

SPECIATION IN STICKLEBACK FISH:
INTERACTIONS BETWEEN SEXUAL SELECTION AND ECOLOGY CAN MAKE OR BREAK SPECIES

By

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ABSTRACT

SPECIATION IN STICKLEBACK FISH: INTERACTIONS BETWEEN SEXUAL SELECTION AND ECOLOGY CAN MAKE OR BREAK SPECIES

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We investigate how ecology and sexual selection interact to shape the speciation process using threespine stickleback species pairs (*Gasterosteus* spp.). We examine how reproductive isolation can evolve in the forward direction and break down through reverse speciation. We first focus on one reproductive barrier, sexual isolation, which reduces gene flow between species through differences in mate preferences and mating signals and is likely important for species formation and maintenance. We provide the first evidence that sexual isolation has been lost in a species pair that has recently collapsed into a hybrid swarm. We also show that preferences females have for conspecific mates and the traits they use to distinguish con- and heterospecific males contribute to this loss. This work highlights the fragility of isolation between young species pairs and considers the role of sexual isolation in speciation.

Second, we explore how sexual selection and ecological differences can contribute to speciation via male competition. We find that selection via male competition in one habitat would promote trait divergence and reproductive isolation, while in another habitat, selection would hinder divergence. Other behavioral mechanisms in male competition that might promote divergence, such as avoiding aggression with heterospecifics, are insufficient to maintain separate species. This work emphasizes the importance of mating habitats in male competition for both sexual selection and speciation.

Third, we explore how environmental differences might mediate the expression, and current maintenance, of sexual isolation. Surprisingly, we find that the expression of female discrimination was fairly insensitive to habitat, despite the significance of habitat differences for sexual isolation to evolve. Female sensitivity to habitat was only shown by the ecotype being subsumed by hybridization, suggesting this plasticity may have contributed to reverse speciation. Also, habitat sensitivity in the expression of male courtship would further erode sexual isolation. Thus, environmental differences may play very different roles in the evolution versus maintenance of sexual isolation and the forward versus reverse process of speciation.

Lastly, we ask how patterns of reproductive isolation in stickleback species pairs that represent early to late stages of the speciation process reveal how isolation might evolve both in the forward and reverse directions. The types of barriers that contribute most to isolation differ along the speciation continuum, thus the primary barriers that initiate speciation differ from those that complete it. Premating isolation, especially habitat and sexual isolation, likely plays an especially important role in initiating speciation. The loss of sexual isolation in reverse speciation and absence in halted movement along the speciation continuum highlights its potential importance for movement along the speciation continuum. Intrinsic postmating isolation is likely necessary to complete and maintain speciation. Asymmetrical barriers may reveal selection that acts differently on each taxon and could predict the likelihood of forward, halted, or reverse movement along the continuum as well as the direction of introgression if reversal does occur. This study, and others that look at most or all potential reproductive barriers in systems that span the speciation continuum, can generate important insights into how new species evolve, what maintains them, and when and how they might collapse.

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INTRODUCTION

How do new species arise? This question has been of intense interest since Darwin first wrote about “that mystery of mysteries” (1859). Species are defined as populations that can potentially interbreed but do not because of barriers to reproduction (Mayr, 1942).

Understanding the formation of new species, i.e. the speciation process, is important for determining what affects biodiversity. This aim becomes even more important considering the potential for global change as well as recent and impending losses of species (Rhymer & Simberloff, 1996; Seehausen, 2006).

Great strides have been made in our understanding of speciation, but many questions still remain. A primary question is: how does reproductive isolation evolve? To answer this, we should explore the forms of isolation that initiate speciation and reduce gene flow between taxa as well as isolation that completes speciation and stops gene flow between taxa. We should also study the evolutionary forces that underlie different forms of isolation, and how quickly and extensively these evolutionary forces can result in isolation.

There is particular interest to determine the role of ecology in speciation. To what extent do environmental differences affect isolation and divergence between taxa? In other words, how much of the speciation process can be explained by ecology? The importance of environmental differences may be pervasive during the speciation process (Schluter, 2001; Sobel et al., 2010). Indeed, environmental differences may facilitate speciation under a broad range of circumstances: from complete spatial isolation in allopatry to complete overlap in sympatry, and from where natural selection plays a primary role in divergence to where sexual selection plays a primary role.

Sexual selection, both on its own and in interaction with ecology, may be particularly important for speciation (Panhuis et al., 2001; Maan & Seehausen, 2011). Sexual selection can directly cause rapid divergence in female mate preferences and male mating traits between taxa, which in turn may result in sexual isolation (Lande, 1981; Lande & Kirkpatrick, 1988; West-Eberhard, 1983; Ritchie, 2007). Questions still remain about how much sexual selection may drive the speciation process as well as how natural and sexual selection interact during speciation (Panhuis et al., 2001; Maan & Seehausen, 2011).

Research on sexual selection in speciation has focused primarily on female mate choice and neglected the role of male competition in speciation (Seehausen & Schluter, 2004; Dijkstra & Groothuis, 2011; Qvarnstrom et al., 2012). Despite the known importance of male competition within populations for rapid evolutionary change (Andersson, 1994), we know little about how male competition may act between populations and how environmental differences might play a role in this process. Because male competition can sometimes oppose female mate choice (Candolin, 2004; Sih, 2002; Hunt et al., 2009), we need to study both female mate choice and male competition to correctly predict evolutionary change and understand the causes, consequences, and interactions between these forms of sexual selection in the context of speciation.

In addition to thinking about how reproductive isolation evolves in the forward direction, as taxa diverge to become new species, we also need to consider the reverse process, where isolation erodes and distinct species are lost. On a basic level, we can determine how quickly and easily species might be lost and which factors, such as environmental conditions and the strength and types of isolation, play a major role in that loss. On an applied level,

understanding what facilitates and hinders the reverse process, may help us to limit the loss of species. This is especially important as human impacts on habitats and population distributions become more severe and widespread (Rhymer & Simberloff, 1996; Seehausen, 2006).

To answer questions about the forward and reverse speciation process and the roles of ecology and sexual selection, I use stickleback fish, which are a model system of ecological speciation (McKinnon & Rundle, 2002; Schluter, 1998) in addition to a well-cited example of the interactions between sexual selection, ecology, and speciation (Maan & Seehausen 2011; Boughman 2002). Much of my work focuses on the limnetic-benthic species pairs of sticklebacks that live in freshwater lakes in British Columbia (McPhail, 1994). Seven pairs have evolved in parallel (Boughman, 2006; Gow et al., 2008) and thus effectively represent replicate populations. Divergence between limnetic and benthic fish has been very rapid, occurring over the past 13,000 years (McPhail, 1994; Bell & Foster, 1994). Thus, current isolation in these species pairs was likely important for the later stages of the speciation process. I also broaden my study of stickleback fish to taxa pairs that span early to late stages of the speciation process, including lake-stream pairs and anadromous-freshwater pairs, that likely evolved in less than 15,000 years (McPhail 1994; Bell & Foster 1994), as well as the oldest pair of stickleback species, the Japan Sea-Pacific Ocean pair, that likely diverged over the past 1.5 million years (Kitano et al., 2007). Throughout my research, I have also taken advantage of the recent collapse of one limnetic-benthic species pair that occurred in the past 30 years, presumably due to drastic, human-induced environmental change (Taylor et al., 2006). Thus, I can examine how isolation has been lost in this pair and compare it to the forward process of speciation evident

in the intact limnetic-benthic pairs as well as other stickleback taxa pairs that span early to late stages of the speciation process.

The biology of threespine stickleback fish makes them both interesting and manageable to study questions of speciation, sexual selection, and ecology. First, most threespine stickleback fish live one year in the wild, though some populations may live up to two years but rarely longer (Baker, 1994). Fish of each species use distinct feeding habitats; in limnetic-benthic species pairs, limnetics feed in the open water while benthics feed along the lake bottom near the shore (McPhail, 1994). In the breeding season, fish migrate into mating habitats, with males migrating before females to establish territories and build nests (Ostlund-Nilsson, 2007). Once females develop eggs, they search for mates. Males attract females with a courtship dance, and courtship involves an elaborate series of back-and-forth interactions between males and females. Females can choose whether to deposit eggs in a male's nest without coercion by the male. After spawning, the female immediately leaves, and the male provides all parental care to the eggs and fry. Females are the choosier sex, and there is no evidence of male choice in most systems (Kitano et al., 2007, Kozak et al., 2009, Raeymaekers et al., 2010, but see Hay & McPhail, 1975). Major sources of selection that act on the phenotypes of these fish include natural selection for efficient feeding morphology and from predators and parasites as well as sexual selection on female preferences and male traits that is mediated by environmental differences between mating habitats (McPhail, 1994).

Here, I investigate how ecology and sexual selection interact to shape the speciation process. I first zoom in on sexual selection due to female mate choice and its importance for maintaining species. In Chapter 1, I ask whether sexual isolation has been lost in a collapsed

limnetic-benthic pair relative to another limnetic-benthic pair with strong reproductive isolation. I use mating trials within and between lakes to distinguish between whether an overall loss of sexual isolation was due to a loss of female preferences for mates of their own species or a loss of distinct, species-specific male mating traits. This work sheds light on how sexual isolation might be lost as species collapse and identifies the essential components that maintain sexual isolation in species with strong reproductive isolation.

Next, I turn to male competition, the form of sexual selection often neglected for its role in speciation, and consider the potential interaction between male competition and the presence of different environments in favoring or hindering divergence between species as well as maintaining species differences that already exist. In Chapter 2, I measure male competition within and between species where different environments are present or absent. This work determines whether male competition can contribute to reproductive isolation and whether this depends on environmental differences. This research also explores how male competition may have played a role in the collapse of one species pair.

I then ask how sexual selection and ecology can interact to affect female mate choice. In Chapter 3, I examine how environmental differences can affect female mate discrimination between males of each species. The divergence of female mate preferences and male mating traits, and thus the evolution of sexual isolation, relied on environmental differences (Boughman, 2001; Boughman et al., 2005). Here, I test whether those environmental differences are needed for the current expression of sexual isolation, and thus its maintenance. This suggests how early and late stages of the speciation process might differ. This work also

determines whether environmental change may have been the immediate and direct cause of loss of sexual isolation in the collapsed species pair.

Finally, I broaden my look at reproductive isolation to all potentially contributing forms of isolation, such as use of different habitats, incompatibilities between gametes of different species, and low relative fitness of hybrids compared to parental forms. I also expand the study system to include many stickleback taxa pairs that represent early to late stages of the speciation process. In Chapter 4, I ask how patterns of reproductive isolation in these systems reveal how isolation might evolve both in the forward and reverse directions. I can also evaluate the relative importance of various barriers for initiating speciation, completing it, and maintaining taxa that do not lose isolation.

In the following four chapters, I use 'we' to describe the research I completed with the guidance and input of my research advisor, Janette Boughman.

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CHAPTER 1

Loss of sexual isolation in a hybridizing stickleback species pair

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Introduction

The process of speciation has been studied for decades, and we have learned much about how selection and ecology shape speciation (Coyne and Orr, 2004; Nosil et al., 2009; Schemske, 2010; Sobel et al., 2010; Maan and Seehausen, 2011). Yet key questions still remain. One essential question is how specific reproductive barriers contribute to the speciation process (Mayr, 1963; Coyne and Orr, 2004; Schemske, 2010; Sobel et al., 2010). Researchers have only begun to examine the relative magnitudes of individual barriers, the order in which barriers evolve, and the forces that drive barrier evolution. Exploring these ideas is complicated, in part, because the speciation process can take thousands to millions of years from start to finish.

Multiple strategies can address this difficulty. One productive approach is to study speciation at different stages of the process from differentiated populations to incipient species to fully isolated species and then compare barrier presence and strength across these stages (Hendry et al., 2009; Nosil et al., 2009; Merrill et al., 2011). A less commonly used approach is to study formerly isolated species that begin to hybridize. In these cases, barriers that break down were likely necessary to maintain distinct species. This strategy is even more powerful for young species (Seehausen et al., 1997; Grant and Grant, 2008). Current barriers between young

species were likely important for generating species because additional barrier evolution is limited by time since divergence relative to species that diverged millions of years ago (Schemske, 2010).

Young species are particularly susceptible to species collapse because reproductive isolation between young species may be incomplete or even reversible. When reproductive isolation is incomplete, premating barriers may be the primary barriers present because they tend to evolve early in the speciation process (Coyne and Orr, 2004). Such reproductive isolation may be particularly fragile because premating barriers often rely on environmental differences, making them potentially reversible if environments change. A number of studies suggest that an environmental disturbance can cause species to collapse (Gow et al., 2006; Taylor et al., 2006; Hendry et al., 2009; Nosil et al., 2009; Schemske, 2010; Vonlanthen et al., 2012), and a few studies have shown the loss of premating isolation after environmental change (Seehausen et al., 1997; Gilman and Behm, 2011).

One premating barrier, sexual isolation, reduces mating between species due to differences in mating signals and mate preferences (Coyne and Orr, 2004). Sexual isolation is often important for initiating speciation (Coyne and Orr, 2004) and maintaining separate species (Mayr, 1963), especially in taxa with strong sexual selection (Lande, 1981; Lande and Kirkpatrick, 1988; Butlin and Ritchie, 1994; Mendelson, 2003; McPeck and Gavrillets, 2006). Relative to other barriers, sexual isolation is often among the strongest barriers to reproduction in animals because it can evolve early in the speciation process and act early in the life cycle (Jiggins et al., 2001; Mendelson et al., 2007; Matsubayashi and Katakura, 2009; Dopman et al.,

2009). Barriers that act early in the life cycle limit the number of hybrids produced, which weakens selection for later-acting barriers (Coyne and Orr, 2004; Schemske, 2010).

In species with female mate choice, females must prefer conspecific mates for sexual isolation to occur. Female conspecific mate preference requires three things: males of each species differ in mating signals, females can discriminate species differences in mating signals, and females prefer to mate with conspecifics over heterospecifics. In the absence of any of these components, sexual isolation by female mate choice cannot occur.

Here we examine the importance of sexual isolation to species maintenance and the speciation process using young limnetic-benthic threespine stickleback species pairs (*Gasterosteus* spp.). We compare the strength of sexual isolation in a species pair that recently collapsed into a hybrid swarm (Gow et al., 2006; Taylor et al., 2006) to another species pair that maintains strong reproductive isolation. Stickleback species pairs are ideal for testing questions of reproductive isolation and mate choice (McKinnon and Rundle, 2002; Rundle and Schluter, 2004; Boughman et al., 2006). Species pairs have evolved in parallel in seven lakes in coastal British Columbia within the past 15,000 years (McPhail, 1993; Taylor and McPhail, 2000; Gow et al., 2008). Pre- and postmating reproductive isolation is strong in all lakes. Sexual isolation minimizes hybridization between species within and across lakes due to parallel speciation (Ridgway and McPhail, 1984; Nagel and Schluter, 1998; Rundle et al., 2000; Boughman, 2001; Boughman et al., 2005). In other words, females of each species accept conspecifics and reject heterospecifics from their own lake as well as other lakes. Additionally, ecological postmating isolation reduces hybrid survival and reproduction (Bentzen and McPhail, 1984; Gow et al.,

2007; Hatfield and Schluter, 1999; McPhail, 1992; Schluter, 1993, 1995; Vamosi and Schluter, 1999; but see Taylor et al., 2012).

Historically, the stickleback species pair in Enos Lake was strongly isolated. Sexual isolation was strong (Ridgway and McPhail, 1984; Table 1.1), and all the components necessary for female conspecific mate preference existed. Females preferred conspecific and rejected heterospecific mates, and males of each species differed in mating traits of color, size, and shape (McPhail, 1984; Ridgway and McPhail, 1984; Taylor et al., 2006; Table 1.2). However, very recently the frequency of hybrids in Enos Lake increased dramatically to 24% (Gow et al., 2006). Both morphological and microsatellite data have confirmed that the species pair has dissolved into a hybrid swarm, where parental forms are rare compared to hybrid and backcrossed individuals (Kraak et al., 2001; Gow et al., 2006; Taylor et al., 2006). The precise causes of the species collapse are not fully known.

Here we test whether sexual isolation has been lost in Enos fish to determine if breakdown of this reproductive barrier contributes to the collapse of this formerly distinct species pair. We predicted reduced sexual isolation in Enos fish for two reasons. First, sexual isolation could break down after an environmental change because it is based on ecologically-mediated traits under divergent selection between distinct mating habitats; these traits include color (Boughman, 2001), size (Nagel and Schluter, 1998), odor (Rafferty and Boughman, 2006), and shape (Vines and Schluter, 2006, Head et al., 2013). Second, the loss of sexual isolation would increase heterospecific matings, which is likely necessary to fully explain the very rapid and drastic increases in hybrid frequencies and extent of introgression. Previous work has confirmed that postmating isolation is reduced; fitness of hybrid and parental forms is now

equal (Behm et al., 2010). Yet, this is insufficient to explain the existence of a hybrid swarm. If only postmating isolation were lost, we would expect more hybrid and backcrossed individuals to survive to adulthood, but parental forms would not decrease due to maintained sexual isolation. Only the loss of premating isolation predicts such extensive hybridization and introgression and the eventual loss of parental limnetic and benthic forms.

We hypothesized that sexual isolation in Enos fish would be weaker than sexual isolation in another species pair in Paxton Lake, which remains strongly isolated. Necessarily, we compared limnetic-like and benthic-like hybrid morphs in Enos Lake to pure species in Paxton Lake. Using the most limnetic-like and benthic-like Enos fish maximizes our ability to detect any remaining sexual isolation. However, it also makes our estimates of change in isolation conservative. Hereafter, we use the word ‘type’ to refer both to species from Paxton Lake and morphs from Enos Lake, and we use ‘homotypic’ for fish of the same type and ‘heterotypic’ for fish of different types.

We distinguished between two factors that could weaken sexual isolation: loss of female preference for homotypic mates and loss of male species-specific mating traits. Determining which factor was lost informs how sexual isolation may have broken down. If females still strongly prefer homotypic mates and reject heterotypic mates, this could favor species divergence. However, if females no longer prefer homotypic mates, then they will mate randomly with respect to male type and produce more hybrids. If species-specific male traits are lost, then females will be unable to distinguish between male types even if females maintain strong preferences for homotypic mates. To detect the loss of either or both of these requirements for sexual isolation in Enos fish, we use between-lake mating trials with Paxton

females that strongly prefer homotypic mates and Paxton males that have distinct species-specific traits. Previous work has shown strong sexual isolation in limnetics and benthics between these lakes (Rundle et al., 2000). We predicted that if Enos females lack preferences for homotypic mates, then Enos females will not discriminate between Paxton limnetics and benthics, despite the distinct species-specific mating traits between Paxton male types. We also predicted that if Enos males do not have distinct species-specific mating traits, then neither Paxton nor Enos females will discriminate between Enos male types.

We next compared current Enos female preferences and Enos male traits to expectations from previous work to evaluate how altered preferences or traits could explain changes to Enos sexual isolation. Here we briefly summarize prior research on limnetic-benthic species pairs from three lakes, including Paxton and Enos, to explain our predictions for Enos female preferences for male color, size, and shape. Prior work has shown that females preferred redder males, although this preference was strong in limnetic females and weak in benthic females (Boughman et al., 2005). No previous work has tested female preferences for melanistic color, although Enos benthic males were black while limnetic males were red (McPhail, 1984), so color-based mate discrimination between species seems possible. Previous work on size preferences showed that females were more likely to accept heterospecific mates when they were similar in length (Nagel and Schluter, 1998; Boughman et al., 2005). This size preference was stronger in benthic than limnetic females (Boughman et al., 2005). Shape preferences may have influenced assortative mating by environment in allopatric stickleback populations (Vines and Schluter, 2006), a recent test in a limnetic-benthic species pair showed that limnetic females preferred limnetic-shaped males, while benthic females had no shape

preference (Head et al., 2013). For male traits, we predicted that trait differences between male types should be larger in Paxton than Enos because previous work shows that male redness, length (Boughman et al., 2005, Table 1.2), and shape (Taylor et al., 2006) differed significantly between male types.

This work is the first to directly test whether sexual isolation has been reduced in Enos fish. Loss of sexual isolation has been the suspected cause of hybridization between the Enos limnetic and benthic sticklebacks (Taylor et al., 2006). More broadly, we examine how sexual isolation contributes to species maintenance and how species collapse provides insight into the speciation process.

Materials and Methods

Study populations

In March and April 2008, we used minnow traps to collect limnetic and benthic threespine stickleback fish from Enos and Paxton Lakes on Vancouver and Texada Islands, BC. Due to high hybridization rates in Enos Lake, pure limnetics and benthics are rare (Gow et al., 2006; Taylor et al., 2006), so we selected the most limnetic- and benthic-like fish using well-established differences in body shape (for males and females) and nuptial color (for males) (McPhail, 1984, 1992; Hatfield, 1997). Previous studies have used shape to identify species (e.g., McPhail, 1984; Schluter, 2003; Schluter and McPhail, 1992). In one study using Enos fish from the extremes of the limnetic-benthic spectrum, fish categorized by morphology and genetics matched with a 97% success rate (Taylor et al., 2006). In Paxton Lake, collection of limnetic and benthic fish was straightforward as this species pair is strongly reproductively

isolated and no intermediate fish were encountered during collection. We transported fish to the University of Wisconsin-Madison. Fish were housed in groups of the same lake, type, and sex and maintained on a 14:10 L:D cycle at 18°C. All fish were fed brine shrimp (*Artemia* sp.) and bloodworms (*Chironomus* sp.) once per day.

Mating trials

We used no-choice trials to measure female preferences and mating interactions between a male and a female all the way to spawning. We wanted to measure female preferences in the absence of male-male and female-female interactions. Conducting trials with more than one male or female would obscure measures of female preference (Wagner, 1998). We selected males in reproductive condition (displaying nuptial colors and territorial behaviors) from holding tanks and placed each male in a 101-L aquarium with nesting materials (plastic tray of sand and filamentous algae). To entice males to build a nest and perform courtship behaviors, we presented them with a gravid female from their own lake once every other day. We alternated whether a male saw a homotypic or heterotypic female during enticement. We used a male in courtship trials after he finished building a nest, which is a prerequisite for spawning.

We selected gravid females for mating trials and randomly assigned each female to a pair of nested males (one of each type seen in random order) from either the female's lake or the other lake. Each female had two trials in one day with at least two hours of rest between trials. We conducted courtship trials for 20 minutes or until the female entered the nest to spawn. If a female entered the nest, we removed her before she could deposit her eggs so both

the male and female could have a second trial. Female type and lake varied across male trials based on when females were gravid. The amount of male courtship did not differ based on female type, female lake, or their interaction (all $F_{1,378} < 1.15$, all $P > 0.28$). Most males had two trials, but some males had only one trial if they failed to court in their second trial (five males) or if no females were available for trials near the end experiment (three males). We never reused the same pair of males. We only reused a small number of fish in a second set of trials (6 males and 25 females) to maintain experimental balance across all treatments. Fish were only reused after spending at least two weeks in tanks with other fish from the same lake, type, and sex. Thus, if reused, males had to build a new nest and females had to develop a new clutch of eggs. We found no effect of reuse in our statistical analyses. Females did not respond differently to reused versus non-reused males ($F_{1,89} = 0.19$, $P > 0.6$). Reused females did not differ in their discrimination between homo- and heterotypic males compared to non-reused females ($F_{1,183} = 0.01$, $P > 0.9$).

