true pattern.

Another interesting comparison is between the predicted gene flow based on RI(mating) and the observed hybridization rate in the wild. The $\frac{H}{H+C}$ term in equation 2.1 represents the fraction of offspring of females of a given species that are hybrid if only the barrier in question operates and equal opportunities for each cross type exist. For Paxton fish (using the Nagel data), this value is .07 for benthics and .21 for limnetics. If we assume equal population sizes and clutch sizes of the two species (admittedly an unlikely scenario), and RI(mating) is the only isolating barrier, the next generation would contain 14% hybrids. This is substantially higher than the at most 5% hybrids observed in nature (McPhail 1992; Gow et al. 2006). 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 seems likely that other isolating mechanisms are important for maintaining benthics and limnetics as distinct species.

2.4.3 Interactions among isolating barriers

The potential for different isolating mechanisms to interact has been noted often (e.g. Martin and Willis 2007; Lowry et al. 2008) but rarely studied. This study quantified habitat and mating isolation independent of other mate choice cues. By using only conspecific trials, I assessed habitat isolation in the absence of other species-specific mate choice cues. This approach could be applied to other barriers and to other systems, but only when the trait in question can be manipulated to cover the full range of conspecific and heterospecific values—e.g. here, males were forced to nest in their preferred or “heterospecific” habitat.

My approach to looking for interactions with size differences and male nuptial colour was to show that females’ responses to these traits differed depending on the male species. Such a finding means that the

presence of other, unspecified species-specific traits alters that component of mating isolation—different components of mating isolation interact. In stickleback, these interactions act mostly to strengthen isolation due to size differences and male nuptial colour. However, this approach does not provide evidence as to which traits might cause these interactions. Furthermore, because only spawning rates, not hatching success or offspring survival or fertility, were investigated, all these interactions involve only premating barriers. Whether these barriers also interact with postmating mechanisms is still unknown.

Interactions among isolating barriers could increase or decrease total reproductive isolation. An interaction between body size and habitat choice for both males and females seems plausible. The smallest fish are most vulnerable to predation by insects in vegetation (Marchinko 2009), so only the largest limnetic females may even consider a benthic male in nature. Meanwhile, territorial interactions may force the smallest benthics to nest in the open (A. Lackey, pers. comm.), where, because of their size, they are even more likely to hybridize. Both of these interactions would decrease reproductive isolation.

2.4.4 Conclusion

I investigated several aspects of premating reproductive isolation between sympatric stickleback species. Despite strong divergence in male nest site preference, habitat isolation between benthics and limnetics is weak. Based on previous studies, mating isolation was found to be substantial and depends strongly on differences in body size and male nuptial colour. However, it is not sufficient to maintain the species pairs alone. Mating isolation is also asymmetric, particularly in the case of male colour, which has a stronger effect on mate choice in limnetic females than in benthics. Interactions among isolating barriers increase premating isolation to varying extents, but further study is needed to determine what traits cause these interactions. These results suggest that strong premating barriers maintain the stickleback species pairs but must work in concert with other isolating mechanisms.

Chapter 3

General Discussion

Identifying and quantifying different barriers to interbreeding are important steps in studying both the origin and maintenance of species. I measured two such isolating barriers, habitat and mate choice, in a threespine stickleback species pair. Although the existence of these barriers was previously either known or suspected, this study quantified their strength for the first time. Additionally, I took two novel approaches to the potential for interactions among barriers. First, I used only conspecific mating trials to evaluate habitat choice in the absence of other species differences. Second, I used generalized linear models to evaluate whether male species identity modifies the relationship between female spawning probability and size difference or male colour, which would indicate interactions among traits influencing mate choice.

The measurements of habitat and mating isolation provided by this study will affect our view of the evolution and conservation of stickleback species pairs. Most surprising is the finding that habitat isolation is weak despite strongly divergent nest site preferences in males. Females seem not to modify their spawning probability depending on the habitat of the male's nest. Therefore, habitat isolation alone cannot make a major contribution to the maintenance of the Paxton Lake species pair. Notably, the absence of a strong female habitat preference also implies that it probably was not a major selective force causing divergence in male nest site preference between the species, or reducing interspecific gene flow in the early stages of speciation.