We recorded male and female courtship behaviors (Ridgway and McPhail, 1984; Wootton, 1976 pp. 187–193) with Observer behavioral recording software (Noldus Technologies, Wageningen, The Netherlands). For males, we recorded zig-zag, bite, chase, lead, and show. For females, we recorded receptive behaviors (approach, angle, and head-up) and preference behaviors (follow, examine, and spawn) (Kozak et al., 2009). We discarded a pair of trials for only one female, who did not perform any receptive or preference behaviors to either male. In total, we analyzed results from 382 trials from 166 females (38 Enos limnetic-like, 48

Enos benthic-like, 48 Paxton limnetic, and 32 Paxton benthic females) and 191 males (49 Enos limnetic-like, 49 Enos benthic-like, 50 Paxton benthic, and 43 Paxton limnetic males).

For all males, we measured multiple traits of known or potential importance in mate choice: color, size, and shape. Before and after each behavioral trial, we scored male redness and darkness. Historically, Enos limnetic males displayed red nuptial throat color and benthic males expressed black throat and body color (McPhail, 1984; Boughman, 2001). In our study, benthic-like males also expressed some red in addition to black throat color (see discussion). Both species of Paxton males express red throat color, but limnetics are redder than benthics (Boughman, 2001, Boughman et al., 2005, Table 1.2). Despite these color differences, sexual isolation was historically strong between limnetics and benthics from Paxton and Enos Lakes (Rundle et al. 2000). For redness, we scored the area and intensity of red throat color on a scale from 0 (no color) to 5 (large area of color with high intensity) using a standardized scoring method developed by our lab group that yields results comparable to reflectance data (Boughman, 2001, 2007). We scored body darkness on a scale from 0 (absence of melanic color) to 5 (intense melanic color) (Lewandowski and Boughman, 2008). For body size, we measured the standard length of each fish before behavioral trials using Vernier calipers accurate to 0.02 mm. We also photographed the left side of all fish for shape analysis. We used a Kodak DX4330 digital camera arranged at a stationary location above the fish, and we used ambient light.

Sexual isolation analyses

For each trial, we calculated three indicators of female response to a male. First, we calculated female inspection, which is the number of times a female examined the nest for

every time a male showed the nest (Kozak et al., 2009). This measure accounts for the dependence of female preference behaviors on male courtship behaviors. We also calculated female preference score, which assigns each trial a value from 0 to 4 depending on whether a female responded at each level of stickleback courtship. Scores were assigned as follows: 0 (no response), 1 (approach, angle, or head-up), 2 (follow), 3 (examine the nest), and 4 (enter the nest to spawn) (Kozak and Boughman, 2009). This preference score encompasses how far a male and female proceeded with courtship. Results for female preference score were very similar in direction and magnitude to results for female inspection. We present the results for female inspection and preference score to allow comparison to other studies, but we focus our interpretation on female inspection. Lastly, we recorded whether or not a female entered the nest to spawn with a male, which we used to calculate spawning probabilities for particular pairings across types and lakes. We also included spawning probabilities from previous work (Table 1.1) to put our results in context of historical data.

To measure the strength of sexual isolation and factors affecting it, we analyzed female inspection (continuous) using ANOVA, preference score (count) using a generalized linear model with a poisson distribution and log link function, and spawning (binary) using a generalized linear model with a binomial distribution and logit link function. For all models, we included the following factors: female type [limnetic(-like) or benthic(-like)], male type (homotypic or heterotypic relative to female's type), male lake (same or different from female's lake), and their two-way interactions. Higher-order interactions were not significant, so we removed them and report results from reduced models. As each female had two trials, we included female identity as a repeated measure with a compound symmetry covariance

structure, which assumes that the female's two trials are correlated. We square root transformed female inspection to improve normality; no other transformations were necessary. Analyses were run separately by female lake because of different expectations for Paxton and Enos female responses to male types from each lake. We also found statistical support for this expectation; females from each lake responded differently to males from their own or the other lake (female lake*male lake: inspection $F_{1,372} = 4.10$, $P = 0.0436$; preference score: $F_{1,376} = 5.88$, $P = 0.0157$). Means and significance of differences are virtually identical when we ran analyses separately by female lake and with female lakes combined, so we present only the separate lake analyses here.

To examine differences in isolation within and between Enos and Paxton Lakes, we calculated a measure of mate discrimination (response to homotypic males minus response to heterotypic males) for each female for inspection, preference score, and spawning. We tested for significant differences using one-tailed *t*-tests because we had an *a priori* expectation that Paxton female conspecific mate preference for Paxton males would be stronger than or equal to conspecific mate preference in all other within- and between-lake pairings.

We conducted all analyses in SAS software v9.2 (SAS Institute Inc., 2010). For all post-hoc comparisons, we used false discovery rate (FDR) to adjust p-values for multiple comparisons (Benjamini and Hochberg, 1995; Verhoeven et al., 2005), and we provide both raw and FDR-controlled p-values. We also calculated effect sizes using Cohen's *d* to illustrate the magnitude of both significant and nonsignificant findings.

Shape analyses

To analyze male shape, we placed 19 landmarks on digital images of the left side of each fish (Figure 1.1). We based our landmarks on those used by Taylor et al. (2006). We adjusted or removed some of their landmarks because particular landmarks visible on photographs of preserved and stained specimens, as used in Taylor et al. (2006), were difficult to locate on our photographs of live fish. We imported landmark coordinates into the program PAST (<http://folk.uio.no/ohammer/past>) and used the Procrustes transformation to center, scale, and align the coordinates. We used canonical variate analysis to visualize how distinct limnetic(-like) and benthic(-like) fish were from Paxton and Enos Lakes.

Female preference and male trait analyses

We also tested whether females preferred particular trait values of color, size, and shape using female inspection as our measure of preference in ANCOVAs and generalized linear models. We added each male trait singly to models including female type, female lake, male type, male lake and their two-way interactions. We also included interactions between male trait covariates and these categorical model terms and interactions. We reduced models by removing nonsignificant terms. We used two measures of size: standard length and centroid size. Standard length is measured from the anterior tip of the lower lip to the posterior tip of the caudal peduncle (See Figure 1.1). Centroid size is the geometric mean of the distance between each landmark and the centroid point of all of the landmarks (Zelditch et al., 2004 pp. 12–13). To test for female preferences for shape, we used discriminate function analysis (DFA) to create a single shape score. First, we used Paxton fish to generate a limnetic-benthic axis. We

then applied the Paxton discriminant function to Enos fish so that the axis of greatest discrimination in Enos fish would be relevant to limnetic- and benthic-specific shape characteristics. Second, we generated a discriminant function based on Enos males alone. This accounts for shape differences between Enos male types that females may have used in discrimination that were not encompassed by the Paxton discriminant function. We tested for female shape preferences for all fish along the Paxton-generated axis as well as for Enos fish along the Enos-generated axis.

Results

Female discrimination between male types and preference for homotypic males

We first measured the strength of sexual isolation *within lakes* for Enos and Paxton fish to determine if females discriminate between male types and prefer homotypic males from their own lake. Enos females lacked strong sexual isolation. Enos females did not discriminate between Enos male types; these females responded highly to homo- and heterotypic males (Table 1.3: male type*male lake and male type terms are not significant, Figure 1.2A, C). In contrast, Paxton females had strong sexual isolation; females strongly discriminated between Paxton male types and preferred homotypic males (Table 1.3: male type*male lake term is significant, Figure 1.2B, D).

We then tested whether the strength of sexual isolation in Enos fish was weaker than that in Paxton fish. We found that Enos sexual isolation was significantly weaker than Paxton sexual isolation as measured by inspection (Table 1.4A). Preference score showed the same pattern, but the difference was not significant after correction for multiple tests (Table 1.4B).

These findings indicate that Paxton fish have maintained strong sexual isolation while Enos fish have lost it.

Next we measured female discrimination and preference *between lakes* to test for two factors that could weaken sexual isolation: loss of female preferences for homotypic mates and loss of male species-specific mating traits. We tested for loss of Enos female preferences for homotypic mates by asking if Enos females no longer discriminated between male types and no longer preferred homotypic males even when provided with distinct Paxton male types. Indeed, Enos females did not discriminate between Paxton male types and did not prefer homotypic over heterotypic males (Table 1.3, Figure 1.2A & C). Further, Enos female discrimination of Paxton male types was significantly weaker than Paxton sexual isolation as measured by inspection (Table 1.4A). This evidence indicates that Enos females lacked the preferences needed to impart sexual isolation.

We tested for loss of Enos male species-specific mating traits by asking if Paxton females, who have the ability to discriminate between types, responded differently to homo- and heterotypic Enos males. We found that Paxton females did not discriminate between Enos male types and did not prefer homotypic Enos males (Table 1.3, Figure 1.2B & D). Also, Paxton females discriminated between Enos male types significantly less than between Paxton male types (Table 1.4A). These results suggest that Enos males lacked traits that would have allowed females to discriminate between them.

Female preferences and male traits

We tested Enos female preferences for male morphological traits to explore why Enos female preferences no longer imparted sexual isolation. We examined male redness, darkness, size, and shape because of the known or suspected importance of these traits for sexual isolation (see introduction). Based on this previous work, we expected Enos limnetic-like females to prefer redder males of either type and Enos benthic-like females to have weak or no red preference. Instead, we found that both types of Enos females preferred redder limnetic (-like) males but did not prefer redder benthic(-like) males (Table 1.5). For darkness, we predicted Enos benthic-like females might prefer darker benthic males. However, we found no preference for black (all $F_{1,91} < 0.76$, all $P > 0.38$). For size, we expected females to accept heterotypic males similar in size to the female. Consistent with this prediction, Enos females did prefer males more similar in size to themselves ($F_{1,94} = 4.81$, $P = 0.031$). Yet, Enos females applied this preference to homo- and heterotypic males, which was more broadly than expected. We expected that females might prefer males with homotypic shape scores, but we did not find any preferences for shape using the Paxton discriminant function (all $F_{1,76 \text{ or } 87} < 3.23$, $P > 0.08$) or the Enos discriminant function (all $F_{1,43 \text{ or } 47} < 1.78$, $P > 0.18$).

Next, we examined trait differences between male types to determine why Paxton females discriminated between Paxton but not Enos male types. We expected that the mean absolute trait difference between male types would be greater in Paxton than Enos for male redness, length, and shape scores. Indeed, Paxton male types differed significantly more than Enos male types in all three male traits (Table 1.6), which means females should have been able

to discriminate between Paxton male types more easily than Enos male types. Our canonical variate shape analysis also suggests that Paxton male types were more distinct than Enos male types. Paxton male types fell into two distinct shape clusters, while Enos male type clusters overlapped (Figure 1.3). The axis of discrimination between limnetic(-like) and benthic(-like) fish in Figure 1.3 appears to be very similar between Paxton and Enos Lakes.

Discussion

Our study shows that Enos fish lacked sexual isolation. Enos females did not discriminate between male types and did not prefer homotypic over heterotypic males. In contrast, Paxton fish maintained strong sexual isolation.

Contributions of female preference and male traits to overall loss of sexual isolation

Loss of female conspecific mate preference or male species-specific traits could cause an overall loss of sexual isolation. We found evidence that Enos females lacked preferences for conspecific mates. Even when presented with Paxton males, which were the most morphologically distinct male types, Enos females responded highly to both homo- and heterotypic males. Thus, Enos females either could not distinguish between male types or did not prefer one type over the other. We also found that Paxton females responded similarly to both Enos male types, which indicates that Enos males were not distinct enough for Paxton females to distinguish. Interestingly, Paxton females rejected heterotypic males from their own lake but accepted both Enos male types. These data suggest that Paxton females have broad acceptance criteria and narrow rejection criteria, only rejecting males with certain

combinations of traits and/or extreme values of a single trait. Selection on preferences to exclude heterospecific traits could have initiated speciation (McPeck and Gavrillets, 2006) or completed speciation via reinforcement (Rundle and Schluter, 1998; Servedio and Noor, 2003). Our findings indicate that both changes in female preferences and male traits likely contributed to the loss of sexual isolation: Enos females did not prefer homotypic males or discriminate between male types, and Enos male types were not distinct enough to allow females to discriminate between them.

Divergent female preferences can generate sexual isolation, but in Enos females, existing preferences for male traits would not contribute to sexual isolation. Previous work has shown that sexual isolation between limnetics and benthics likely requires both size and color (Boughman et al., 2005). For size, females are more likely to mate with heterospecifics when males are closer in size to the female (Nagel and Schluter, 1998). For color, Enos limnetic females are expected to prefer redder males while Enos benthic females should have no red preference (Boughman, 2001; Boughman et al., 2005). In our study, Enos females seem to have maintained historic size preferences; they preferred males similar in size to themselves. However, for color preferences, both Enos female types preferred red in limnetic (-like) males but not in benthic(-like) males. This result was unexpected for benthic-like females, which historically did not prefer red males, regardless of the male's type (Boughman, 2001; Boughman et al., 2005). It is interesting that Enos females responded differently to red depending on male type despite the fact that the range of redness expression in both male types overlapped considerably (limnetic(-like) males: 0.4 - 4.9; benthic(-like) males: 0 - 4.8). This suggests that Enos females may be able to discriminate male types but do not prefer to mate

with one type over the other. Overall, Enos female types appeared to share preferences for size and color, which would impede sexual isolation.

Male types must have distinct traits or trait values for females to be able to distinguish between them. Differences in redness, size, and shape between Enos male types were smaller than those between Paxton male types. Thus, females likely had a harder time discriminating between Enos versus Paxton male types. Historically, Enos benthic males were black with no red nuptial color (McPhail, 1984). However, in our sample, 70% of our 48 Enos benthic-like males expressed at least some redness. Paxton limnetic females have strong preferences for red (Boughman et al., 2005); thus, increased redness expression in Enos benthic-like males may explain why Paxton limnetic females accepted these heterotypic males. Historical data for size ranges in Enos benthics and limnetics overlapped considerably (benthic: 37 - 59 mm, limnetic: 36 - 51 mm, (Bentzen and McPhail, 1984)), and this was also true of our sample of Enos male types (benthic-like: 47 - 60 mm; limnetic-like: 43 - 55 mm). Yet, in our sample, both Enos male types were larger than historical measures. Paxton benthic females should respond more often to heterotypic males when these males are large (Nagel and Schluter, 1998). This may explain why Paxton benthic females were more likely to accept heterotypic males from Enos Lake than Paxton Lake.

Overall, we found that current Enos female preferences did not impart isolation between types. Moreover, the relatively small male trait differences between Enos types would limit female discrimination. Thus, the loss of both female preferences and distinct male mating traits contributed to weakened sexual isolation.

The role of sexual isolation in species maintenance and collapse

This study demonstrates a loss of sexual isolation in Enos fish, which were historically reproductively isolated. We would expect the loss of sexual isolation to increase heterospecific matings and hybrid offspring, which have been observed in the field (Gow et al., 2006; Taylor et al., 2006). Without sexual isolation, Enos fish should continue to hybridize, promoting further breakdown of species differences.

The loss of sexual isolation could also interact with other reproductive barriers to further dissolve reproductive isolation in Enos fish. For example, prior work in Enos fish documented the loss of postmating isolation that historically reduced hybrid growth and survival (Behm et al., 2010). Loss of sexual isolation would produce more hybrids, and loss of postmating isolation would let hybrids survive and reproduce. In combination, the loss of these two barriers could generate a feedback loop that could quickly degrade total reproductive isolation. Research on interactions between multiple barriers is scarce (Martin and Willis, 2007; Lowry et al., 2008) but could be fruitful. Future work on barrier interactions could determine if particular barriers tend to evolve together and facilitate species to diverge or breakdown.

Environmental changes could further weaken sexual isolation by diminishing females' perception of male color differences or by homogenizing male traits. In sticklebacks, distinct light environments in each species' mating habitat mediate female color perception (Boughman, 2001). Additionally, male color, size, and shape are ecologically mediated (Milinski and Bakker, 1990; Schluter and McPhail, 1992; Schluter, 1993, 1995) and phenotypically plastic (Frischknecht, 1993; Day et al., 1994; Day and McPhail, 1996; Candolin, 2000; McKinnon et al., 2004; Lewandowski and Boughman, 2008). Thus, sexual isolation in Enos fish is probably much

weaker in the wild than in our study. Continued work on Enos Lake could illuminate the causal connections between the environment, hybridization, and speciation across many different taxa. Future studies could also determine if environmental differences that facilitate speciation can just as easily degrade species barriers.

Recent theoretical work showed that strong and permanent disturbances to sexual isolation will likely cause species collapse when sexual isolation is the only reproductive barrier (Gilman and Behm, 2011). Current empirical examples of species breakdown demonstrate that other barriers were not strong enough to maintain species once sexual isolation started to dissolve (Seehausen et al., 1997; Richmond and Jockusch, 2007). This suggests that other barriers may have to be particularly strong to maintain species after the loss of sexual isolation.

Species that have recently diverged or collapsed provide ideal systems for understanding how species form and persist. Empirical work on species collapse, including our study, is opportunistic. Lack of replication and pre-collapse data can make it difficult to determine general patterns and processes of species breakdown. Our work serves as a call for researchers to document environmental, phenotypic, and genotypic differences between diverging or recently diverged taxa. Not only will this information provide insight into how divergence occurs but it will also allow us to understand what halts or reverses the speciation process. Research on multiple taxa pairs across different stages of divergence, including collapsing pairs, will identify the necessary components for individual reproductive barriers to function and the forces that shape how barriers evolve. Additional empirical and theoretical studies on species collapse may reveal that the same processes that can promote rapid speciation can also facilitate species collapse.

APPENDIX

APPENDIX: Chapter 1 tables and figures

Table 1.1 **Current and historical spawning proportions.**

Lake	Female-Male type	Previous Studies Spawning proportion (N)	Present Study Spawning proportion (N)
Enos	L-L	0.38 (24)	0.67 (15) ³
Enos	L-B	0.25 (24)	-----
Enos	B-B	0.38 (26)	0.53 (15) ³
Enos	B-L	0.35 (26)	0.38 (8) ³
Paxton	L-L	0.54 (24)	0.65 (20) ¹ , 0.54 (54) ²
Paxton	L-B	0.33 (24)	0.20 (15) ¹ , 0.25 (32) ²
Paxton	B-B	0.04 (23)	0.31 (66) ²
Paxton	B-L	0.04 (23)	0.13 (32) ²

We present the proportion of no-choice trials where spawning occurred from the present study and three previous studies: (1) Hatfield and Schluter 1996, (2) Rundle et al. 2000, (3) Ridgway and McPhail 1984. We include sample sizes (N) in parentheses next to spawning proportions. We denote limnetic(-like) fish with L and benthic(-like) fish with B.

Table 1.2 **Historical trait differences between male types across multiple lakes.**

Trait	Difference of Means (L-B)	t	DF	P
Redness	1.44	3.06	265	0.0024
Length	-7.26	7.00	269	<0.0001

Differences of mean trait values for male limnetics and benthics from three lakes, including Paxton and Enos, are calculated using data from Boughman et al., 2005. Redness was measured by eye on a scale from 0 - 5 like in our study. Length is standard length measured in millimeters. Significant p-values are in bold.

Table 1.3 Enos and Paxton female discrimination between male types.

A. Inspection

Source of Variation	Enos			Paxton		
	DF	F	P	DF	F	P
female type	95	0.77	0.3829	88	37.22	<0.0001
male type	96	0.02	0.8782	89	3.64	0.0595
male lake	95	0.06	0.8051	88	7.61	0.0071
female type*male type	96	0.00	0.9595	89	1.78	0.1851
female type*male lake	95	3.39	0.0688	88	0.21	0.6457
male type*male lake	96	0.20	0.6537	89	8.45	0.0046

B. Preference Score

Source of Variation	Enos			Paxton		
	DF	χ^2	P	DF	χ^2	P
female type	1	0.18	0.6694	1	26.65	<0.0001
male type	1	1.48	0.2234	1	1.03	0.3103
male lake	1	0.46	0.4992	1	3.55	0.0594
female type*male type	1	1.42	0.2334	1	1.03	0.3101
female type*male lake	1	2.91	0.0883	1	0.42	0.5179
male type*male lake	1	0.02	0.8961	1	6.02	0.0141

Analysis of variance for the effects of female type [limnetic(-like) or benthic(-like)], male type (homo- or heterotypic), male lake (same or different from female's lake) and their two-way interactions on (A) female inspection and (B) preference score. Higher order interactions were not significant. Enos and Paxton Lake females were analyzed separately. Significant p-values are in bold. We were particularly interested in the significance of two of the model terms: male type and male type*male lake. If male type is significant, then females discriminated between male types from her own lake and from the other lake. If male type*male lake is significant, then females likely discriminated between male types from one lake but not the other. If neither term is significant, then females did not discriminate between male types from either lake.

Table 1.4 Tests for absence of Enos sexual isolation, female mate discrimination, and distinct male traits.

A. Inspection

Comparison	Difference	S.E.	DF	T	P	P _{FDR}	<i>d</i>
Pax SI - Enos SI	0.407	0.168	95	2.24	0.0087	0.0131	0.460
Pax SI - Enos fem, Pax male	0.451	0.157	90	2.87	0.0026	0.0077	0.605
Pax SI - Pax fem, Enos male	0.384	0.188	87	2.04	0.0221	0.0221	0.437

B. Preference Score

Comparison	Difference	S.E.	DF	T	P	P _{FDR}	<i>d</i>
Pax SI - Enos SI	0.213	0.187	46	1.14	0.1310	0.1310	0.336
Pax SI - Enos fem, Pax male	0.255	0.210	46	1.22	0.1147	0.1310	0.360
Pax SI - Pax fem, Enos male	0.444	0.222	44	2.01	0.0255	0.0765	0.606

C. Spawning

Comparison	Difference	S.E.	DF	T	P	P _{FDR}	<i>d</i>
Pax SI - Enos SI	0.202	0.114	1	0.175	0.4448	0.4448	0.350
Pax SI - Enos fem, Pax male	0.025	0.105	1	0.238	0.4256	0.4448	0.476
Pax SI - Pax fem, Enos male	0.140	0.129	1	1.085	0.1957	0.4448	2.170

For each lake (Paxton and Enos) female discrimination (averaged across female types) of homotypic and heterotypic males was calculated for (A) inspection, (B) preference score, and (C) spawning probability. Each line of the table compares mean Paxton female discrimination of homotypic and heterotypic Paxton males (Paxton sexual isolation) to mean female discrimination of homotypic and heterotypic males for the other within- and between-lake pairings. Pax SI - Enos SI, the difference between Paxton sexual isolation and Enos sexual isolation, tests for the absence of Enos sexual isolation. Pax SI - Enos fem, Pax male, the difference between Paxton sexual isolation and Enos female discrimination of Paxton male types, tests for the absence of Enos female mate discrimination. Pax SI - Pax fem, Enos male, the difference between Paxton sexual isolation and Paxton female discrimination of Enos male types, tests for the absence of Enos male distinct traits. A positive difference indicates that Paxton sexual isolation is stronger than the other within- or between-lake pairing. We used one-tailed *t*-tests because we expected Paxton sexual isolation to be greater than or equal to discrimination in other within- and between-lake pairings. Significant differences are in bold, and both raw and FDR-controlled *p*-values are shown. Effect sizes, as calculated by Cohen's *d*, are also included.

Table 1.5 Enos female color preference.

Female Type	Male Type	Red Slope	S.E.	DF	T	P	P _{FDR}	<i>d</i>
Benthic-like	homotypic (B)	0.035	0.058	47	0.59	0.5556	0.7215	0.172
	heterotypic (L)	0.316	0.077	46	4.12	0.0002	0.0008	1.215
Limnetic-like	homotypic (L)	0.193	0.081	46	2.38	0.0213	0.0426	0.702
	heterotypic (B)	-0.022	0.062	46	0.36	0.7215	0.7215	0.106

We present the slopes for the relationship between Enos female inspection and male redness in homotypic and heterotypic males. We also list whether males are (L) limnetic(-like) or (B) benthic(-like) to highlight the preference parallels between female types. This interaction of female inspection for red by female type and male type was significant in Enos females ($F_{1,92} = 11.92$, $P = 0.0008$). Significant slopes are highlighted in bold. We show raw and FDR-controlled p-values as well as Cohen's *d*.

Table 1.6 **Trait differences between male types.**

A. Redness differences

Lake	N	Mean difference	S.E.			
Paxton	95	2.04	0.09			
Enos	95	1.21	0.08			
Lake difference		Difference	S.E.	DF	T	P
Paxton - Enos		0.83	0.12	188	6.94	< 0.0001

B. Length differences

Lake	N	Mean difference	S.E.			
Paxton	96	7.15	0.45			
Enos	95	4.08	0.32			
Lake difference		Difference	S.E.	DF	T	P
Paxton - Enos		3.07	0.56	189	5.52	< 0.0001

C. Shape differences

Lake	N	Mean difference	S.E.			
Paxton	92	19.41	0.70			
Enos	95	13.71	0.89			
Lake difference		Difference	S.E.	F	T	P
Paxton - Enos		5.71	1.14	185	5.02	< 0.0001

For males from each lake (Paxton and Enos), we show the mean of the absolute trait difference between males types for (A) red, (B) standard length, and (C) shape scores based on the Paxton discriminant function. We also tested whether mean trait differences in Paxton were greater than those in Enos. For red, standard length, and shape, mean trait differences between male types in Paxton are significantly greater than those in Enos.

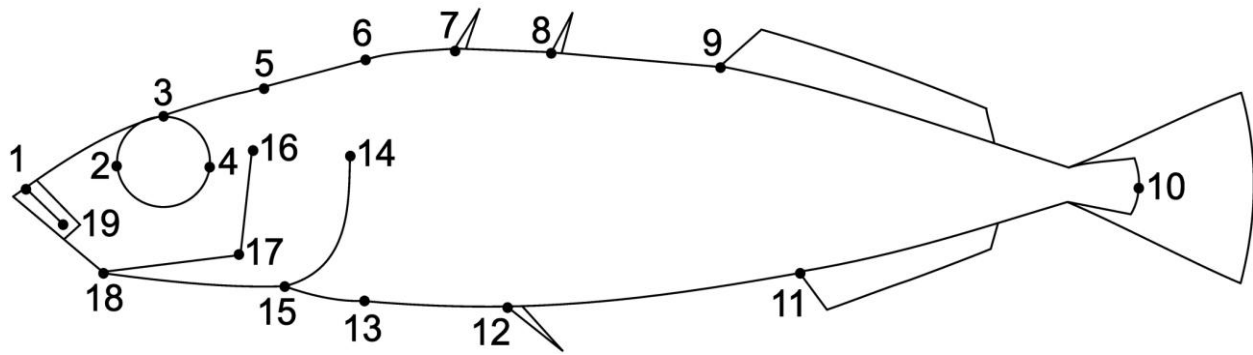


Figure 1.1. **Morphometric landmarks.** Location of the 19 landmarks used in morphometric analysis of threespine sticklebacks, based on the consensus configuration: (1) anterior tip of upper lip; (2) most anterior point of left eye; (3) most dorsal point of left eye; (4) most posterior point of left eye; (5) midpoint of the line posterior to the top of the eye and the intersection with dorsal midline; (6) point of intersection between the dorsal midline and the line posterior to the top of the eye; (7) anterior junction of first dorsal spine with the dorsal midline; (8) anterior junction of second dorsal spine with the dorsal midline; (9) anterior insertion of anal fin membrane with the dorsal midline; (10) caudal border of hypural plate at the lateral midline; (11) anterior insertion of anal fin membrane with the ventral midline; (12) anterior junction of pelvic spine on ventral midline; (13) point along ventral midline directly ventral to point 6; (14) posteriodorsal extent of opercular aperture; (15) posteroventral extent of opercular aperture; (16) dorsal point of angular; (17) posterior edge of angular; (18) anterior edge of angular; (19) posterior extent of maxilla.

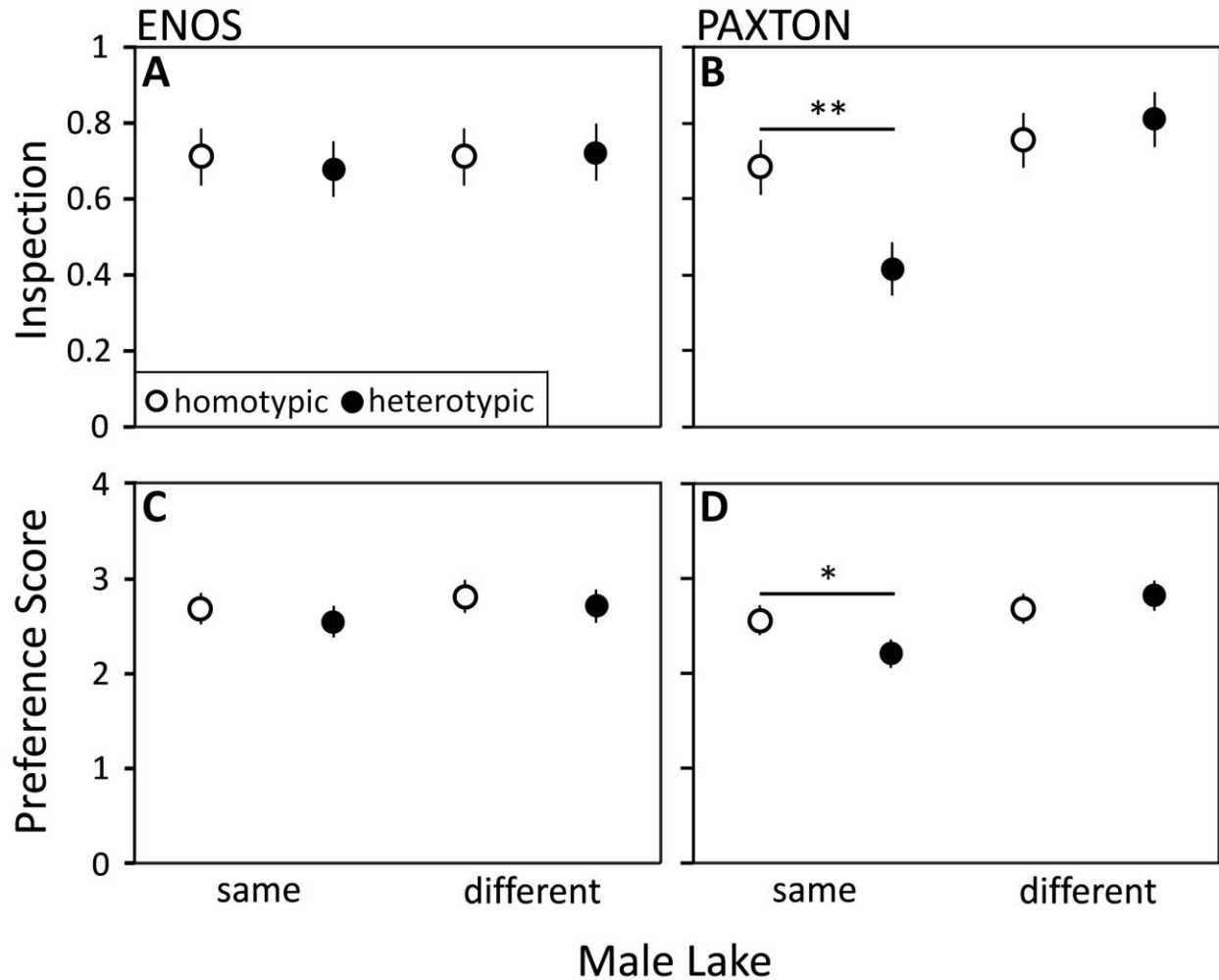


Figure 1.2. **Female response to males of each type from each lake.** Mean female response with standard error bars for female inspection and preference score for (A, C) Enos and (B, D) Paxton females of homotypic (open symbols) and heterotypic (filled symbols) males from either the same or different lake. Model effects and their significance are shown in Table 1. Significant differences in least-squared means for all pair-wise comparisons are shown with FDR-controlled p-values. All other pair-wise comparisons are nonsignificant. ** P < 0.01, * P < 0.05. Data presented for female inspection are square root transformed as analyzed.

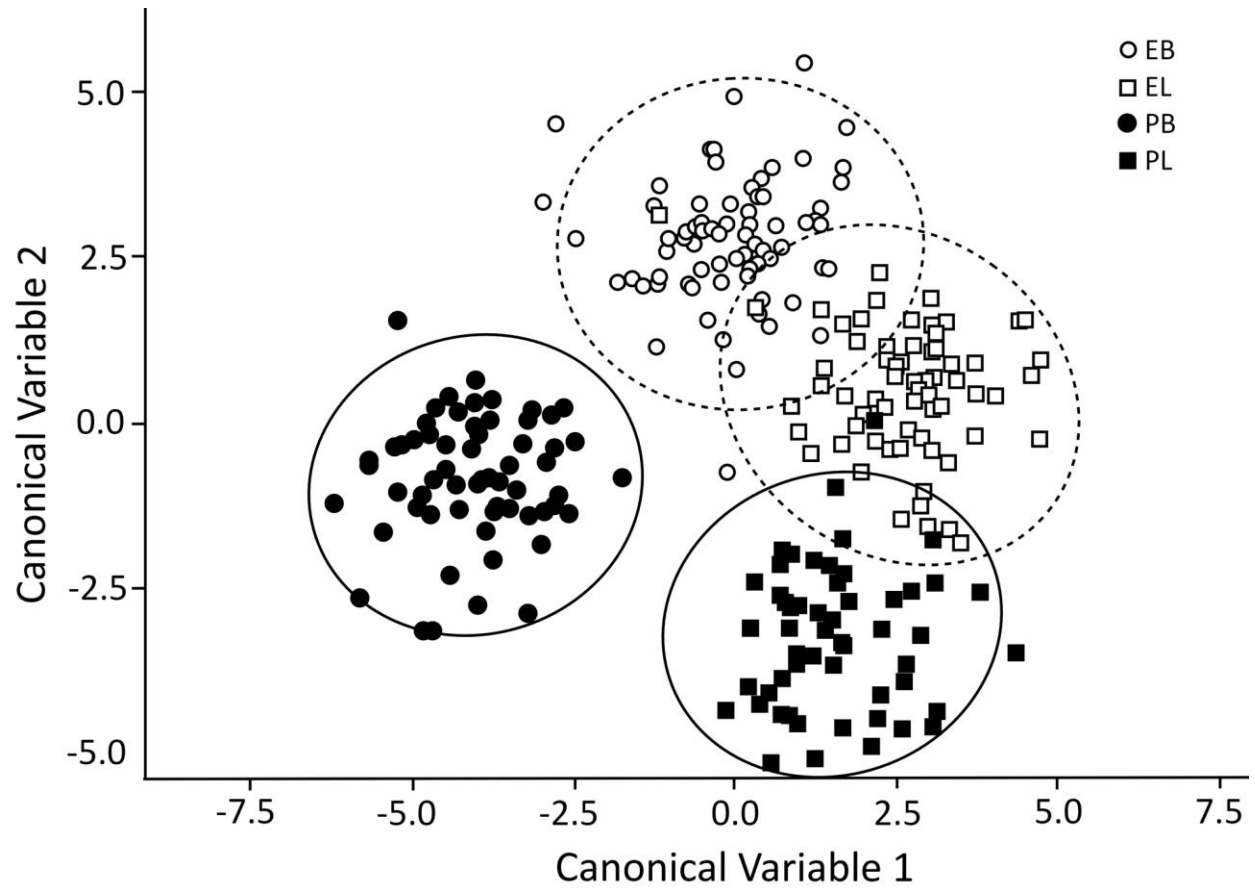


Figure 1.3. **Canonical shape scores for Paxton and Enos male types.** Shape scores are plotted along the first and second canonical variable axes. Ellipses show 95% confidence around the cluster mean. Letters refer to Paxton (P), Enos (E), limnetic(-like) (L), and benthic(-like) (B).

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CHAPTER 2

Divergent sexual selection via male competition: ecology is key

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Introduction

Sexual selection is an important evolutionary force in speciation (Lande, 1981; Lande and Kirkpatrick, 1988; Panhuis et al., 2001; Coyne and Orr, 2004). This is largely due to the ubiquitous divergence sexual selection causes in female preferences and male display traits that subsequently reduces mating between species (Turelli et al., 2001). Studies of sexual selection in speciation typically focus on female mate choice, leaving the role of male competition in speciation understudied (Grether et al., 2009). Recent studies of male competition in speciation have explored how behavioral interactions, particularly biased aggression toward similar competitors, can cause disruptive selection that facilitates speciation (Seehausen and Schluter, 2004; Dijkstra and Groothuis, 2011). Few studies, however, have considered how the environment affects the intensity of male competition, the traits that mediate such competition, or the contributions of competition to speciation (Patten et al., 2004; Robertson and Rosenblum, 2010; Sullivan-Beckers and Cocroft, 2010; Vallin and Qvarnstrom, 2011).

Environments could easily impact how male competition contributes to sexual selection. We know from work in female choice that the environment can affect which traits matter to sexual selection and how they matter. For example, environmental differences can influence

which traits females use to select mates when genetic benefits to females vary across environments (Welch, 2003) or when environmental differences affect signal transmission (Schluter and Price, 1993; Endler and Basolo, 1998; Boughman, 2002; Maan et al., 2006). In male competition, environmental differences can similarly affect which traits make successful competitors. For example, when the availability of breeding resources differs between environments, this can change the relative importance of male morphological and behavioral traits (Baird et al., 1997; Reichard et al., 2009).

Ecological differences can also enhance the importance of sexual selection to speciation (Lande and Kirkpatrick, 1988; Ritchie, 2007; Maan and Seehausen, 2011; Weissing et al., 2011) probably because mating trait divergence is even more likely when environments differ (e.g., Boughman et al., 2005). Although most of the work examining ecological effects on sexual selection and speciation has been done in the context of female choice and focused on male display traits (reviewed in Maan and Seehausen, 2011), ecological differences can also affect traits involved in male competition. Such dynamics might contribute to speciation when environmental differences cause male traits to diverge between populations via male competition (Patten et al., 2004; Robertson and Rosenblum, 2010; Vallin and Qvarnstrom, 2011).

If environmental differences drive divergence between species through male competition, then environmental change can alter the role of male competition in speciation. In the documented cases of reverse speciation, changing environments eroded reproductive isolation that depended on environmental differences (Seehausen et al., 1997; Taylor et al., 2006; Vonlanthen et al., 2012). In male competition, environmental change could modify which

traits are important to male success or how males compete within and between species. Either of these changes could reduce reproductive isolation through male competition.

Most previous work on male competition in speciation has focused on how aggression within and between species can favor divergence and maintain species. Avoiding interspecific aggression, or biasing aggression to conspecifics, generates frequency dependent and disruptive selection that can facilitate speciation (van Doorn et al., 2004) and coexistence of species instead of allowing one species to outcompete the other (Mikami et al., 2004; Seehausen and Schluter, 2004; Dijkstra and Groothuis, 2011). Selection against heterospecific aggression may arise when two species do not share the same limiting resources because the costs of aggression between species are not offset by the benefits of resource acquisition. However, even when resources are shared, dominance asymmetry between species can still select against heterospecific aggression in one direction. In this scenario, males of the less dominant species should avoid heterospecific aggression (Dijkstra and Groothuis, 2011). Prior work has also explored how selection against heterospecific aggression could favor outcomes that enhance divergence, such as species recognition or habitat segregation (Seehausen and Schluter, 2004; Grether et al., 2009; Dijkstra and Groothuis, 2011). Species recognition is expected to promote character displacement of male traits (Grether et al., 2009). Habitat segregation is expected to reduce encounter rates between heterospecific males as well as between heterospecific males and females, enhancing sexual isolation. Habitat segregation could also increase ecological differences between species.

Here we ask whether mating habitats affect sexual selection and reproductive isolation via male competition within and between species. We also test predictions of selection against

heterospecific aggression to determine how aggression within and between species could contribute to reproductive isolation. We use limnetic-benthic species pairs of threespine stickleback fish that live in multiple lakes in British Columbia, Canada (McPhail, 1984, 1992). Stickleback species pairs are ideal for testing questions in sexual selection and speciation. First, sexual selection via male competition is likely in sticklebacks and could impact reproductive isolation. Males gain territories to build nests and court females, and females require nests for depositing their eggs. Territories are limited and aggressively defended (Black and Wootton, 1970; Ostlund-Nilsson, 2007). Thus, competition can determine which males have access to females. Second, ecology could be important for sexual selection and speciation through male competition. Limnetic and benthic males tend to occupy different microhabitats, with limnetics nesting in the open and benthics nesting in dense vegetation (Ridgway and McPhail, 1987; McPhail, 1994). These distinct habitats may favor different traits in male competition. Competition in each habitat could favor different traits or trait optima because certain traits are more detectable or reliable (Schluter and Price, 1993) or because different attributes or strategies help males secure a territory. Competition is likely within and between stickleback species because males of each species tend to segregate into separate habitats, but limnetic and benthic males are likely territorial neighbors (Ridgway and McPhail, 1987). Open and vegetated habitats are distributed along the lake shoreline in a mosaic pattern, and patches of each habitat are small compared to the potential travel distance of a single fish (Boughman, 2006). The cause of habitat segregation is unknown, but here we explore whether male competition could be a potential cause.

We compare stickleback populations from two lakes that differ in the type of mating habitat and in the strength of reproductive isolation. Paxton Lake has mixed habitat with both open and vegetated areas, and the species are strongly reproductively isolated (McPhail, 1992; Rundle et al., 2000; Boughman et al., 2005). Enos Lake historically had mixed habitat but now has only open habitat following the introduction of an invasive crayfish (Taylor et al., 2006; Behm et al., 2010). After this environmental disturbance, limnetics and benthics began hybridizing and now constitute a hybrid swarm (Gow et al., 2006; Taylor et al., 2006). We ask whether environmentally-induced changes in male competition may have contributed to this loss of reproductive isolation.

We measure competition in mixed and open habitats in males from both lakes to assess how mating habitats affect male competition within and between species, and we consider how these dynamics could contribute to reproductive isolation. We made the following predictions. If mating habitats affect male competition, then morphological and behavioral traits that predict male nesting success should differ between mixed and open habitats. If mixed habitats strengthen the contribution of male competition to reproductive isolation, then male competition should favor divergence in mixed habitat. If male competition differs between Paxton and Enos males, then the current environmental conditions in each of these lakes could affect male competition. In the context of recent events in Enos Lake, this work can also address how habitat loss may affect male competition and species maintenance. Lastly, we test how patterns of aggression within and between species could contribute to reproductive isolation, and we look for evidence of species recognition and habitat segregation.

Materials and Methods

Study populations

We collected limnetic and benthic threespine stickleback fish from Enos and Paxton Lakes from Vancouver Island and Texada Island, British Columbia in April 2009 using minnow traps. We identified limnetics and benthics using species-specific characteristics of body shape for males and females. We distinguished between the sexes using presence of nuptial color for males and eggs for females. In Enos Lake, pure limnetics and benthics are rare due to hybridization (Gow et al., 2006; Taylor et al., 2006), so we selected the most limnetic-like and benthic-like fish based on species differences in body shape and nuptial color (McPhail, 1984, 1992; Hatfield, 1997). Categorizing limnetic-like and benthic-like fish via body shape has very accurately classified Enos fish in other studies (e.g., Taylor et al., 2006), where fish categorized by morphology and genetics matched with a 97% success rate. Using the most morphologically divergent Enos fish provides information about the maximum remaining isolation in this population. We use the word “type” to refer both to Enos morphs and Paxton species. For instance, two limnetic or two limnetic-like males are homotypic, whereas a pair of limnetic and benthic or limnetic-like and benthic-like males are heterotypic.

We transported fish to the University of Wisconsin-Madison and housed them in tanks by lake, type, and sex. Fish rooms were maintained on a 14:10 L:D cycle at 18°C. We fed fish brine shrimp (*Artemia* spp.) and bloodworms (*Chironomus* spp.) once per day.

Male competition trials

Male competition trials were conducted in twelve 75-gallon tanks. Each tank had a one-inch thick layer of fine-grain natural-colored sand as substrate for nest building. We randomly assigned tanks to one of two habitat types: mixed and open. The mixed habitat type was split in half; the vegetated half had sixteen evenly spaced plastic plants and the open half had no plants. The open habitat type had two open halves. We added one small plastic plant to the back corner of each open half in both habitat types as refuge from extended aggressive interactions.

When males developed moderate nuptial color, we set up two males of each type (two benthic(-like) and two limnetic(-like) males) for a total of four males per tank. With this design, we can compare interactions within and between species. All males in a tank were from the same lake (Enos or Paxton). There were no partitions in the tanks, so all males could interact and establish territories and nests in any location. To examine the importance of size differences in male competition, we selected a larger and a smaller male within each type that differed in standard length on average by $4.25 \pm 0.24\text{mm}$ (approximately 6 - 11% of a male's total body length). We measured standard length using Vernier calipers accurate to 0.02mm. Within each tank, we ranked all four males by length with 1 as the largest and 4 as the smallest male. Thus, length rank is a relative length measure. To identify males within treatment tanks, we randomly assigned each male one of four elastomer colors. We marked males along the back between the spines and the dorsal anal fin. We found no correlation between a male's elastomer color and his red nuptial color, length, length rank, aggression, or proportion of days a male had a territory or nest (all correlation coefficients $|R| < 0.11$ and all $P > 0.13$).

We observed each tank of four males daily until three days after one male finished building a nest or up to 14 days, whichever came first. We conducted three-minute focal observations of each male in a tank sequentially and in randomized order. We also randomized the order in which we observed each tank of males. During each observation, we recorded any aggressive behaviors the focal male performed and received to get an overall aggressive activity measure. Aggressive behaviors were bite, chase, and charge (Irsel, 1953). Each day, we also recorded whether aggression occurred within each pair of males as well as the directionality of that aggression. We conducted observations on a total of 56 tanks and 224 males with 14 replicate tanks for each habitat type in each lake. We did not reuse any males.

Before daily observations of fish within a tank, we recorded each male's color scores for throat, eye, and body using standardized methods developed by our research group (Boughman, 2001; Lewandowski and Boughman, 2008). Throat redness has been linked to male competition success in previous work (Bakker and Sevenster, 1983; Rowland, 1984; Bakker and Milinski, 1993; Baube, 1997). We measured throat redness area and intensity each on a scale from 0 to 5 with 0.5 increments. We averaged area and intensity scores to get overall redness. A redness score of 0 indicates no red, and a score of 5 indicates a large area of intense red. Eye and body color measures were not significant factors in our models (all main effect and interaction terms had $P > 0.10$), so we focus solely on reporting results for throat redness.

After daily observations in a tank, we recorded the location of all male territories and nests. A centimeter scale running along the bottom of the tank allowed observers to record locations consistently across observation days and tanks. We determined the boundaries of a male's territory by observing where a male defended his territory against other males by

aggression. We categorized a male's territory size as large, small, or absent. Large territories took up over one-third of the tank volume, while small territories took up less than one-third. We scored territory sizes as 2 for large, 1 for small, and 0 for absent.