My habitat choice experiment aimed to estimate habitat isolation in the absence of any other species differences by using only conspecific pairs. Thus my results indicate that, if habitat choice has any role in maintaining the species pairs, it is because of an interaction with other mate choice cues rather than as a strong barrier per se. This finding is unexpected, because the collapse of the species pair in the Enos Lake coincided with a loss of macrophyte cover due to introduced crayfish, which suggests that the homogenization of the lakebed removed a major component of reproductive isolation. Nevertheless, my findings do not mean that macrophyte cover in the species pair lakes is unimportant from a species maintenance or conservation perspective. In nature, the barriers and their interactions together determine the hybridization rate, and loss of appropriate nest habitat could still be disastrous if there are interactions between other barriers and habitat.

My investigation of data from previous studies confirms the role of body size and male nuptial colour in reproductive isolation, but this is the first time that their contribution to RI has been quantified. Isolation due to the combined effect of differences in these two traits is as strong as the observed mating isolation between these species. However, these contributions are not the same in both species, with colour alone having a limited effect on RI for benthic females. In addition, the strong effects of size and colour do not necessarily mean that they are the only major components of mating isolation. Effects of olfactory cues, behaviour, and other morphological traits are known (Ridgway and McPhail 1984; Albert and Schluter 2004; Boughman et al. 2005; Rafferty and Boughman 2006), and these effects could contribute to RI alone or via interactions with size or colour.

Few studies, if any, have investigated interactions among reproductive isolating mechanisms. I looked for the presence and strength of interactions between components of mating isolation by comparing additive models to models with interaction terms between specific traits and male species. A significant interaction would indicate that other, unspecified factors interact with the trait in question to change RI. There is, indeed, evidence that such interactions exist and increase RI, but this approach could not identify the traits that cause the interactions. However, identifying interactions and determining how much they affect RI are necessary first steps in determining their prevalence and importance. The next step, identifying the traits that cause the interactions, will also facilitate the investigation of interactions with other isolating mechanisms. For example, once we know that the probability of spawning with a heterospecific male correlates with size difference, we could look for correlations between (parental) size difference and habitat choice, time of spawning, hatching success, progeny growth or survival, or progeny fertility. Measuring correlations between the traits that are known to contribute to one barrier and the probability of passing through other barriers would enable the study of interactions between pre- and postmating barriers, something that my calculations could not assess.

Ultimately, understanding the traits that contribute to RI is also one approach to identifying the genes that cause RI. Finding these genes would allow us to measure RI on a per-gene level. The idea that a single gene can have an effect on interspecific gene flow links Ernst Mayr's biological species concept to more recent views of a species' genome as porous, with neutral alleles readily shared among interbreeding taxa. This knowledge would also directly contribute to our understanding of the genetic architecture of speciation with gene flow—how many genes, and with what effects, are necessary to maintain species boundaries.

As noted earlier, identifying contemporary RI due to various barriers does not necessarily explain whether those barriers were important in earlier stages of speciation. However, barriers that do not play a role now are very unlikely to have contributed to divergence (Coyne and Orr 2004). Thus, it seems that habitat isolation by itself was at best a minor player in stickleback speciation. The components of mating isolation that I studied make, in most cases, a large contribution to present-day reproductive isolation, and hence could also have been important in the past. Future work on these traits in the “ancestral” stickleback, the marine population, could be informative as to how they evolved when the species pairs were formed. For example, there is already evidence for character displacement of male nuptial colour between benthics and limnetics from comparisons with solitary populations (Albert and Schluter 2004). Studying male colour and

female preference for it in marine fish would help us understand how much these traits changed during the initial colonization of freshwater, when proto-benthics and limnetics were allopatric from one another.

One way in which the present strength of isolating barriers could be informative about the early stages of speciation is in the case of interactions among traits that cause mating isolation, the presence of which could be evidence for reinforcement. More specifically, divergence in multiple mate choice cues strictly due to genetic correlation with ecological divergence would be expected to produce an additive effect on RI. An interaction among mate choice cues implies that the effect of multiple cues is more or less than additive, and thus that sexual selection based on one trait is altered in the presence of another. This response suggests active species recognition rather than sexual selection against low-quality mates, something that could only be the result of reinforcement or direct selection on mating preferences.

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