We summarized a male's morphological traits, behavior, and territory and nest characteristics across all observation days. First, we averaged each male's standard length from before and after all observations. For male color, we averaged a male's redness scores across all observation days. We looked at plots of redness across observation days to evaluate if averaging male redness was appropriate. Male redness scores were relatively stable after the first few days of observation. Thus, average redness scores capture both how red the male was for most of the observations as well as initial color changes. Additionally, average redness is a more comprehensive measure than redness at the start or the end of the observation days. Average aggression performed by each male is the sum of the number of bites, chases, and charges given in a focal observation, divided by the observation time in seconds, and averaged across all observation days. We performed similar calculations for average aggression received by each male. Net aggression for each male is the difference between average aggression performed and received. Positive net aggression values indicate a male performed more aggression than he received. Territory success is the proportion of days a male had a territory, and nesting success is the proportion of days a male had a nest. We also averaged a male's territory size across all observation days. For each tank of males, we calculated the proportion of days homo- and heterotypic aggression occurred, and finally, we calculated the difference between homo- and heterotypic aggression proportions.

Statistical analysis

We used path analysis and selection gradient analysis as complementary approaches to (1) evaluate how mating habitats affect the direction and magnitude of selection via male competition on male morphological traits of redness and size and (2) explore causal relationships between male traits and nesting success in male competition.

(a) Path analysis

We used path analysis to test hypothesized relationships between the following variables: average redness, average body length, average net aggression, the proportion of days a male had a territory, and the proportion of days a male had a nest. For simplicity, we refer to these variables respectively as redness, length, aggression, territory success, and nesting success. We arcsine square root transformed territory and nesting success to improve normality. As males within each tank are not independent, we used number of tanks as our sample size. Additionally, we tested models with and without tank as a variable affecting nesting success. Tank has no impact on the magnitude or significance of the paths in our model, so we exclude tank in the models we present here. We conducted these analyses in SAS 9.2 using PROC CALIS (SAS Institute Inc., Cary, NC, USA), which uses structural equation modeling to estimate parameters using maximum likelihood.

We hypothesized two path diagrams *a priori* to consider two alternate ways that male redness and length could affect territory success. We tested which of these two models best fit the data, and we show the best supported model in Figure 2.1. Both models hypothesized that (1) male redness and length affect aggression, (2) redness and length are correlated, and (3)

higher aggression increases territory success, which in turn increases nesting success. Previous work has shown in other stickleback populations that redder and larger males tend to be more aggressive (Bakker and Sevenster, 1983; Rowland, 1983a; Bakker and Milinski, 1993). We allowed for a correlation between redness and length because previous work found that limnetics and benthics differ in both size and color with limnetics being smaller and redder (Boughman et al., 2005). Work in other stickleback populations suggested positive relationships between aggression, territory size, and having a nest (van den Assem, 1967). Prior work also suggests that redness and length can impact aggression and territory success somewhat independently (Bakker and Sevenster, 1983; Rowland, 1984; Rowland and Sevenster, 1985; Bakker, 1994; Rowland et al., 1995; Baube, 1997). The two models we proposed differ in the hypothesized relationships from redness and length to territory success. The best supported model (shown in Figure 2.1), allows both direct and indirect relationships from redness and length to territory success (i.e., redness and length could directly affect territory success and/or directly affect aggression, which could then indirectly affect territory success). We also tested a reduced model that only allows indirect relationships from redness and size to territory success through aggression.

Our primary goal was to use path analysis to evaluate how relationships between male morphological traits, aggression, and territory and nesting success might differ between habitats. Thus, we ran path analyses for males in mixed habitats separately from males in open habitats (N = 28 tanks for each habitat type). We tested the fit of the two models discussed above for each habitat type. In mixed habitat, the model in Figure 2.1 fit significantly better than the reduced model according to chi-squared model fitting criterion (testing differences in

models' chi-squared statistics: $\chi^2_2 = 14.23$, $P = 0.0008$). For open habitat, both models fit equally well ($\chi^2_2 = 2.90$, $P = 0.23$).

To assess whether it was appropriate to pool males across lakes and male types for our models testing habitat effects, we tested the model in Figure 2.1 for Paxton and Enos males and then for benthic(-like) and limnetic(-like) males. Across all four of these models, the signs of all causal paths were the same, and the path magnitudes differed only slightly (< 0.25). The only difference between lakes was that the correlation between redness and length existed in Paxton males (0.39 ± 0.16 , $t_{16} = 2.39$, $P = 0.0295$, $P_{FDR} = 0.0413$) but was absent in Enos males (-0.23 ± 0.18 , $t_{16} = 1.26$, $P = 0.2257$, $P_{FDR} = 0.2633$). The correlation between redness and length was not significant for either male type (both $P > 0.48$). The similarity of relationships across these models gives us confidence to pool lakes and male types in our analyses of habitat effects.

(b) Selection gradient analysis

We followed the methods of Lande and Arnold (1983) and Janzen and Stern (1998) to estimate selection gradients for our dichotomous fitness measure of whether a male built a nest. We performed logistic regression on the standardized traits of length and redness. We ran regressions separately for males in each of the two habitat types: mixed and open. We transformed logistic coefficients and their standard errors using a constant: the average of $W(z)[1-W(z)]$, where $W(z)$ is the average gradient of the predicted selection surface for trait z (Janzen and Stern, 1998). This transformation generates coefficients and standard errors based

on relative fitness, and these coefficients are comparable to those from linear regression analyses.

We also determined selection gradients using nesting success, which is our fitness measure in the path analyses. Significance of gradients is highly similar when we used nesting success or the dichotomous nest measure. For simplicity, we only present the results for the dichotomous measure.

(c) Multiple regression analysis

We used multiple regression to explore the effects of male lakes and types in aggression, territory size, and nesting likelihood. These analyses expose details and relationships unapparent from the path or selection gradient analyses. Using ANOVA, we tested models with net aggression or average territory size as the response variable. For nest likelihood, we used a generalized linear model with a binomial distribution and logit link function. For the proportion of days homo- or heterotypic aggression occurred, we used a generalized linear model with a Poisson distribution and log link function. The difference between the proportion of days homo- and heterotypic aggression occurred was normally distributed, so we tested models with this response variable with ANOVA. In each model, we included categorical variables of male type (limnetic(-like) and benthic(-like)), habitat (mixed or open), and lake (Paxton or Enos). We also included the continuous covariates of redness or length in separate analyses. We included all interactions in initial models and then removed nonsignificant terms. We controlled for the fact that males within a tank are not independent by including a random effect of tank for all models except when the response variable was the

difference between homo- and heterotypic aggression; in that model, we controlled for tank effects by calculating aggression at the level of the tank. In SAS 9.2, we tested normally distributed models using PROC MIXED and binomial- and poisson-distributed models using PROC GENMOD. For post-hoc tests, we controlled p-values for multiple comparisons using false discovery rate (FDR) (Benjamini and Hochberg, 1995; Verhoeven et al., 2005), and we report both raw and FDR-controlled p-values.

Results

Our path analysis revealed that the relative importance of male length and redness for nesting success differed between males in mixed (open plus vegetated) versus open habitat. Redness, but not length, predicted aggression and territory success in mixed habitat (Figure 2.2A). In contrast, in open habitat, length, but not redness, predicted aggression, which in turn influenced territory success (Figure 2.2B). Across habitat types, the relationships were consistent among aggression, territory success, and nesting success (Figure 2.2A, B). More aggressive males had higher territory and nesting success.

Our selection gradient analysis agreed with the path analysis results and added additional insight into favored trait combinations of redness and length. Consistent with our path analysis, we found moderate and positive directional selection for one trait in each habitat type: redness in mixed habitat and length in open habitat (Table 2.1). Selection gradient analysis revealed that the trait not under directional selection in each habitat type experienced nearly significant quadratic selection (Table 2.1). Additionally, negative correlational selection occurred- in mixed but not open habitat (Table 2.1). The combined effects of these selection

pressures are shown in Figure 2.3. Across both habitat types, small males with little redness were disfavored.

The major difference between habitat types was that mixed habitat favored two trait combinations, while open habitat favored only one (Figure 2.3). Importantly, mixed habitat favored trait combinations we see in species pairs: small size with lots of red (limnetic) or large size with little red (benthic). Open habitat favored only one trait combination, large size with lots of red, which is a unique trait combination compared to what we typically see in limnetic and benthic males for intact species pairs.

Results from our path and selection gradient analyses predict different targets of directional selection in each habitat. Our sampled lakes differ in currently available habitat, and so males from each lake may differ in redness and length due to previous generations of selection in these habitats. Paxton Lake has mixed habitat, which should favor redder males, while Enos Lake has only open habitat, which should favor larger males. We tested whether mean trait values of redness and length differed between males from each lake. We pooled males across male types and experimental habitat types to look for evidence of past selection at the lake level. As predicted by our path analysis and directional selection gradients, Paxton males were significantly redder than Enos males (difference in least squares means for average redness = 0.90 ± 0.14 , $t_{222} = 6.28$, $P < 0.0001$), and Enos males were significantly larger than Paxton males (difference in least squares means for average length = 1.58 ± 0.69 , $t_{222} = 2.27$, $P = 0.0242$). We also report mean redness and length for males from each lake separately by male type in Table 2.2.

Next, we used multiple regression to take a closer look at how male length and redness influence net aggression. We found that larger males aggressed more than smaller males across habitats, but the slope of this relationship was significantly steeper in open than mixed habitats, as revealed by the significant interaction between length and habitat (Figure 2.4A). These patterns were also true for relative length ranks within tanks (Figure 2.4B). The largest males aggressed significantly more in open than mixed habitats. Additionally, in open habitat, the largest males aggressed significantly more than all other male ranks. Higher redness also consistently predicted higher aggression ($F_{1,154} = 33.48$, $P < 0.0001$), but this pattern did not differ between habitat types ($F_{1,154} = 0.42$, $P = 0.5196$). None of the above relationships differed between male types (interaction terms of male type with redness or length all have $F < 0.43$ and $P > 0.51$). Also, the total number of males that held nests or territories did not differ between habitat types (all $\chi^2 > 0.5$ and $P > 0.4$).

Our secondary set of analyses tested for evidence of selection against aggression between male types and two potential outcomes of such selection: species recognition and habitat segregation. These analyses evaluate how interactions between male types could affect reproductive isolation via male competition and test specific predictions about dominance asymmetry (Mikami et al., 2004; Seehausen and Schluter, 2004; Dijkstra and Groothuis, 2011). Selection against aggression between male types predicts that males should be more aggressive to homotypic than heterotypic males. Our measure of aggression was the proportion of days males performed aggression to homo- or heterotypic males. We used this measure because it allowed us to distinguish between aggression to homo- or heterotypic males, whereas net

aggression does not. Only one male type in each lake was more aggressive to homotypic than heterotypic males (Table 2.3). Paxton limnetics aggressed more to limnetics than benthics, whereas benthics were equally aggressive to both male types. The reverse pattern with respect to male type occurred in Enos Lake. Enos benthic-like males aggressed more to benthic-like than limnetic-like males, but limnetic-like males were equally aggressive to both male types. These findings are not due to differences in overall levels of homo- or heterotypic aggression between male types, lakes, or their interaction (all $\chi^2_1 < 1.98$, $P > 0.15$).

Evidence for species recognition in male competition requires that males respond differently to phenotypic differences depending on whether rivals are homo- or heterotypic. We tested whether absolute differences in length and redness between male types predicted differences in aggression to homo- and heterotypic males. Only limnetic(-like) males changed their aggression to heterotypic but not homotypic males based on phenotypic differences. As length differences increased, limnetic(-like) males aggressed less to benthic(-like) males, whereas benthic(-like) male aggression to limnetic(-like) males remained unchanged (Table 2.4A). As redness differences increased, only Paxton limnetic, but not Paxton benthic or Enos males, aggressed less to heterotypic males (Table 2.4B). No males in our study changed their aggression to homotypic males based on length or redness (all $\chi^2_1 < 0.05$, $P > 0.81$).

Distributions of redness and length overlap between male types (redness scores (0 - 5): limnetic(-like) 0.11 - 4.36, benthic(-like) 0 - 4.63; length: limnetic(-like) 39.72 - 59.28mm, benthic(-like) 43.95 - 67.91mm). This supports the idea that limnetic(-like) males responded

differently to homo- and heterotypic males based on species recognition and not simply due to larger phenotypic differences between than within male types.

Another potential outcome of selection against heterotypic aggression is habitat segregation. When we looked across all males that had territories in mixed habitat tanks, benthic(-like) males were more likely to hold territories in the vegetation than the open (33 males in vegetation, 11 in open: $\chi^2_1 = 11.00, P = 0.0009$), while limnetic(-like) males were equally likely to have a territory in either habitat (12 males in vegetation, 13 males in open: $\chi^2_1 = 0.40, P = 0.8415$). However, a more exact test of habitat segregation involves looking only at cases where benthic(-like) and limnetic(-like) males had territories in the same tank. We found a pattern of benthic(-like) territories in the vegetation and limnetic(-like) territories in the open, but the trend is not significant (segregation occurred in 13 out of 19 cases, $\chi^2_1 = 2.579, P = 0.108$). In all of the cases where segregation did not occur, both benthic(-like) and limnetic(-like) males held territories in the vegetation. When we compared territory sizes for each male type across habitats, we found that in the vegetation, benthic(-like) males had significantly larger territories than limnetic(-like) males (least squares mean difference = $0.61 \pm 0.17, t_{65} = 3.69, P = 0.0005, P_{FDR} = 0.0020$). Additionally, limnetic(-like) males had much larger territories in the open than the vegetation (least squares mean difference = $0.50 \pm 0.20, t_{65} = 2.52, P = 0.0143, P_{FDR} = 0.0286$).

Previous work predicts that selection against heterospecific aggression can be strong and result in species recognition or habitat segregation, but this prediction only holds if the

costs or benefits of heterotypic aggression are the same between male types. Dominance asymmetry occurs when males of one type are more aggressive to heterotypic males than are males of the other type. In this case, costs or benefits of heterotypic aggression likely differ between male types. If dominance asymmetry occurs, we expect that only the less dominant type will avoid heterotypic aggression, so selection would favor species recognition or habitat segregation only in the less dominant type. Dominance asymmetry between male types could explain our findings that only one male type avoided heterotypic aggression and exhibited species recognition and that habitat segregation seems weak. Thus, we tested for dominance asymmetry between male types using nesting likelihood. Nesting likelihood most directly assesses how dominance asymmetry could affect reproductive isolation compared to other potential measures of male success (e.g., territory success) because nesting is required for spawning to occur. We found evidence of dominance asymmetry between Paxton male types. Paxton benthic males were significantly more likely to have a nest than Paxton limnetic males (Figure 2.5). Of Paxton nested males, 75% (24 of 32, $\chi^2_1 = 8.0$, $P = 0.0047$) were benthic. In contrast, Enos benthic-like and limnetic-like males were equally likely to nest and appear to lack dominance asymmetry (Figure 2.5).

Discussion

In this study, we aimed to determine how mating habitats affect how male competition contributes to sexual selection and speciation. We also tested whether other mechanisms, such as biased aggression toward similar competitors, species recognition, and habitat segregation, affect how male competition contributes to speciation. Overall, we find that mating habitats

strongly impact male competition in ways that can either facilitate or hinder divergence. In contrast, biased aggression, species recognition, and habitat segregation play relatively weaker roles in promoting divergence via male competition.

Our first main finding is that mating habitats altered the relative importance of male traits in male competition. Male competition in mixed habitat (open and vegetated) favored redder males while open habitat favored larger males. Research that measures selection on male sexual signals due to male competition across different environments is relatively rare. One study in gobies showed that complex habitats generated positive sexual selection pressures on male size while open habitats did not (Myhre et al., 2013). In general, we might expect divergent selection on male traits used in male competition directly through selection on male competitive ability or indirectly through selection generated by female mate choice, predation, or parasitism (Qvarnstrom et al., 2012). Selection on male traits through female mate choice may act in concert or opposition to selection from male competition (Hunt et al., 2009), while both predation and parasitism likely select against aggression in male competition due to increased visibility to predators and impaired immune function when testosterone is high (reviewed in Qvarnstrom et al., 2012). Future work on male competition across environments could shed light on the extent to which male competition depends on the environment.

We also found that redness and size differed in how they influenced male competition success. Redness directly affected a male's aggression and territory success, which suggests that redness can signal a male's fighting ability with or without physical contact. Size directly affected male aggression, suggesting that larger males dominate smaller males through physical

contact. A study in lizards found similar results; color signaled resource holding power while size directly predicted aggression (Baird et al., 1997). These results highlight the importance of measuring multiple traits, assessing their relative importance, and determining how they may affect fitness.

Our second main finding is that the shape of fitness functions differed between habitat types, which could explain the maintenance of species differences in one lake with mixed habitat and recent breakdown of species differences in another lake with open habitat. In mixed habitat, male competition favored divergent selection on male traits, which could allow competitor and mate recognition and promote speciation or maintenance of species. Mixed habitat favored two trait combinations, large with little red and small with lots of red, which match what we see in reproductively isolated stickleback species. Benthic males are larger with less red while limnetic males are smaller with more red (McPhail, 1984, 1992; Boughman et al., 2005). In contrast, open habitats favored one trait combination, which should weaken competitor and mate recognition and increase hybridization. Open habitats favor large, red males. This is a unique combination compared to the two species but matches what we currently see in Enos Lake, where vegetation was destroyed and only open habitat remains. In this lake, red has increased in benthic-like males. Historically, benthic males had no red coloration (McPhail, 1984; Boughman, 2001), but in our sample, 82% of benthic-like males (46 out of 56) express at least some red. Hybridization may have generated many new trait combinations, but our work suggests selection from male competition where habitat is currently open may also have favored large, red males and may explain the current abundance of individuals with this trait combination. Our study suggests that loss of vegetated habitat in

Enos Lake likely changed male competition in a way that would undermine reproductive isolation and instead facilitate hybridization.

Male competition is certainly not the only selective force acting on male traits, but our data suggest that its importance has been underappreciated. Earlier work in sticklebacks showed that male competition and female choice should work in concert to favor redder males (Bakker and Sevenster, 1983; Rowland, 1984; Bakker and Milinski, 1993; Baube, 1997; Candolin, 1998; Boughman *et al.*, 2005). Predation should not select against red (Reimchen 1989), although it may reduce current investment in red (Candolin, 1998). These selective forces in sum should always favor redder males. Our work reveals that male competition in mixed habitats can also favor dull males when they are large (Figure 2.3). For male size, previous work suggested that male competition between species could favor larger size (Rowland, 1983a, b). Trout predation may also favor larger fish that can escape gape-limited predators (Reimchen, 1991). Female choice favors larger size only in the larger benthic species but should limit size increases in the smaller limnetic species because hybridization is more likely when heterospecific mates are similar in size (Nagel and Schluter, 1998). Our work suggests that male competition could strengthen divergent selection on size from female choice in mixed habitats, where both large and small males can succeed. In sticklebacks, selection on male traits from male competition has been understudied in the past few decades, especially for male competition between species. Our work highlights how selection from male competition can help to explain the presence or maintenance of trait combinations that differ between species.

Male competition also favors divergent male traits in flycatchers (Alatalo *et al.*, 1994), cichlids (Seehausen and Schluter, 2004), and lizards (Robertson and Rosenblum, 2010). In each

of these systems, male visual signals diverge primarily along one trait axis. In our system, male traits diverge along two trait axes: length and redness. Work on female choice suggests that multiple traits can facilitate species divergence when the relative importance of each trait differs between populations (Candolin, 2003). Female choice may favor multiple traits in males because females assess multiple characters, males use different signals depending on their condition or the signaling context, or different male traits indicate different aspects of male quality (Moller and Pomiankowski, 1993; Andersson, 1994; Johnstone, 1996; Marchetti, 1998; van Doorn and Weissing, 2004). The same processes can apply to male competition, where instead of males signaling to females, males signal to rival males. Our study suggests multiple traits influence sexual selection and speciation via male competition.

Previous work has begun to explore other ways that male competition could promote divergence, including aggression biased to conspecifics, species recognition, and habitat segregation. When males aggress more within than between species, this can help to maintain distinct species because it keeps one species from outcompeting the other (Seehausen and Schluter, 2004). Selection against heterospecific aggression can favor species recognition (Grether et al., 2009) and habitat segregation (Schluter and Price, 1993). If males of both species avoid heterospecific aggression, this symmetrically favors divergence between species and could strongly contribute to speciation. In our study, however, only one male type in each lake was more aggressive to homotypic than heterotypic males: limnetic males in Paxton Lake and benthic-like males in Enos Lake. As only one male type avoids aggression with heterotypic males, we expect weak species recognition and habitat segregation.

If only one male type avoids heterotypic aggression, then only that male type is likely to recognize homo- versus heterotypic competitors. Males from Paxton Lake fit this prediction; Paxton limnetic males avoided heterotypic aggression and showed evidence of species recognition while Paxton benthic males did not. Males from Enos Lake, however, did not show evidence of species recognition as expected by aggression patterns. Benthic-like males avoided aggression with limnetic-like males but showed no signs of species recognition based on redness or size. It is possible that benthic-like males based recognition on a different trait, like shape. Alternatively, aggression patterns in Enos males may not match general predictions because hybridization in Enos Lake could have changed the genetic or learned underpinnings of rival recognition. Hybridization may have eroded genetic preferences for aggression to one male type or trait differences between male types that allowed recognition to occur. If competitor recognition is learned, hybridization could have changed social interactions that affect learning. Additionally, hybridization could have altered costs and benefits of aggression between male types. Perhaps Enos male types currently compete for shared resources more than they did historically.

As both male types did not avoid heterotypic aggression, strong habitat segregation is unlikely. Indeed, we found that habitat segregation between male types was weak, and males of each type held territories in both habitats. However, benthic(-like) males were more likely to hold territories in the vegetation than in the open and maintained larger territories in vegetation than limnetic(-like) males. Limnetic(-like) males were equally likely to have territories in the vegetation or the open but maximized their territory size in open habitat. These patterns may arise because vegetated habitat is preferred by both male types but

benthic(-like) males exclude limnetic(-like) males from the vegetation. Earlier field work in stickleback species pairs showed significant habitat segregation (Ridgway and McPhail, 1987). Our study suggests that male competition can contribute to habitat segregation but is unlikely to be the sole cause.

Dominance asymmetry could explain why only one male type avoided heterotypic aggression. If one type is more dominant than the other, then selection should only favor the less dominant type to avoid aggression with heterotypic males. We found evidence of dominance asymmetry in Paxton males that is consistent with this prediction. Paxton benthics have a nesting advantage over limnetics, and limnetic males avoided heterotypic aggression. In other animal systems, dominance asymmetry often leads to local exclusion or extinction of one species (Kodric-Brown and Mazzolini, 1992; Owen-Ashley and Butler, 2004; Pearce et al., 2011). Despite ongoing heterospecific aggression and dominance asymmetry that could hinder divergence, limnetic and benthic stickleback species in Paxton Lake remain distinct. This suggests that distinct habitats may be necessary for divergence via male competition.

Selection against heterospecific aggression may also be weak, as we observed in our study, if species share resources. Shared resources between species equalize the benefits of competing with males of both species. Although limnetics and benthics have diverged to use different food resources (Bentzen and McPhail, 1984; McPhail, 1984, 1992), males of each species may still compete for territories or mates. Territories are limited both in nature (Ridgway and McPhail, 1987) and in our study; all four males in a tank rarely obtained territory on the bottom for nesting (2 out of 56 tanks). Males of each species may also compete for

access to females. Previous work has shown that males will court females of either species (Kozak et al., 2009). Thus, shared resources may maintain aggression between male types.

Aggression in sticklebacks is unlikely to facilitate divergence because aggression between types still occurs in one direction. In cichlids, aggression bias toward similar phenotypes is also not sufficient to stabilize the speciation process (Dijkstra et al., 2007; Dijkstra and Groothuis, 2011). These studies and our own suggest that aggression patterns alone are unlikely to maintain reproductive isolation between species. Instead, distinct habitats are likely necessary to foster divergence.

Male competition can also interact with female choice to promote divergence. Traits used in male competition are often the same as those important for female choice (Berglund et al., 1996; Hunt et al., 2009). When male competition and female choice act on the same traits, these selective forces tend to strengthen each other (reviewed in Hunt et al., 2009). For example, in flycatchers, male competition between species maintains habitat segregation, which bolsters female learning of mating habitats (Vallin and Qvarnstrom, 2011). Thus, divergence caused by male competition could promote divergence caused by female choice. Future work on the relative importance of male competition and female choice in sticklebacks will generate a fuller picture of how sexual selection contributes to speciation.

In our study, we found that mating habitats change the relative importance of male traits in male competition. We also found that selection through male competition in two habitats favored two trait combinations while selection in one habitat favored a single trait combination. Other potential forces of divergence, including reduced heterospecific aggression, seemed insufficient to maintain distinct species. However, reduced heterospecific aggression,

even if weak or only in direction, could contribute to divergence favored by two mating habitats. Our work highlights the importance of the interaction between ecology and male competition in speciation. Future work should continue to address the role of male competition in ecological speciation.

APPENDIX

APPENDIX: Chapter 2 tables and figures

Table 2.1 Selection gradients for linear, quadratic, and correlational selection.

A. Mixed

Trait	β or γ	S.E.	P
Length	0.167	0.131	0.2036
Redness	0.381	0.143	0.0079
Length ²	-0.526	0.135	0.0514
Redness ²	-0.180	0.146	0.5381
Length*Redness	-0.581	0.211	0.0058

Correlation of length and redness

Pearson's R	P
0.127	0.1805

B. Open

Trait	β or γ	S.E.	P
Length	0.378	0.151	0.0121
Redness	0.101	0.144	0.4829
Length ²	-0.260	0.141	0.3577
Redness ²	0.486	0.142	0.0864
Length*Redness	0.096	0.154	0.5348

Correlation of length and redness

Pearson's R	P
-0.016	0.8622

We calculated selection gradients from multiple regressions on two standardized traits. We used logistic regression for our dichotomous fitness measure of whether a male had a nest. Logistic coefficients and standard errors are transformed by relative predicted fitness (as described in Janzen and Stern 1998 and our methods text) to approximate selection gradients and standard errors similar to those derived from linear multiple regression. We show selection coefficients, standard errors, and p-values for males in mixed (A) and open (B) habitat. Significant coefficients are in bold.

Table 2.2 **Mean redness and length for male types from each lake.**

Lake	Male Type	Male trait means	
		Redness (0 - 5 scale)	Length (mm)
Paxton	Limnetic	2.25 ± 0.13	46.20 ± 0.32
Paxton	Benthic	3.04 ± 0.11	56.49 ± 0.60
Enos	Limnetic-like	2.22 ± 0.14	51.01 ± 0.40
Enos	Benthic-like	1.26 ± 0.15	54.83 ± 0.48

For each male trait, means and standard errors are shown. Redness is measured by eye on a scale from 0-5 that accounts for both intensity and area of color averaged across all observation days. Length is standard body length averaged before and after behavioral trials.

Table 2.3 Test for biased aggression to homotypic versus heterotypic males.

Lake	Male Type	Mean differences in aggression performed (homotypic - heterotypic)				
		Mean	S.E.	T	P	P _{FDR}
Paxton	Limnetic	0.125	0.04	3.11	0.0024	0.0088
Paxton	Benthic	0.014	0.04	0.35	0.7301	0.8309
Enos	Limnetic-like	-0.009	0.04	0.21	0.8309	0.8309
Enos	Benthic-like	0.118	0.04	2.91	0.0044	0.0088

For each male type from each lake, we tested whether males are more aggressive to homotypic than heterotypic males. The interaction of lake and male type is significant ($F_{1,105} = 8.67$, $P = 0.0040$). Positive means indicate homotypic is greater than heterotypic aggression, which could facilitate maintenance of two male types. Degrees of freedom for each test are 105. Significant means are in bold.

Table 2.4 Test of whether males recognize heterotypic males.

A. As absolute differences in length increase

Change in aggression performed to heterotypic males							
Lake	Male Type	χ^2	P	ML estimate	S.E.	χ^2	P
Pooled	Limnetic(-like)	4.46	0.0347	-0.057	0.028	4.14	0.0420
Pooled	Benthic(-like)	1.65	0.1996	---	---	---	---

B. As absolute differences in redness increase

Change in aggression performed to heterotypic males							
Lake	Male Type	χ^2	P	ML estimate	S.E.	χ^2	P
Paxton	Limnetic	4.29	0.0383	-0.533	0.280	3.61	0.0573
Enos	Limnetic-like	0.84	0.3585	---	---	---	---
Pooled	Benthic(-like)	0.04	0.8483	---	---	---	---

If males change their aggression to heterotypic males as absolute phenotypic differences increase, this is evidence of species recognition. We tested whether aggression performed to heterotypic males changed as absolute differences in length (A) and redness (B) increased. Aggression performed is the proportion of total observation days heterotypic aggression occurred. We identify the aggressor under the heading male type. We tested whether male types from each lake differed in their response to phenotypic differences. The interaction between lake and change in redness was significant for limnetic(-like) males ($\chi^2 = 4.96$, $P = 0.0259$), so we list male types from each lake separately. All other lake interactions with changes in phenotype were not significant (all $\chi^2 < 0.07$, $P > 0.78$), so we pooled the lakes. Degrees of freedom for all tests are 1. When the male type effect was significant, we present the maximum likelihood (ML) estimate, standard error, chi-square value, and p-value. Significant chi-square values and ML estimates are in bold. We do not present FDR-controlled p-values because there is only one test of ML estimates for each of the phenotypic traits.

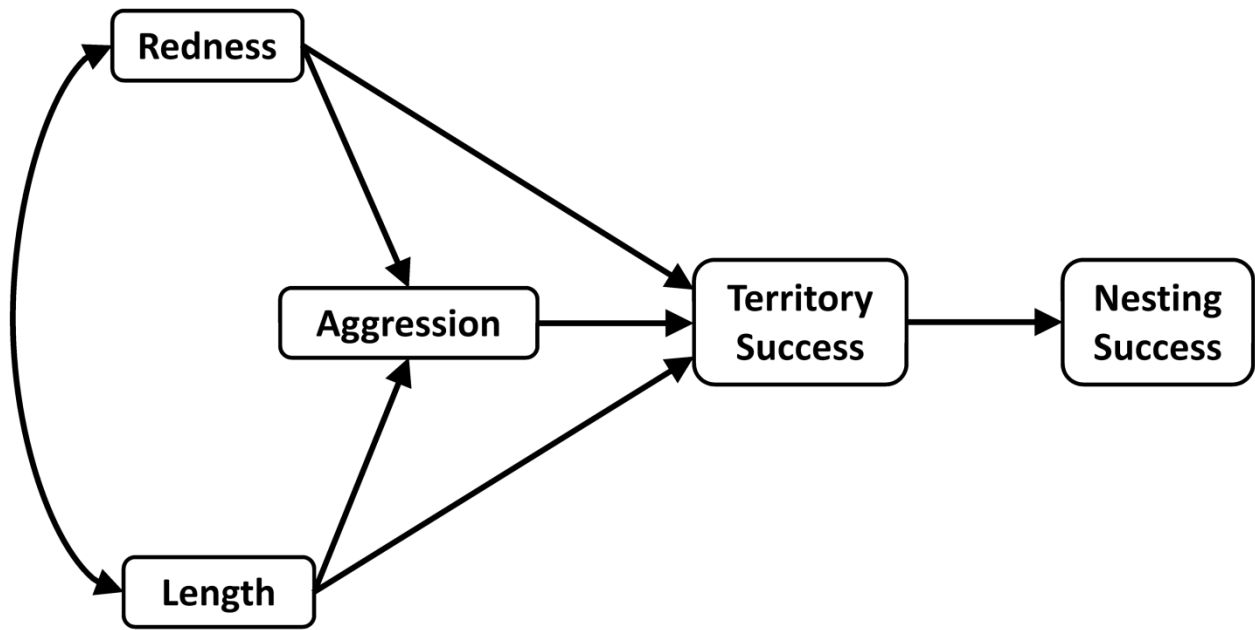
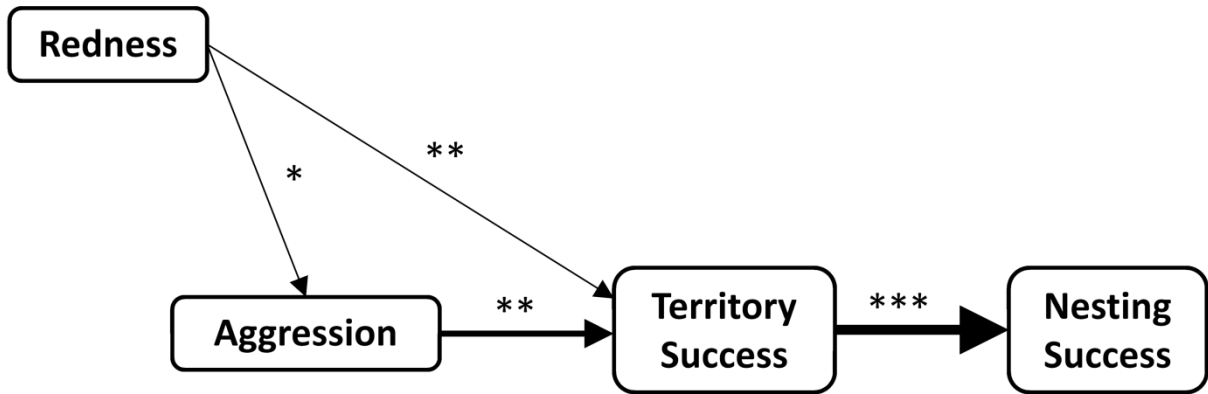


Figure 2.1 **Hypothesized path diagram for relationships between male morphological traits and aggression, territory success, and nesting success.** Single-headed arrows represent causal paths while double-headed arrows indicate correlations.

(A) Mixed



(B) Open

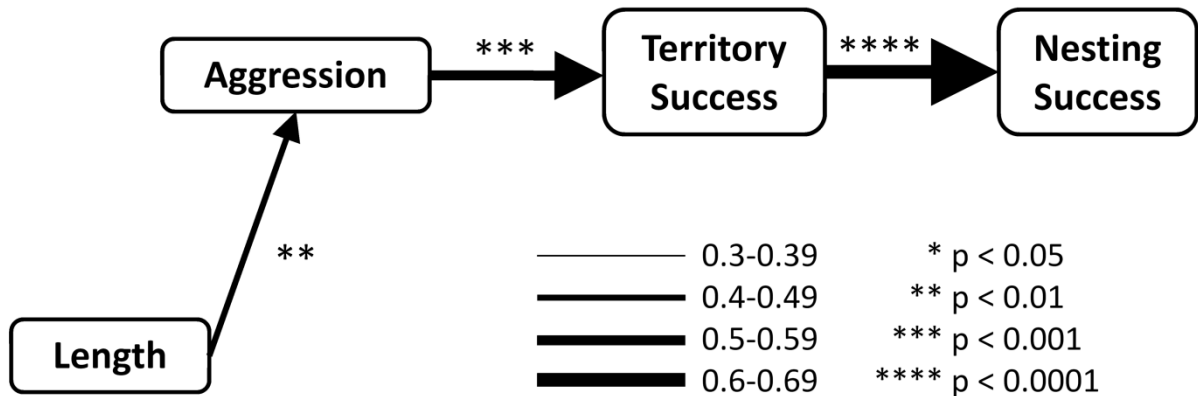


Figure 2.2 **Path diagrams for relationships between male morphological traits and net aggression, territory success, and nesting success.** Results for significant relationships ($P < 0.05$) in mixed (A) and open (B) habitats. All significant paths had standardized magnitudes of 0.3 or higher. Line thickness shows path magnitudes.

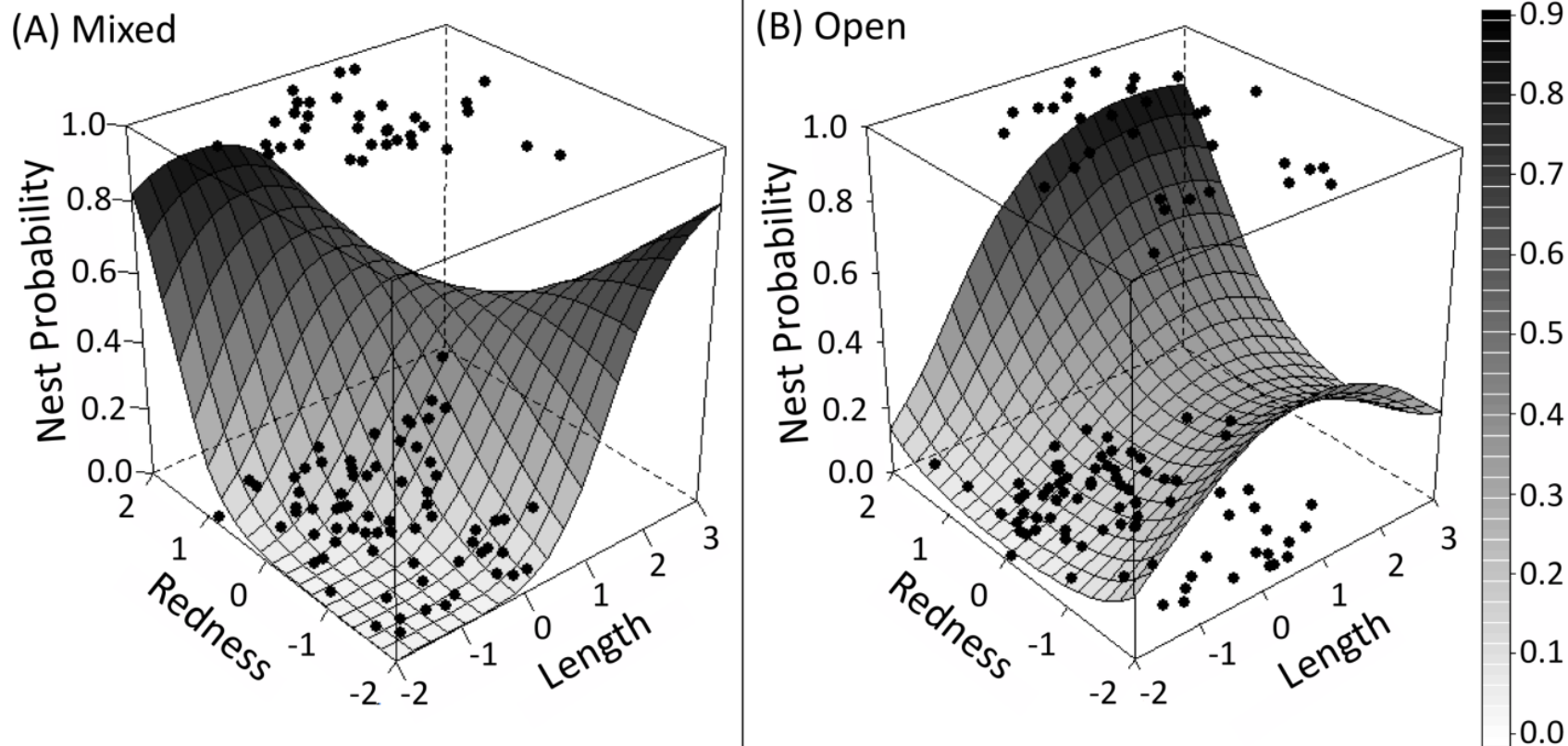


Figure 2.3 **Fitness surfaces for male nesting probability based on redness and length.** For mixed (A) and open (B) habitat types, we show predicted fitness surfaces from quadratic regression analysis. Actual data for nesting probabilities (0 or 1) are also plotted. Redness and length are standardized to mean of 0 and standard deviation of 1.

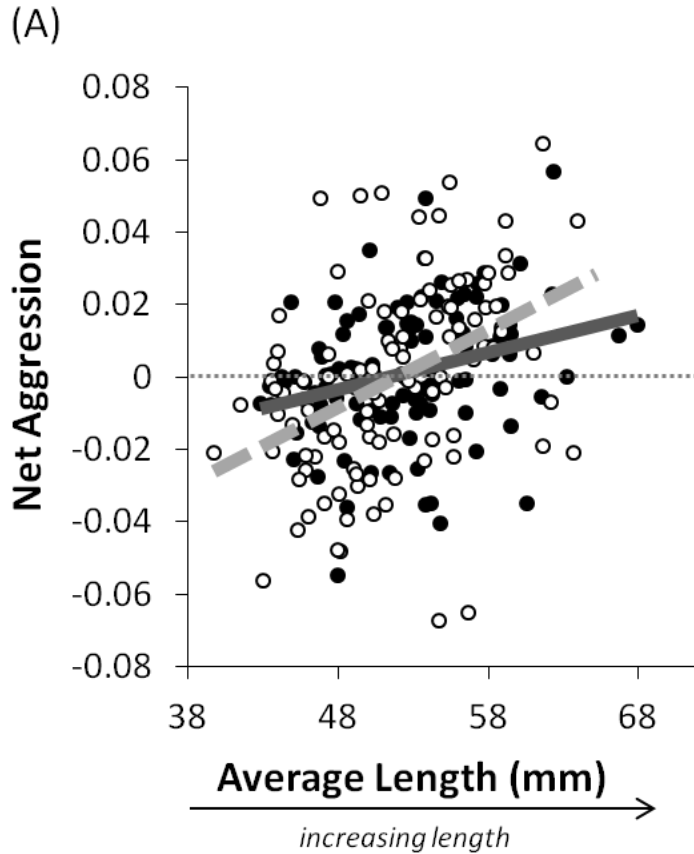
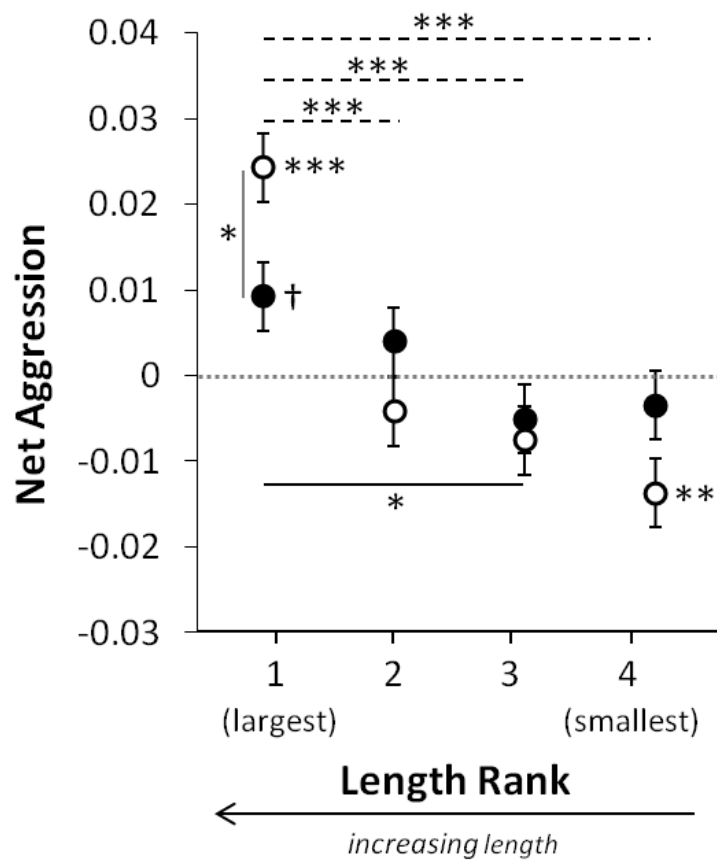


Figure 2.4 **Habitat type differences in net aggression for males of different sizes.** A male's net aggression is the number of aggressive behaviors a male performed minus the number that male received in interactions with any other male. We examined the relationship between net aggression and male size measured as average length (A) and relative length rank within each tank (B). In both (A) and (B), habitat types are shown for mixed (filled circles) and open (open circles). In (A), regression lines are shown for mixed (solid dark grey line) and open (dashed light gray line) habitat types. Both slopes are significantly different than zero (GLM: Mixed slope $\pm$ standard error = 0.0010 ± 0.0003 , $t_{111} = 3.21$, $P = P_{FDR} = 0.0017$; Open slope $\pm$ standard error = 0.0022 ± 0.0005 , $t_{111} = 4.73$, $P < 0.0001$, $P_{FDR} = 0.0002$). The relationship between net aggression and average length differs significantly between habitat types (ANCOVA length and habitat interaction $F_{1,166} = 4.01$, $P = 0.0468$). In (B), the least squares mean $\pm$ standard error of net aggression for males of each length rank is shown. Significant differences are shown for mixed habitat with black lines, open habitat with dashed lines, and comparisons between habitat types with the grey line. Significant differences from zero are indicated directly to the right of the filled or open circle. All p-values are FDR-controlled for multiple comparisons. † $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 2.4 (cont'd)

(B)



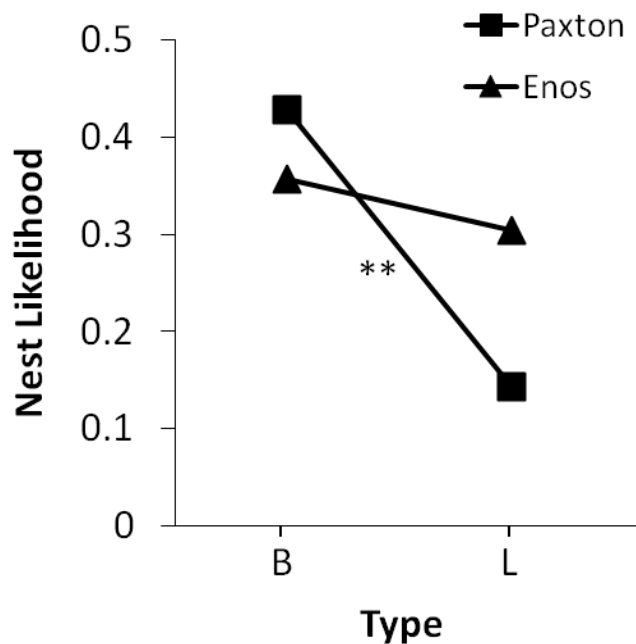


Figure 2.5 **Differences in likelihood to nest for males of each type from each lake.** Nest likelihood is estimated from whether males of each type from each lake had a nest at any time during observations. The interaction of type and lake is significant ($\chi^2_1 = 4.30$, $P = 0.0380$). Types are designated as B for benthic(-like) males and L for limnetic(-like) males. Tests for differences were only run between types within lakes as shown by the black lines. ** $P < 0.01$

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CHAPTER 3

Are environmental differences that favored sexual isolation to evolve

necessary to maintain it?

Introduction

In ecological speciation, environmental differences are important both in furthering the evolution of reproductive isolation and in maintaining isolation that has already evolved. This is the case for multiple components of reproductive isolation, including sexual isolation, where differences in mate preferences and traits used in mate choice reduce mating between species (Lande and Kirkpatrick, 1988, Panhuis et al., 2001, Turelli et al., 2001). Substantial data suggest that sexual selection is especially important to speciation when it interacts with natural selection via ecology and that sexual selection should primarily affect sexual isolation (Ritchie, 2007, Maan and Seehausen, 2011). In the evolutionary role, distinct environments select for divergent phenotypes, and the evolution of those phenotypes causes reproductive isolation as a byproduct (Mayr, 1947, Schluter, 2001, Rundle & Nosil, 2005, Nosil & Harmon, 2009). Female preferences and male mating traits may diverge between environments when natural or sexual selection favor different mating trait values or mating preferences in distinct environments (Lande, 1982, Lande & Kirkpatrick, 1988, Coyne & Orr, 2004, Maan & Seehausen, 2011). In the maintenance role, environmental differences affect the expression of differences in female preferences and male traits that confer reproductive isolation in a facultative or plastic manner, which can alter the magnitude of isolation (Etges et al., 2007, Maan & Seehausen, 2011). Environmental properties can influence how females detect and evaluate mating traits

(Schluter & Price, 1993, Boughman, 2002, Myhre et al., 2013). Environmental factors can also affect how much males court and how they signal, potentially altering which signals transmit well (Endler, 1992), which are preferred by females (Heuschele et al. 2009), and which help males outcompete rival males (Lackey & Boughman, 2013a). Despite the interest in these issues, it remains unclear whether the environment is more critical in its evolutionary or maintenance role, and so whether it has different effects on the evolution or expression of sexual isolation. We explore these issues here.

Sexual isolation plays a central role throughout the speciation process. It is likely to evolve early and thus may help initiate speciation (Mendelson, 2003, Coyne & Orr, 2004). Late in the speciation process, reinforcement can favor increased sexual isolation to avoid costly heterospecific matings (Servedio & Noor, 2003). For both reasons, sexual isolation may contribute substantially to how quickly or completely two taxa progress toward becoming distinct species. Microhabitat differences can alter the expression of courtship behavior, mating signals, and preferences (Schluter & Price, 1993, Boughman, 2002), and by doing so affect the strength of sexual isolation. Therefore, habitat differences can affect the accumulation of reproductive isolation and progress on the speciation continuum from a single population to distinct species. Because sexual isolation appears to commonly depend on the environment, changes in environment can either enhance or undermine its expression throughout the speciation process. Environmental changes that weaken sexual isolation can halt the speciation process early or even reverse it if substantial isolation has already built up. In several cases of reverse speciation for example, anthropogenic environmental change has reduced sexual isolation by undermining the expression of female preference for particular male traits (e.g.,

Seehausen et al., 1997, Fisher et al., 2006, Ward & Blum, 2012). These environmentally induced changes in expression have had evolutionary consequences by fostering hybridization.

A key aspect of the environment that can affect mating interactions and the expression of male and female mating traits is the presence of vegetation. The structural complexity of vegetation can provide safety from predators (Murdoch & Oaten, 1975) and shield individuals from competitors (Hixon & Menge, 1991, Danley, 2011), but also can obscure some mating signals, interfering with the expression of male signals and/or female preferences (Dzieweczyński & Rowland, 2004, Hibler & Houde, 2006, Candolin et al., 2007, Myhre et al., 2013). A lack of vegetation can facilitate transmission of visual signals but increase the intensity of male competition and the risk of predation. Therefore, the presence or absence of vegetation can alter how males court, how females evaluate potential mates both within and between species, and how much sexual isolation results.

Phenotypic plasticity in mating traits can be a key factor in responding to changes in habitat. Early in the speciation process, plasticity can allow individuals to adjust mating behavior and/or mating preference to accommodate novel habitats (Irwin & Price, 1999, Pfennig et al., 2010). Within species, examples have shown that males can quickly adjust their mating traits in response to new environments (e.g., Rodriguez et al., 2008, Halfwerk & Slabbekoorn, 2009), and females can adjust their preferences to accommodate rapid changes in male traits (e.g., Tinghitella & Zuk, 2009). As divergent adaptation proceeds later in the speciation process, loss of plasticity and genetic accommodation becomes more likely, and can actually buffer diverging species from environmental change that could undermine sexual isolation (Pfennig et al., 2010). Therefore, depending on where diverging populations are in the

speciation process, plasticity of mating traits and preferences in different environments may enhance or undermine sexual isolation.

Here we test how the environment can modulate the expression of female preferences and male courtship traits important for sexual isolation. We explore these questions in pairs of limnetic-benthic threespine stickleback species (*Gasterosteus* spp.), a model system for studying ecological speciation (Schluter, 2001, McKinnon & Rundle, 2002) and an excellent system in which to test how the environment affects the expression of sexual isolation.

Different habitats affect both the evolution and current maintenance of various reproductive barriers, including sexual isolation (Boughman, 2006). Abundant evidence shows that diverging natural selection arising from environmental differences is essential for speciation (e.g., Rundle et al., 2000), with ecologically dependent postmating isolation as the hallmark of ecological speciation (Schluter, 2001, Rundle & Nosil, 2005). Moreover, sexual selection and sexual isolation are also ecologically dependent (Boughman, 2001, Boughman et al., 2005, Boughman, 2006). Divergent natural selection between distinct feeding and mating habitats has generated species differences in color, size, and shape (Bentzen & McPhail, 1984, Bentzen et al., 1984, Schluter, 1993, Schluter, 1995, Boughman, 2001), each of which females pay attention to during mate choice either within or between species, or both (Nagel & Schluter, 1998, Boughman, 2001, Boughman et al., 2005, Head et al., 2009, Kozak et al., 2009, Conte & Schluter, 2013, Head et al., 2013). Sexual selection via male competition also generates divergent selection on male traits in different habitats, favoring small, red males in open habitat and large, dull males in vegetation (Lackey & Boughman, 2013a). How female choice and male competition are jointly affected by habitat has not been explored however. In the current

study, we expand on all this prior work by evaluating whether environmental differences affect how females express divergent preferences that produce sexual isolation, or affect how males court. We consider these effects on the expression of sexual isolation in the context of well known effects of environment on the evolution of reproductive isolation for stickleback species.

We compared fish from two lakes that differ currently in the presence of different microhabitats and where they are in the process of speciation. Paxton Lake has two distinct mating habitats, with limnetic males nesting in the open and benthic males in dense plants (McPhail, 1994). Historical sexual isolation was strong and remains so to the present day (Lackey & Boughman, 2013b), and habitat differences are key to the evolution of isolation (e.g., Schluter, 1995, Boughman, 2001, Rundle, 2002). Enos Lake historically had these two distinct mating habitats (McPhail, 1984, Ridgway & McPhail, 1984, McPhail, 1994), but an invasive crayfish destroyed habitats with dense plants, and only open habitat now remains (Taylor et al., 2006). Sexual isolation was strong historically in Enos fish (Ridgway & McPhail, 1984) but has been lost since the introduction of the crayfish (Lackey & Boughman, 2013b). Using Paxton fish, we test whether habitats help maintain sexual isolation currently, in the context of how habitats helped to generate sexual isolation in evolutionary time. Using Enos fish, we test whether changes in mating habitats affect the expression of female preferences and male courtship traits, and whether this would have weakened sexual isolation, contributing to the increases in hybridization observed.

We measured the strength of sexual isolation in dichotomous choice trials with a single female choosing between a benthic and limnetic male across three habitat treatments: native, alternative, and open. Native habitat matched wild nesting patterns (benthics in vegetation and

limnetics in the open) and alternative habitat swapped male ecotypes between habitats. Open habitat lacked all vegetation. We asked if habitats affect how strongly sexual isolation is expressed, and if this depended on having both habitats present, or on whether mating took place in native or alternative habitat. We reasoned that the strongest isolation would be expressed in native habitat given that the species evolved there. First, we asked if habitat affects female preferences and sexual isolation by comparing the magnitude of sexual isolation by each species in each habitat treatment. We focused on the ability of females to discriminate between con- and heterospecific males, and asked whether this discrimination depended on the same trait differences across habitats. Second, we asked if habitat modulates male mating traits by comparing male color, size, courtship, and male-male aggression across habitat treatments. Third, we explored how habitat affected the two components of sexual selection -- female choice and male competition -- by focusing on male-male aggression here and by comparing our female choice results to previous findings on male competition (Lackey & Boughman, 2013a). And last, we compared results across lakes to determine if the extent of plasticity in mating traits affects how vulnerable sexual isolation is to environmental change.

Materials and Methods

We collected wild stickleback fish in mid-April 2011 from Paxton Lake, Texada Island and Enos Lake, Vancouver Island in British Columbia. We identified reproductive males and females by the presence of nuptial color and eggs, respectively. We used species-specific characteristics of body shape, size, and color to identify limnetic and benthic fish in Paxton Lake and the most limnetic-like and benthic-like fish in Enos Lake (McPhail, 1984, McPhail, 1992, McPhail, 1994).

Pure limnetics and benthics in Enos Lake are rare due to recent hybridization (Gow et al., 2006, Taylor et al., 2006). Categorizing fish by body shape has been successful in another study, where identification by body shape and genetics matched at a 97% success rate (Taylor et al., 2006). We use ‘ecotype’ to refer to Paxton species and Enos morphs. We use ‘homotypic’ to refer to fish of the same ecotype [e.g., two limnetic(-like) fish] and ‘heterotypic’ to refer to fish of different ecotypes [e.g., a limnetic(-like) and a benthic(-like) fish]. Fish were transported to our lab and housed in tanks by sex, ecotype, and lake. We maintained fish at summer conditions with 14-hour day lengths and 18°C room temperatures. We fed fish brine shrimp (*Artemia* spp.) and bloodworms (*Chironomus* spp.) once per day.

Mating trials

We set up 75-gallon tanks for female dichotomous choice trials with one female and two males. To each tank, we added a one-inch thick layer of fine-grain sand as nesting substrate. Then we set up one of three habitats in each tank: native, alternative, or open. Both native and alternative habitats had sixteen plastic plants evenly spaced on the “vegetated” half of the tank and nothing added to the “open” half of the tank. Open habitat tanks had two “open” halves. Next, we divided the tank in half with an opaque divider. We selected males that had developed nuptial color and territorial behaviors. Each tank had one male of each ecotype. Native habitat had a benthic(-like) male in the vegetated half and a limnetic(-like) male in the open half. Alternative habitat swapped the male ecotypes with respect to habitat so that a benthic(-like) male was in the open and a limnetic(-like) male was in the vegetation. In open habitat, both halves of the tank were open with a benthic(-like) male in one half and a

limnetic(-like) male in the other. We randomized habitat and male placement with respect to the left or right side of the tank as allowed by our design. To each tank half, we added pieces of aquatic plant material (*Chara* spp.) that males use to build nests in the wild. We enticed males to build nests by removing the divider and placing a gravid female in a clear jar in the middle of the tank for 15 minutes a day. We alternated the ecotype of female seen each day so each male saw equal numbers of homo- and heterotypic females. In the wild, males are very likely to encounter both female ecotypes during the breeding season (Boughman, 2006). During enticements, males could court the female and engage in competition. This reflects how males typically establish territories and nesting sites in the wild because courtship and competition can occur simultaneously (van den Assem 1967). Additionally, males of each species are often territorial neighbors even if they nest in different microhabitats (Ridgway & McPhail, 1987). Our experimental setup best replicates male interactions between ecotypes where open and vegetated habitats meet. Habitat patches in the wild are larger than we use here, and some males in the wild may not nest next to heterotypic males. However, females can easily travel between habitat patches (Boughman, 2006), so it is highly relevant and appropriate to explore how females evaluate males of each ecotype as potential mates.

Once both males built nests, a prerequisite for spawning, we conducted female choice trials. We removed the divider and placed a female in an opaque holding container in the middle of the tank. After a five-minute acclimation period, we released the female. We started the trial when one of the males interacted with the female, and we recorded courtship and male competition behaviors for 25 minutes or until the female entered one of the nests to spawn. For courtship, we recorded the following male behaviors involved in attracting the

female: zig-zag, bite, chase, and lead to the nest (Wootton, 1976, Ridgway & McPhail, 1984, Rowland 1989). For females, we recorded the following: head-up (indicating receptivity), approach, follow a male's lead, examine a male's nest, and enter the nest (Wootton, 1976, Rowland, 1989, Kozak et al., 2009). We did not allow a female to spawn in the nest so that she could be used in a subsequent trial. For male competition, we recorded bites, chases, and charges between males (Iersel, 1953). At the end of the trial, we removed the female and replaced the divider in the middle of the tank.

Most females had two trials, each with a different pair of males. There was a two-hour resting period between a female's trials. For males, each pair of males had up to two trials: one with a limnetic(-like) and one a benthic(-like) female, with at least two hours between trials. We reused some males as part of a new pair because the breeding season is short, about 10 weeks, and we were limited by the number of males that would build a nest. Males took six days on average to build a new nest, but males whose nests have been moved fixed them typically in less than a day. If we reused males, we moved the male and his nest into a new tank with a new male partner. We kept the habitat half where he built his nest (open or vegetated) the same. Before using a male in female choice trials, we ensured that the male's nest had a visible entry hole and that the male was guarding and tending his nest. See the Statistical Analyses section below for details on how we accounted for multiple trials with each male and female in our models. We ran a total of 231 female choice trials with 121 unique pairs of males. For Enos females, we ran 46 native, 46 alternative, and 45 open habitat trials. For Paxton females, we ran 32 native, 30 alternative, and 32 open habitat trials.

We recorded a number of morphological traits for males. We measured each male's standard length before and after all of his trials using Vernier calipers accurate to 0.2 mm. We averaged these two measurements to determine a male's average standard length. Before and after each trial, we recorded a male's nuptial throat color. We used a standardized color scoring method developed in our lab group (Boughman, 2001, Boughman, 2007, Lewandowski & Boughman, 2008) that closely matches reflectance data (Albert et al., 2007, Boughman, 2007). We measured male red throat color area and intensity each on a scale of 0 - 5, where 0 indicates no color and 5 indicates maximum color area or intensity. We summed area and intensity scores to get a red index that ranged from 0-10. We averaged the red index before and after each trial to determine the male's average red index for that trial.

For each trial, we quantified female preference, male courtship, and male competition. First, we calculated the strength of a female's preference using preference score, which measures the extent of interest in a particular male by using how far a female progressed in courtship on a scale from 0 - 4. A male received a score of 0 if the female responded to none of the male's courtship behaviors (was completely non responsive), 1 if she approached the male (indicating initial interest), 2 if she followed the male (indicating sustained interest), 3 if she examined the nest (the last step before actual mating), and 4 if she entered the nest (final acceptance of the male for mating). Then we calculated the difference between a female's preference scores for homo- and heterotypic males (homo minus hetero). This preference score difference ranged from -4 to 4, and positive values indicate that she showed more interest and proceeded farther in courtship with the homotypic than the heterotypic male. This difference in preference score is our measure of female discrimination; hereafter, we refer to it as such. For

each male, we calculated the rate per minute of three types of courtship by summing the relevant courtship behaviors directed toward the female divided by total trial time. We calculated courtship vigor and its two components: aggressive courtship and display courtship. Previous work has shown that males perform more aggressive or display oriented courtship depending on the female ecotype he is courting (Kozak et al., 2009). Calculations for courtship vigor include all male courtship behaviors: zig-zags, leads, bites, and chases (Kozak et al., 2009). Aggressive courtship includes just bites and chases, and display courtship includes just zig-zags and leads (Kozak et al., 2009). Next, we calculated the difference in courtship vigor, aggressive courtship, and display courtship between male ecotypes (homo minus hetero). Positive values indicate that the homotypic male courted the female more vigorously, aggressively, or with more display than the heterotypic male. We also calculated the rate of male-male aggression for each male by summing the number of bites, chases, and charges directed toward the other male and dividing this sum by the total trial time. We calculated the male-male aggression difference between homo- and heterotypic males, where positive values indicate the homotypic male was more aggressive to his rival than vice versa.

Statistical analyses

We analyzed our response variables of female discrimination, courtship difference (vigor, aggressive, or display), and male-male aggression difference using mixed models. All differences were homo- minus heterotypic. We expected that patterns of female discrimination might vary between females from each lake, so we tested whether female discrimination was explained by female ecotype [benthic(-like) or limnetic(-like)], lake (Paxton or Enos), habitat

(native, alternative, or open), or their interactions. We found a significant interaction between lake and female ecotype (Figure 3.1), so we ran all subsequent analyses by lake. All of these 'by lake' models included female ecotype and habitat as categorical factors. Each female had up to two trials, so we used repeated measures with a compound symmetry covariance structure that assumes each female's trials were correlated. We also included a random effect of male pair. For female discrimination, we included all of the following continuous covariates: red difference, length difference, courtship difference (vigor, aggressive, or display), and male-male aggression difference. We tested for collinearity between our continuous covariates using the variance inflation factor (VIF). Values greater than 10 suggest strong collinearity, and all our VIF values were less than 1.9. We included all possible interactions between categorical variables. For continuous covariates, we included all two-way interactions with continuous and categorical variables. We reduced models by removing nonsignificant terms.

To determine how male behavior could influence female discrimination across habitats, we asked whether differences between male ecotypes in male courtship (vigor, aggressive, or display) or male-male aggression behavior was affected by lake, habitat, and whether the female was homo- or heterotypic. We included all possible interactions terms and removed nonsignificant terms. Here, we used repeated measures with male as the subject and female as a random factor. We analyzed the data in SAS 9.2. For post-hoc tests, we controlled for multiple comparisons using false discovery rate (FDR) (Benjamini & Hochberg, 1995, Verhoeven et al., 2005), and we report raw and FDR-controlled p-values.

We next estimated sexual isolation using I_{PSI} (Rolan-Alvarez & Caballero, 2000) in the JMATING program that accommodates data from different choice designs, including the female

choice design we used here (Carvajal-Rodriguez & Rolan-Alvarez, 2006). This program uses the number of mating interactions out of total trials between males and females of different ecotypes. We used whether a female examined a nest to estimate I_{PSI} because spawning happened too infrequently to run statistical tests for females from each lake and in each habitat. We used bootstrapping to estimate I_{PSI} , its standard deviation, and significance. P-values derived from bootstrapping are conservative (Carvajal-Rodriguez & Rolan-Alvarez, 2006), so we do not control these p-values for multiple comparisons.

We used path analysis to estimate the relative contributions of red color, body length, courtship vigor, and male-male aggression differences between male ecotypes to female discrimination. We wanted to understand the effect these traits had on female discrimination and also to compare the effects of male competition and female choice. We tested three models. In all models, we allowed a correlation between red color and length differences and between courtship and aggression differences. Prior work suggests that red color and length are negatively correlated between limnetic and benthic species as limnetic males are smaller but redder than benthic males (Boughman et al., 2005), however, we did not know if color differences between male ecotypes would correlate with size differences. We allowed for a correlation between courtship vigor and male-male aggression differences because a male's relative competitive ability could affect how much he could court the female. Also, previous work found that courtship and aggression were correlated (Rowland, 1984). In all models we also predicted that differences in red and length between male ecotypes would increase male courtship and male-male aggression differences as well as female discrimination. Previous work has shown relationships between male traits of color, size, and aggression (Bakker & Sevenster,

1983, Rowland, 1983, Bakker & Milinski, 1993). Additionally, female preferences are strongest when male color, size, and courtship traits are present (Kunzler & Bakker, 2001). Many studies have also shown that females choose mates based on color and size (Nagel & Schluter, 1998, Boughman, 2001, Boughman et al., 2005, Conte & Schluter, 2013). The differences between the three models involve the relationships of courtship and aggression differences with female discrimination. The baseline model predicted that both courtship and aggression differences directly increased female discrimination. The second model predicted that larger courtship vigor differences between male ecotypes strengthened female discrimination while larger male-male aggression differences did not. The third model predicted the alternative; larger male-male aggression differences between male ecotypes strengthened female discrimination while larger differences in courtship vigor did not.

We then tested these three models to parse out the relative importance of ecotype differences in courtship vigor and male-male aggression for female discrimination. The best fit model predicted that greater differences in male courtship vigor but not male-male aggression would increase female discrimination (AIC = 28.51, Goodness of Fit Index = 0.9991). An alternative model with a relatively poorer fit (AIC = 123.58, Goodness of Fit Index = 0.8803) predicted that greater differences in male-male aggression but not courtship vigor would increase female discrimination. Both of these models were significantly better than the baseline model that predicted that both differences in courtship vigor and male-male aggression would affect female discrimination (differences in chi-square fitting criterion test: both $\chi^2_{10} > 273$, and $p < 0.0001$). We pooled males from Paxton and Enos lakes because path diagrams for each lake were nearly identical, except that the relationship between differences in red and male-male

aggression was marginally significant in Enos ($p = 0.07$) but not Paxton ($p = 0.22$) fish. We performed these analyses in SAS 9.2 using PROC CALIS.

Results

First we examined female discrimination and sexual isolation pooled across habitats. Both female ecotypes from Paxton Lake discriminated strongly between male ecotypes and preferred homotypic males (Figure 3.1A), which resulted in strong sexual isolation (Figure 3.1B). In contrast, only benthic-like females in Enos Lake discriminated between male ecotypes and preferred homotypic males, while Enos limnetic-like females did not (Figure 3.1A). Enos females also lacked sexual isolation (Figure 3.1B). The significant interaction between female ecotypes across lakes ($F_{1, 106} = 4.01$, $P = 0.0477$, Figure 3.1A) warranted running subsequent analyses separately by lake.

Next we tested the strength of female discrimination and sexual isolation across habitats to determine whether habitats modulate the expression of discrimination and isolation. Habitat had only minor effects for both Paxton female ecotypes and Enos benthic-like females. In contrast, the strength of discrimination and direction of preference changed across habitats for Enos limnetic-like females (Figure 3.2). Discrimination and sexual isolation in Paxton fish neared significance in native habitat but not in alternative or open habitat, perhaps because only one species preferred homotypic males in each of these habitats (Figure 3.2B). Habitat altered how Enos limnetic-like, but not benthic-like, females expressed discrimination (Figure 3.2C). Enos benthic-like females tended to prefer homotypic males in all habitats, with strongest effects in native habitat. Unexpectedly, Enos limnetic-like females slightly preferred

heterotypic males in both the native and open habitats with a nonsignificant trend to prefer homotypic males in alternative habitat. Sexual isolation was absent in Enos females across all habitats (Figure 3.2D).

We then turned to testing which trait differences influenced female discrimination between homo- and heterotypic males. We considered differences between male ecotypes in red color, body size, courtship vigor, and male-male aggression (Table 3.1A). The primary factor across both lakes was difference in courtship vigor. Females preferred the more vigorously courting male and discriminated between male ecotypes more strongly with large differences in vigor (Table 3.1A). Only in Enos females did this effect depend on habitat, and the effects were strongest for Enos limnetic-like females (Table 3.1B, Figure 3.2C). Enos males changed how aggressively they courted in alternate habitat, especially so when courting limnetic-like females (interaction of habitat with female ecotype: Enos: $F_{2, 63} = 3.90$, $P = 0.0254$, Paxton: $F_{2, 41} = 2.62$, $P = 0.0846$, Figure 3.3). These differences parallel the pattern of discrimination for Enos limnetic-like females (Figure 3.2C). In native and open habitats, Enos benthic-like males courted limnetic-like females more aggressively than did limnetic-like male rivals. In alternative habitat, Enos male ecotypes courted females with equal aggression. Importantly, Enos females discriminate more strongly with larger differences in aggressive courtship between male ecotypes (Enos: $F_{1, 54} = 8.20$, $P = 0.0059$, Paxton: $F_{1, 32} = 1.96$, $P = 0.1716$).

Finally, we used path analysis to examine how interactions between female choice and male competition might influence female discrimination. We predicted that larger differences in red color and body length would affect discrimination directly and would also have indirect

effects by leading to larger differences in courtship vigor (a target of female choice) and in male-male aggression (a target of male competition). These differences in turn would yield stronger discrimination in females. The best fit path model is shown in Figure 3.4 (see methods for details of model fitting). We pooled males from Paxton and Enos lakes because path diagrams for each lake were nearly identical. We found that females discriminated between male ecotypes more strongly with larger differences in courtship vigor, and this was the only significant path to female discrimination (Figure 3.5). Surprisingly, females did not discriminate between male ecotypes based directly on male differences in red color or body length, consistent with findings in Table 3.1. Instead, males that differed more in red and/or length differed more in courtship vigor, which indirectly led to stronger female discrimination. Habitat had very little effect on these relationships, with the single change that red color had no direct effects on vigor or aggression in open habitat, leaving body length as the sole determinant. We also found that male competition had no direct effects on female discrimination.

Discussion

Habitat sensitive expression of female discrimination and the evolution of reproductive isolation

Differences in environment figure prominently in ecological speciation because contrasting environments generate divergent selection. As phenotypic traits diverge, various isolating barriers arise as a pleiotropic consequence, and speciation proceeds. Given this central role of environment in the evolution of reproductive isolation, we asked whether contrasting environments affect the current expression and thus, the maintenance of reproductive isolation, focusing specifically on sexual isolation. Our results are surprising. In contrast to these

expectations, habitat had little effect on the expression of female discrimination, and these effects were confined to limnetic-like females from Enos Lake. These were also the only females to slightly prefer heterotypic males, except in alternate habitat.

The small effects we found of habitat on the expression of female discrimination and maintenance of sexual isolation contrast markedly with substantial effects of habitat on the evolution of these traits. Earlier work has found that environmental differences are critical to the evolutionary change in male mating signals, female preferences for those traits, and the sexual isolation that results in sticklebacks (Boughman, 2001, Boughman et al., 2005) as well as other taxa (reviewed in Maan & Seehausen, 2011). For example, water color and vegetation affect the transmission of male color, favoring the evolution of conspicuous signals in several fishes (Boughman, 2001, Fuller, 2002, Seehausen et al., 2008). Water color also affects the evolution of female color perception and color preference (Boughman, 2001, Carleton et al., 2005, Seehausen et al., 2008). So, contrasting environments generate divergent selection on male signaling traits and female preferences, leading to their evolutionary divergence and enhanced female discrimination between con- and heterospecific males. These evolutionary changes in male and female traits have been shown to enhance sexual isolation in multiple groups of fishes (Boughman, 2001, Craig & Foote 2001, Maan et al., 2006, Maan et al., 2008, MacColl, 2009). Thus, for female discrimination, the environment appears to be more critical in its evolutionary role than in its maintenance role.

Habitat plays a minor role in the expression of sexual isolation but a major role in the expression of other important isolating barriers, especially ecologically dependent postmating isolation and immigrant inviability. For the expression of immigrant inviability, selection acts

against migrants that leave their native habitat, causing reduced survival generally (Nosil et al., 2005) and for sticklebacks (Schluter, 1993, Schluter, 1994, Schluter, 1995, Rundle, 2002, Vamosi, 2002). The evolutionary response might be to reduce dispersal, and the consequence for speciation is to limit gene flow from one environment to another. The hallmark isolating mechanism of ecological speciation is ecological selection against hybrids. The expression of this barrier occurs when hybrids suffer reduced fitness in distinct parental habitats because their intermediate phenotypes are poorly adapted to either environment (Schluter, 2000, Schluter, 2001). The expression of this barrier has been tested extensively in many taxa (e.g., Nosil et al., 2005), including sticklebacks (Schluter, 1995, Hatfield & Schluter, 1999, Rundle, 2002, Gow et al., 2007, Behm et al., 2010, Taylor et al., 2012). The evolutionary response is that phenotypes diverge to enhance local adaptation, and the consequence for speciation is to reduce gene flow between diverging ecotypes. Clearly, environmental differences are critical both to the expression and evolution of these isolating barriers.

Habitat sensitive expression of male courtship

Despite weak effects of habitat on female discrimination, we found that habitat affects the expression of courtship behavior in males, primarily by altering how aggressively they court females. The presence of vegetation could change how male ecotypes interact with females and with each other during courtship, potentially reducing simultaneous and competitive courtship (Myhre et al., 2013). The pattern of plasticity in response to habitat in aggressive courtship mirrors habitat effects on aggressive male competition (Lackey & Boughman, 2013a). Changes in Enos limnetic-like female discrimination seem to follow these changes in male

courtship. This makes sense in the light of our surprising results from path analyses. Male color and size affect female discrimination only indirectly through their effects on courtship behavior; they have no direct effects. Thus, difference in courtship is the key to whether females discriminate between homotypic and heterotypic males. These differences are sensitive to habitat. Both body size and red color affect male courtship behavior and thus female discrimination in the presence of vegetation, yet in open habitat only size mattered, consistent with prior findings for habitat effects on male competition (Lackey & Boughman, 2013a). Given that female discrimination depends strongly on courtship vigor and courtship aggressiveness in all habitats, this suggests that female discrimination is responding to variation in male courtship, rather than habitat per se. Thus, the expression of male aggressive behavior appears to be modulated by environment to a greater extent than the expression of female discrimination behavior.

Given that we found the traits that made a male more successful than his rival in courtship – larger size and more red color -- also made him more successful in male competition suggests that female choice and male competition may work in concert and select similarly on male traits. This is not because they interact directly. For example, male competition does not necessarily interfere with female discrimination, nor do females appear to choose based on observing competitive interactions. Instead, these two sources of sexual selection appear to favor similar traits. Much of the work exploring interactions between male competition and female choice has been within species (reviewed in Wong & Candolin, 2005, Hunt et al., 2009). In some cases, male competition and female choice oppose each other (Bourne, 1993, Sih, 2002, Candolin, 2004). If so, this opposing selection would slow the rate of

evolutionary change. Yet, there are cases where they act in concert and this should accelerate evolutionary change (Andersson, 1994, Berglund et al., 1996, reviewed in Hunt et al., 2009). Extending these ideas to the context of speciation would be fruitful. We suggest that opposing selection by male competition and female choice would likely slow the rate of divergence in male traits and limit the evolution of sexual isolation. In contrast, synergistic selection should speed up the divergence process thereby enhancing sexual isolation between diverging populations. Most work on sexual selection and speciation focuses on the effects of female choice; we suggest our understanding will broaden by incorporating male competition.

Plasticity and speciation

By and large, with respect to habitat there was little plasticity in discrimination against heterotypic males. Only Enos limnetic-like females showed plastic discrimination, and this plasticity weakened sexual isolation. This may partly explain the asymmetric reproductive isolation in this species pair, where limnetic alleles are introgressing into the benthic genome as hybridization proceeds (Gow et al., 2006). This plasticity, therefore, appears to be non-adaptive. The plastic expression of female discrimination can affect both the direction and rate of speciation; in this case, plasticity would move the speciation process backwards, rather than forwards, by reducing the magnitude of reproductive isolation. It seems the limited plasticity in discrimination, which increases acceptance of heterospecifics, and the greater plasticity in male courtship, which decreases differences between species, has made the Enos species pair more vulnerable to environmental change. In contrast to the plastic expression in Enos limnetic-like females, discrimination in both Paxton species and Enos benthic-like females was insensitive to

habitat. The lack of plasticity in discrimination at the fairly advanced stage of speciation shown by the Paxton pair would be likely to enhance continued divergence in the Paxton Lake pair. The relative paucity of plastic female discrimination coupled with known differences between species in the targets and strength of female preference (Boughman, 2001, Boughman, 2006) suggests a genetic basis to preference and discrimination.

Habitat induces little plasticity in female discrimination. In contrast, several traits that are the basis for that discrimination show plasticity, including body size (Nagel & Schluter, 1998, McKinnon et al., 2004, Boughman et al., 2005), body shape (Day et al., 1994, Day & McPhail, 1996, Head et al., 2013), male nuptial color (Boughman, 2001, Lewandowski & Boughman, 2008), and male courtship (Kozak et al., 2009, this study). The morphological traits body size and body shape are divergent adaptations and contribute also to ecologically dependent postmating isolation and immigrant inviability. Whether such traits typically show plasticity is unclear at present, but this is an intriguing possibility. Typically, one expects that behavior will show high plasticity compared to morphology. Our results suggest that the reverse can be true, at least for the key behavioral trait of female conspecific discrimination. Whether limited behavioral plasticity acts as an accelerator or brake on speciation deserves further study, as it fits into the ongoing debate about whether plasticity accelerates or retards evolutionary change generally (reviewed in Pfennig et al., 2010).

Environmental change and reverse speciation

In this and previous work, we have discovered several factors that appear to cause increased and asymmetric hybridization leading to reverse speciation in Enos Lake. First, only

limnetic-like females have plastic preferences, and they appear to favor heterotypic males except when in vegetation. Second, sexual isolation is weak in the Enos pair, shown both here and in our earlier work (Lackey & Boughman, 2013b), even though historically this was a strong isolating barrier (Ridgway & McPhail, 1984). Third, size alone affects male courtship vigor differences in open habitat, which in turn mediate female discrimination; thus, the historically strong effect of differences in male color and female color preference in generating sexual isolation (Boughman, 2001) do not appear to be in force any longer, mediated in part by the loss of vegetation in the lake and consequent changes in light environment. Fourth, earlier work found that size alone influenced success in male competition in the absence of vegetation, with larger males winning (Lackey & Boughman, 2013a). Given that benthics are larger than limnetics, benthic males would be more likely to establish territories, build nests, and court females in the open habitat remaining in the lake. This could increase the encounter rates between species as well as limit opportunities for limnetic-like females to choose limnetic-like males as mates. And last, hybrids that are formed no longer experience low fitness (Behm et al., 2010), suggesting that the historically strong ecologically dependent postmating isolation has weakened and therefore, no longer removes hybrids from the population. The invasion of the lake by signal crayfish (*Pacifiscus leniusculus*) and subsequent loss of vegetation is thought to have triggered reverse speciation in this case (Taylor et al., 2006). As female discrimination is relatively insensitive to changes in habitat, this suggests that some other environmentally-sensitive mechanism likely initiated the loss of reproductive isolation (e.g., changes to male traits favored by male competition (Lackey & Boughman, 2013a)).

Conclusions

A key conclusion from our results is that environmental differences play a central role in the evolution of sexual isolation, but only a supporting role in its current expression and maintenance. This was somewhat surprising, in particular because maintaining sexual isolation - even when it has evolved due to differences in environment -- does not appear to depend on habitats being different. This pattern contrasts with other components of reproductive isolation, which depend on environmental differences for both their evolution and maintenance. Our results suggest that divergent selection generated by environmental differences could be more central to the speciation process than plasticity, even when that plasticity is adaptive.

APPENDIX

APPENDIX: Chapter 3 tables and figures

Table 3.1 **Effects of trait differences between male ecotypes on female discrimination and preference for homo- and heterotypic males.**

A. Model effects

	Model effects for female discrimination (homo-hetero)			
	Paxton		Enos	
Male trait difference (homo-hetero)	F	P	F	P
red	0.80	0.3766	0.11	0.7398
length	0.01	0.9160	0.74	0.3918
courtship vigor	26.03	<0.0001	85.60	<0.0001
courtship vigor x habitat	2.65	0.0859	4.34	0.0178
courtship vigor x female type	0.16	0.6930	4.94	0.0304
male-male aggression	0.01	0.9241	1.70	0.1978
male-male aggression x female ecotype	8.10	0.0076	1.39	0.2437

Separately by lake (Paxton and Enos), we ran models to test for effects of continuous covariates on female discrimination between male ecotypes. In (A), we show model effects for continuous covariates and their interactions with categorical variables of habitat (native, alternative, open), female type [limnetic-(like) or benthic-(like)]. The models also included main effects of habitat and female type as well as their interaction. Denominator degrees of freedom are 33 for Paxton and 55 for Enos fish. In (B), we present the slopes and associated test statistics for significant interaction effects. Significant p-values are in bold.

Table 3.1 (cont'd)

B. Slopes of significant model effects

Male trait difference	Lake	Habitat	Female Ecotype	Slope	S.E.	DF	T	P
courtship vigor x habitat	Enos	alternative		38.69	6.87	15	5.63	< 0.0001
	Enos	native		25.33	6.94	15	3.65	0.0024
	Enos	open		25.20	6.54	15	3.85	0.0016
courtship vigor x female type	Enos		limnetic-like	27.90	6.51	53	4.29	< 0.0001
	Enos		benthic-like	12.46	4.33	49	2.88	0.0059
male-male aggression x female type	Paxton		limnetic	12.96	6.06	32	2.14	0.0401
	Paxton		benthic	-15.78	7.20	26	2.19	0.0377

(A)

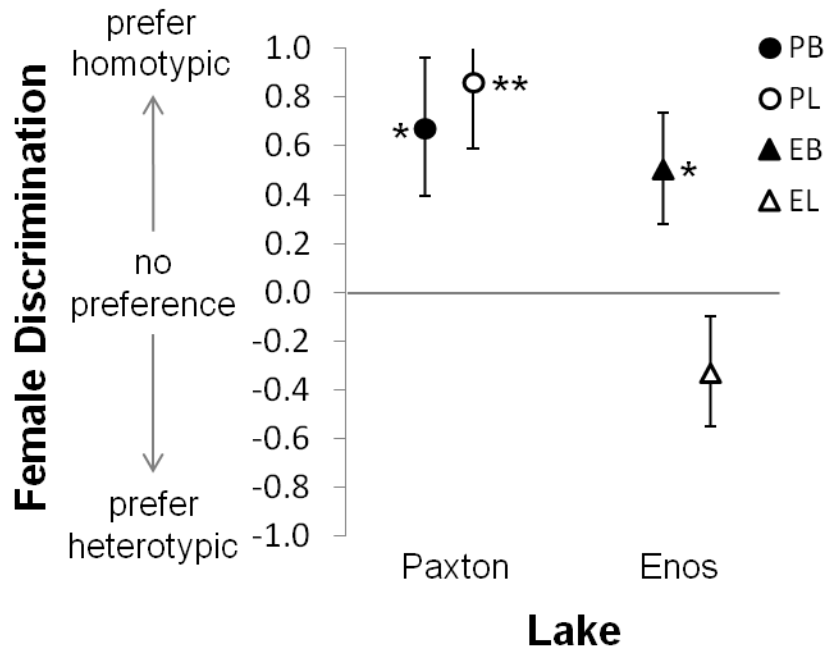
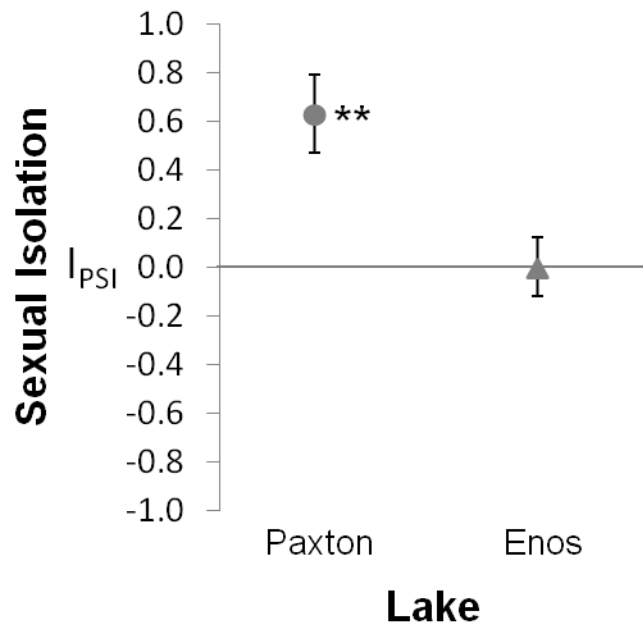


Figure 3.1 **Female discrimination, preference, and sexual isolation across lakes.** In (A), for each female ecotype from each lake, we show female discrimination between and preference for male ecotypes as measured by the difference in preference scores for homo- and heterotypic males. Positive values indicate that females preferred homotypic males more, while negative values show that females preferred heterotypic males more. Values not different from zero indicate that females preferred both males equally. We plot least squares (LS) means $\pm$ standard errors for female discrimination for Paxton benthic (PB), Paxton limnetic (PL), Enos benthic-like (EB), and Enos limnetic-like (EL) females. In (B), we plot sexual isolation for females from each lake using the metric I_{PSI} with standard deviations estimated from bootstrapping. In (A), p-values show significant differences of LS means from zero controlled with false discovery rate, and in (B), p-values are calculated from bootstrapping, with ** $P < 0.01$ and * $P < 0.05$.

Figure 3.1 (cont'd)

(B)



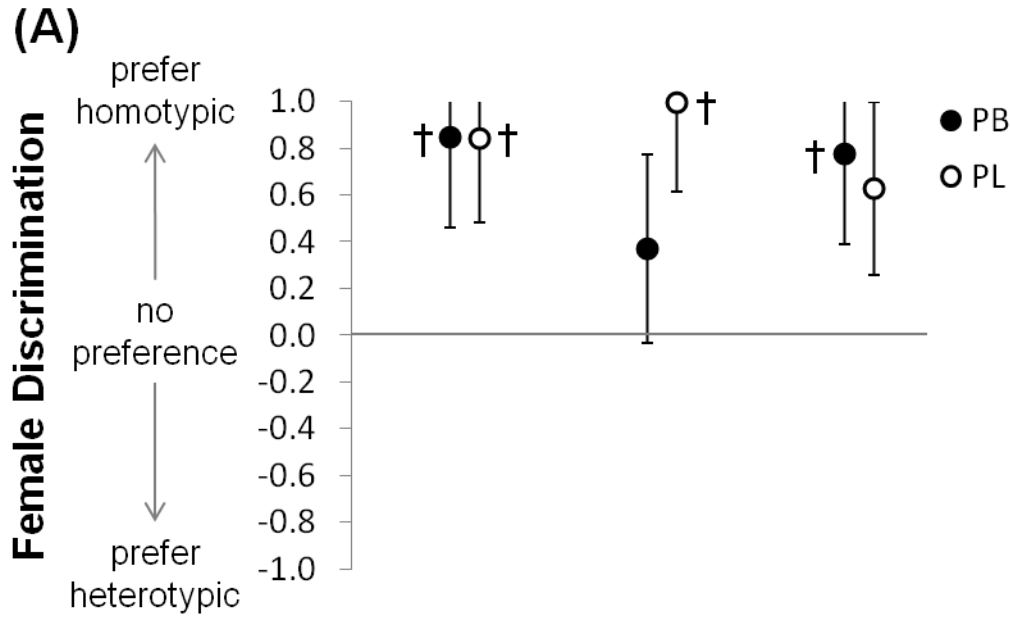


Figure 3.2 **Discrimination, preference, and sexual isolation across habitats and lakes.** We ran analyses for Paxton (A, B) and Enos (C, D) females separately because the interaction of lake and female ecotype was significant (Figure 1). We plot least squares means $\pm$ standard errors for Paxton benthic (PB) and Paxton limnetic (PL) females in (A) and for Enos benthic-like (EB) and Enos limnetic-like (EL) females in (C). We note significant differences from zero directly to the right or left of each symbol. We also show significant differences between female types with brackets and between habitats with dashed lines. All plotted p-values are FDR-controlled for multiple comparisons. We plot I_{PSI} , standard deviations, and p-values derived from bootstrapping for Paxton females in (B) and Enos females in (D). In all panels, * $P < 0.05$, † $P < 0.10$. Before comparing for multiple comparisons, all significance values in (A) and (B) indicated with † were $P < 0.036$ except for Paxton benthic females in open, where the uncontrolled p-value was 0.053.

Figure 3.2 (cont'd)

(B)

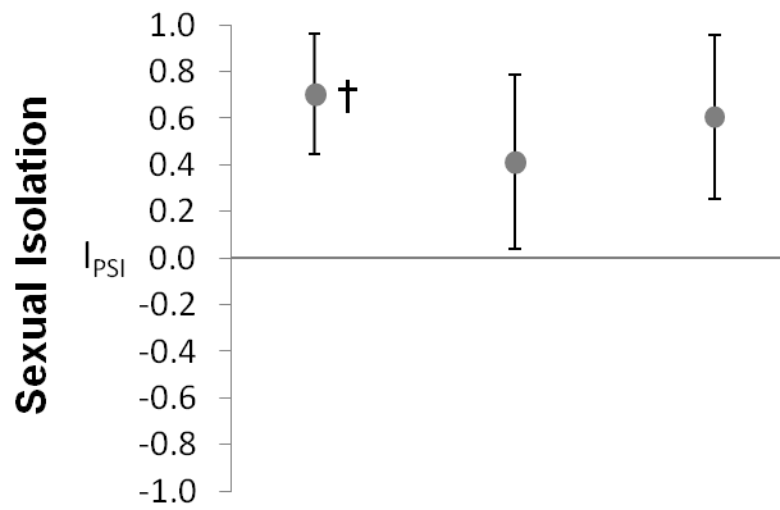
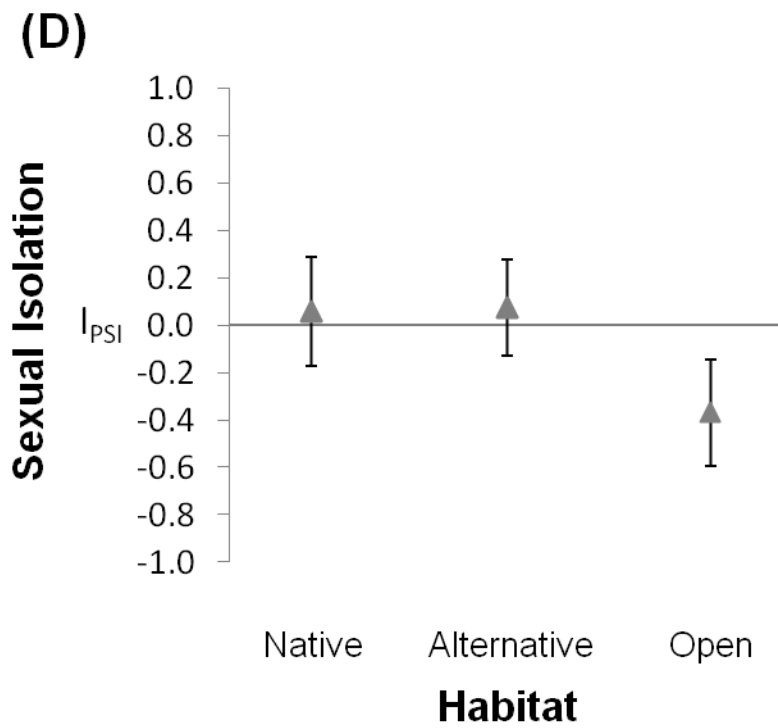
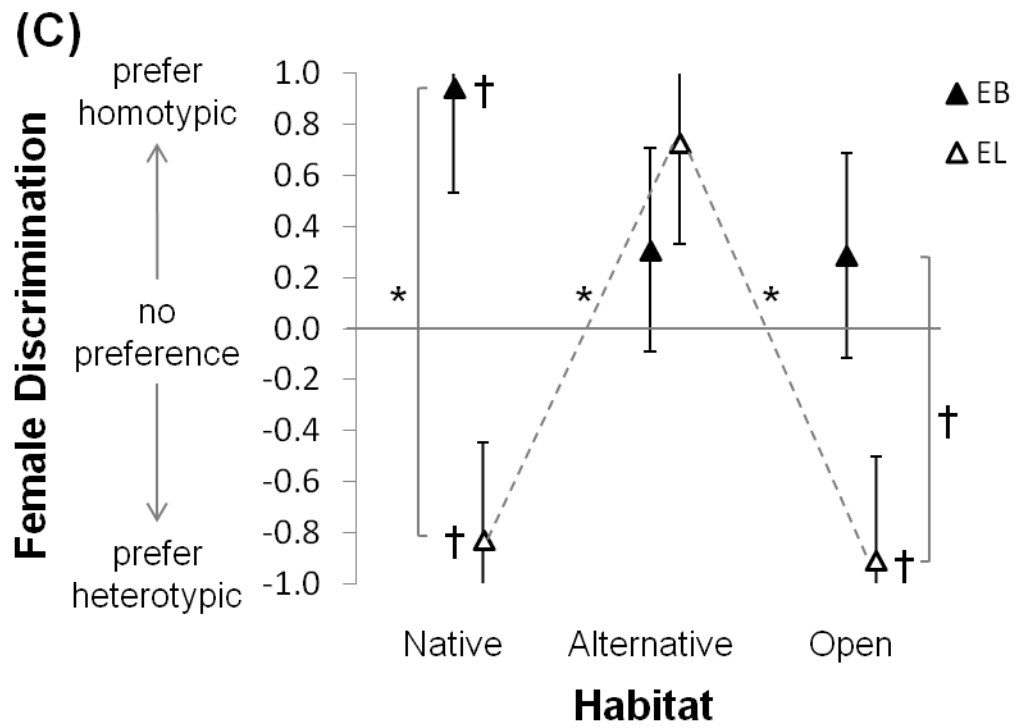


Figure 3.2 (cont'd)



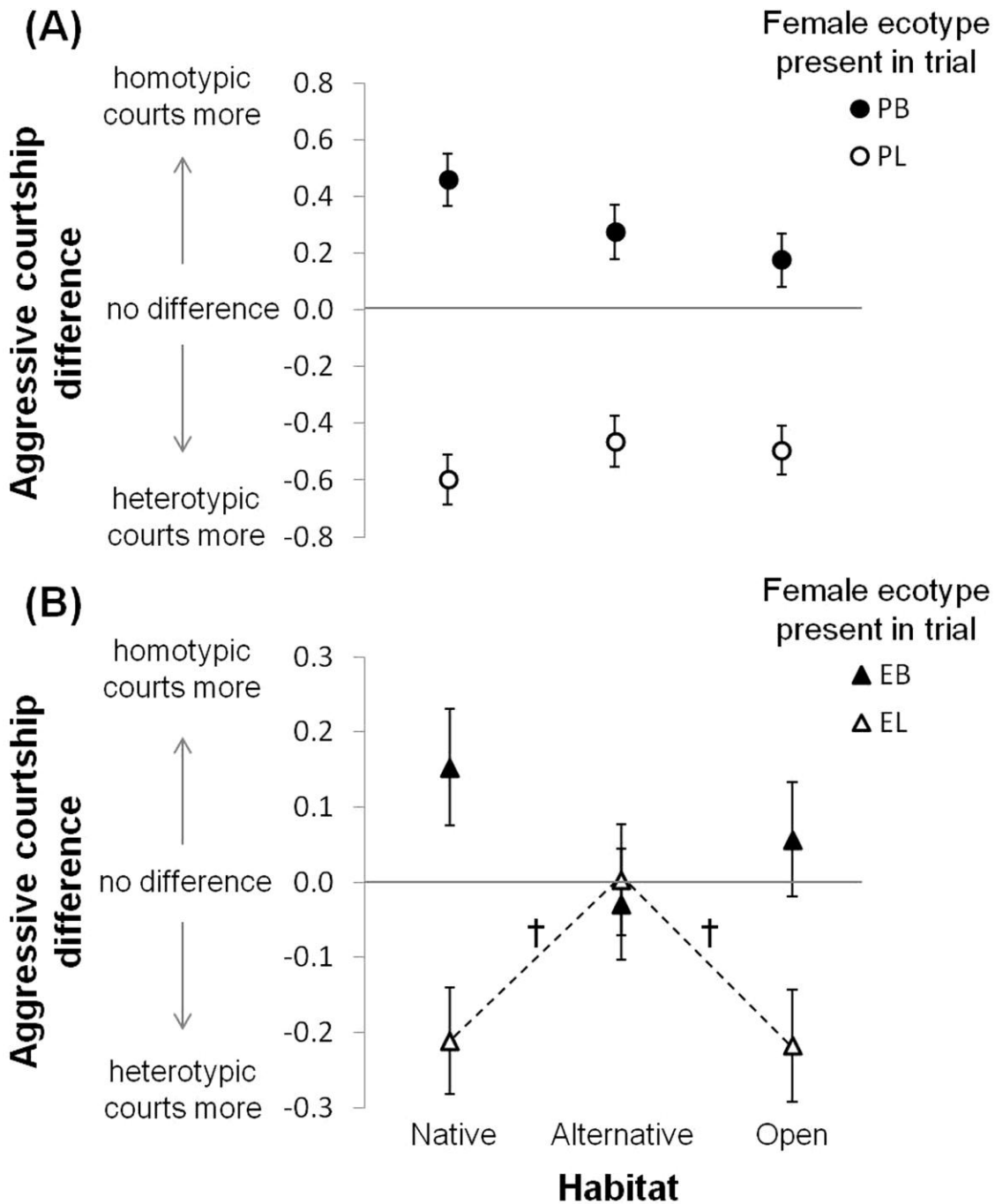


Figure 3.3 **Aggressive courtship differences between male ecotypes across habitats.** We plot least squares means $\pm$ standard errors for mating trials with Paxton benthic (PB) and Paxton limnetic (PL) females in (A) and for Enos benthic-like (EB) and Enos limnetic-like (EL) females in (B). For each female ecotype, we tested whether aggressive courtship differs between homo- and heterotypic males and if this varied across habitats. All plotted p-values are FDR-controlled for multiple comparisons, $\dagger P < 0.10$.

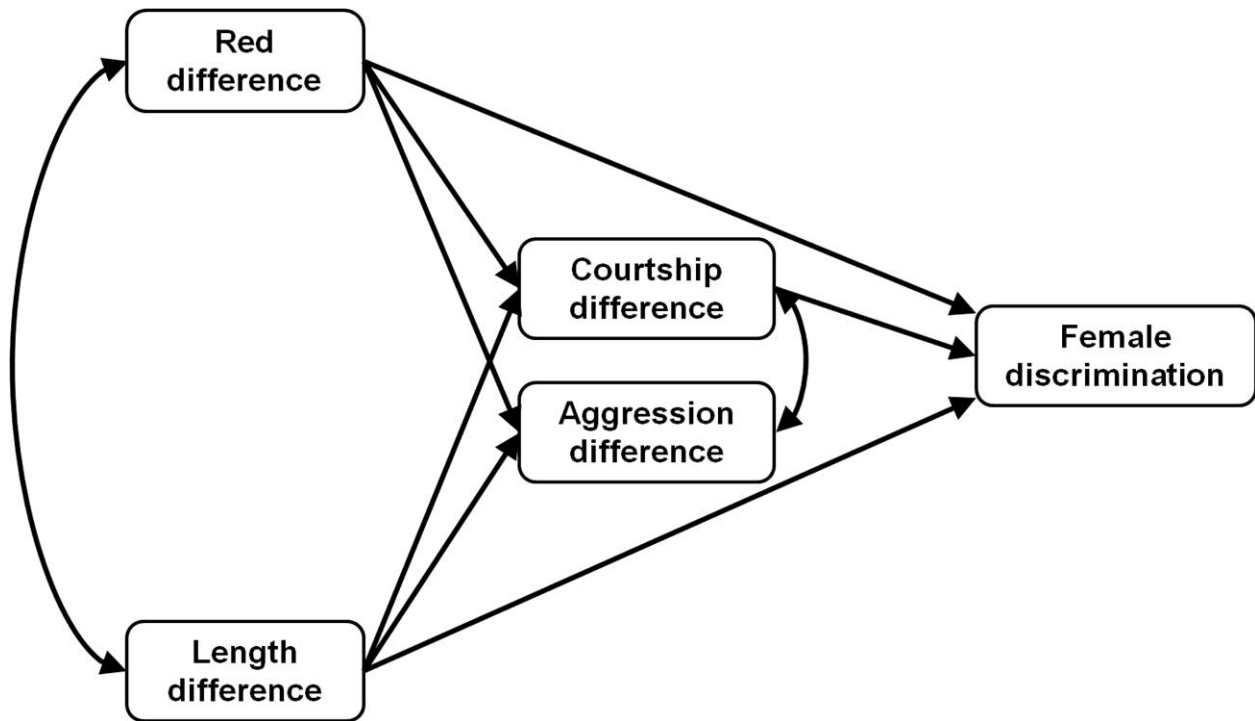
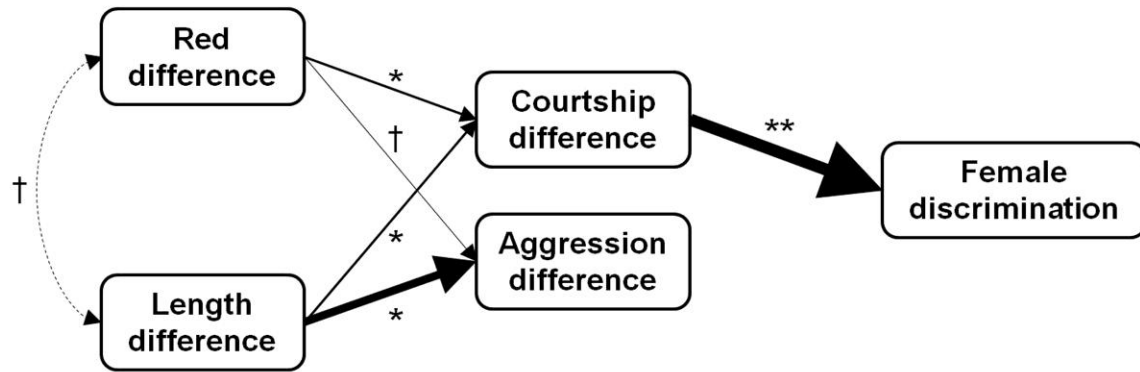
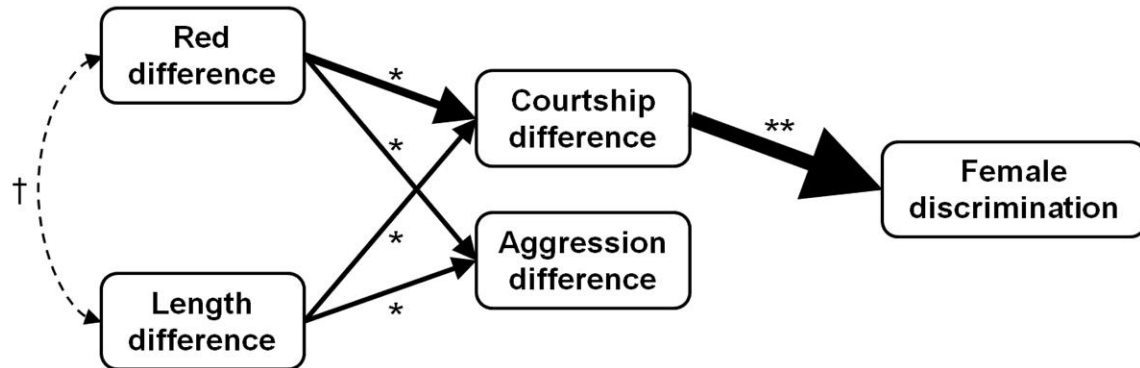


Figure 3.4 **Best fit hypothesized path diagram for relationships between female discrimination and male morphological and behavioral traits.** We show causal paths with straight, single-headed arrows and correlations with curved, double-headed arrows.

(A) Native



(B) Alternative



(C) Open

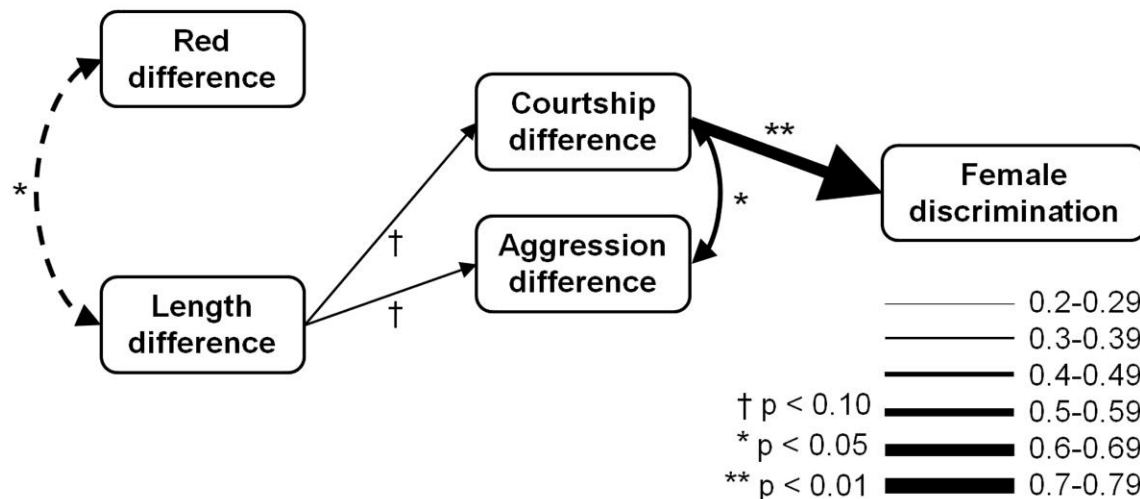


Figure 3.5 **Tested relationships among female discrimination and male morphological and behavioral traits across habitats.** We show significant paths for Paxton and Enos fish combined in native (A), alternative (B), and open (C) habitats. Line thickness shows the strength of the

Figure 3.5 (cont'd)

standardized path coefficient. Straight lines depict causal relationships, while curved lines show correlations. Solid lines indicate positive relationships between variables, while dashed lines indicate negative relationships. Paths are estimated from the best fit model shown in Figure 4. See methods text for other models tested.

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CHAPTER 4

How reproductive isolation evolves along the speciation continuum in stickleback fish

Introduction

To understand how divergence between taxa begins, accumulates, and potentially leads to new species, we examine how diverging taxa move along the speciation continuum from a single panmictic population, to diverging populations that are partially distinct but still exchange genes, to distinct species with little or no gene flow (Figure 4.1). As taxa move forward along the continuum, reproductive isolation accumulates, gene flow decreases, and new species may evolve. However, taxa do not always move forward along the continuum; they may move backward or even get 'stuck' leaving speciation incomplete. Reverse movement involves loss of isolation and increased gene flow and may result in loss of species through hybridization. The reverse process may (or may not) be different from the forward process. Losing isolation seems to occur much faster than accumulating it (Seehausen et al., 1997, Taylor et al., 2006, Gilman & Behm, 2011, Vonlanthen et al., 2012). Yet, we still know little about whether the forms of isolation that maintain species boundaries differ from those that underlie initial divergence. Some taxa appear to move neither forward nor backward but instead seem halted along the speciation continuum. These taxa have stopped accumulating reproductive isolation, and the extent of gene flow remains stable. Such taxa represent examples of incomplete speciation (Nosil et al., 2009). Based on these different directions and rates of movement, it follows that total isolation may be a better indicator of where taxa fall along the speciation continuum than divergence time.

To examine the speciation process, we look at how reproductive isolation evolves. The entire speciation process is typically too long to observe, so we select representatives along the continuum. By measuring the current isolation present between a pair of taxa, we can determine which barriers are important for isolation at the current stage on the speciation continuum. A number of studies have comprehensively examined most or all of the reproductive barriers that contribute to total isolation between a single pair of taxa. This has primarily been done in plants (e.g., Chari and Wilson, 2001, Ramsey et al., 2003, Husband and Sabara, 2004, Kay, 2006, Martin and Willis, 2007, Lowry et al., 2008, Sambatti et al., 2012) but also in a few insect systems (Dopman et al., 2010, Matsubayashi & Katakura, 2009, Sanchez-Guillen et al., 2012). Because the relative importance of individual barriers present and evolutionary forces acting may differ between early and late stages of the speciation process (Nosil et al., 2009, Schemske, 2010), we cannot necessarily generalize findings in one taxa pair across all stages of the speciation process. An alternative approach is to measure a few barriers for taxa across a range of divergence values to determine the order and rate of barrier evolution (Coyne & Orr, 1989, Tilley et al., 1990, Coyne & Orr, 1997, Presgraves, 2002, Mendelson, 2003, Christianson et al., 2005). Yet studying only a few barriers can skew our understanding of the relative importance of different barriers and restrict how accurately we can measure total isolation (Schemske, 2010). In this study, we combine the best aspects of these approaches and examine a comprehensive number of barriers across many taxa pairs that range along the speciation continuum in a model vertebrate system for ecological speciation: stickleback fish. With this approach, we can determine which barriers are important for initiating speciation, accumulating additional isolation (not getting stuck), and completing

speciation (not reversing along the continuum) (Coyne & Orr, 2004, Schemske, 2010). We can then infer which evolutionary forces, including selective and genetic mechanisms, are most important at different stages of the speciation process.

Open questions remain about the types and numbers of reproductive barriers that evolve early and late in the speciation process and how they contribute to overall reproductive isolation.

Barrier types include premating isolation that reduces hybridization before mating (e.g., habitat use and reproductive timing) and postmating isolation that reduces the fitness of hybrids after mating. Postmating isolation can be split into intrinsic barriers that arise due to gametic or genetic incompatibilities between taxa and extrinsic barriers that result from natural or sexual selection against hybrids. Questions still remain about the relative importance of these various types of barriers at different stages of the speciation process. Additionally, we do not know if particular types of barriers or the sheer number of barriers present are more important at different stages of the speciation process. Particular types of barriers might be important if one type of barrier tends to initiate speciation while another type completes it (Coyne & Orr, 2004, Nosil et al., 2009, Schemske, 2010). Moreover, a particular barrier might be very effective at limiting substantial gene flow whereas another may routinely limit only a small amount. For numbers of barriers, we can ask if a single barrier can impart strong and persistent isolation or if multiple barriers are required. In some cases of plant speciation, a single barrier, like mating system, can impart complete isolation (reviewed in Rieseberg and Willis, 2007). However, it is generally thought that many barriers would generate stronger isolation than a single barrier (e.g., Mayr 1947, Price & Bouvier, 2002) because often a single barrier is incomplete or asymmetric, and so allows gene flow (Coyne & Orr, 2004).

We may also find that the importance of types and numbers of barriers are intertwined; particular pre- and postmating barriers may arise together because they result from similar sources of selection. Divergent natural selection can result in divergent, locally-adapted taxa. Such selection would act against migrants moving between habitats as well as intermediate hybrids poorly adapted to either habitat, which could result in two forms of isolation: immigrant and hybrid ecological inviability (Rundle & Whitlock, 2001, Rundle, 2002, Nosil et al., 2005). Divergent sexual selection, sometimes interacting with divergent natural selection, can generate differences in female preferences and male mating traits that limit breeding between species (West-Eberhard, 1983, Lande & Kirkpatrick, 1988, Panhuis et al., 2001, Maan & Seehausen, 2011) as well as with hybrids (e.g., Stratton & Uetz, 1986, Wells & Henry, 1994, Naisbit et al., 2001). This selection could result in both sexual isolation and sexual selection against hybrids (e.g., Jiggins et al., 2001). Thus, we could predict that barriers that result from the same sources of selection might evolve to the same strength and at roughly the same rate and so contribute to the speciation process at roughly the same point in the continuum.

Previous work suggests premating isolation may evolve first and thus initiate speciation while postmating isolation may evolve later and more slowly, contributing to completing and maintaining speciation (Coyne & Orr, 1989, Coyne & Orr, 1997, Mendelson, 2003, Lowry et al., 2008, Rieseberg & Willis, 2007). However, this is by no means a universally held view (reviewed in Coyne & Orr 2004, p. 65-69). Extrinsic postmating isolation may be important for speciation because the environmental differences that restrict hybrids from contributing to future generations also favor further divergence between taxa (Schluter, 1998). Intrinsic postmating isolation may be necessary to complete and maintain speciation, restricting reversal along the

speciation continuum (Seehausen et al., 1997, Taylor et al., 2006, Vonlanthen et al., 2012). The genetic mechanisms that cause intrinsic postmating isolation, including genic incompatibilities and chromosomal inversions, should be robust to environmental changes, which could undermine other forces that generate isolation, because the effects of intrinsic isolation do not depend on the environment (reviewed in Coyne & Orr, 2004). Looking for patterns of intrinsic postmating isolation across the speciation continuum will provide additional insight into the stage at which this type of isolation evolves and how essential it may be to maintain species.

Debate still occurs on the relative importance of pre- and postmating barriers at different stages of the speciation process for several reasons. First, the current relative importance of different barriers does not reveal the order in which barriers evolved (Coyne & Orr, 2004, Schemske, 2010), i.e., the strongest barrier now may or may not have initiated speciation. However, we can say that barriers that do not currently contribute to total isolation likely did not contribute to isolation at earlier stages of the speciation process. Second, when isolation is studied in ancient species, the relative importance of barriers is obscured. Species that diverged millions of years ago may have continued to accumulate reproductive isolation after speciation was essentially complete, so some of the isolation seen currently may not have been involved during the speciation process *per se*. The most relevant data for isolating barriers that initiate speciation come from incipient species, even though they will not always continue accumulating isolation sufficient to become fully distinct species. Recently diverged species are ideal for determining which barriers complete speciation. We can circumvent many of the problems underlying debate about the relative importance of different barriers during speciation by studying related taxa that span the speciation continuum. With this approach, we

can infer the evolutionary order and relative importance of barriers at different stages of the speciation process.

To determine which barriers are important for completing speciation and maintaining distinct species once they evolve, we can study recently diverged species that have begun to move backward along the continuum, hybridizing and losing isolation (Coyne & Orr, 2004). Barriers that collapse when distinct species are lost are likely important to maintain species and limit reverse movement along the speciation continuum. Such loss of isolation can result from environmental changes that alter selective regimes (Schluter & McPhail, 1992, Boughman, 2002, Seehausen, 2006, Seehausen et al., 2008, Behm et al., 2010, Gilman & Behm, 2011, Vonlanthen et al., 2012). By understanding the ecological and evolutionary processes that generate and maintain species, we may be able to prevent species loss (Martin & Willis, 2007, Vonlanthen et al., 2012), which is especially relevant as human-induced alterations are increasingly prevalent (e.g., Rhymer & Simberloff, 1996, Ricciardi & Rasmussen, 1999).

Reproductive isolation may not always accumulate symmetrically because evolutionary forces may act on each taxon differently. Asymmetries in premating barriers have been frequently found (Kaneshiro, 1976, Kaneshiro, 1980, Arnold et al., 1996, Kay, 2006, Kitano et al., 2007, Martin & Willis, 2007, Sanchez-Guillen et al., 2012) and may occur for multiple reasons. Perhaps one taxon is more locally adapted whereas the other taxon has relatively high fitness in both environments. Additionally, one taxon may be more discriminating of mates than the other, though reasons for why sexual selection might act differently on each taxon vary (Kaneshiro, 1976, Kaneshiro, 1980, Arnold et al., 1996). Postmating isolation can be asymmetrical as well (Tiffin et al., 2001, Takami et al., 2007, Turelli & Moyle, 2007), sometimes

even substantially more than premating isolation (Lowry et al., 2008). Extrinsic postmating isolation may be asymmetric if hybrids do worse in one parental environment than the other (e.g., Kuwajima et al., 2010) or are more unattractive to one parent than the other. The likelihood of asymmetric intrinsic postmating isolation depends on the causal mechanism. Intrinsic postmating isolation caused by deleterious epistatic nuclear incompatibilities and chromosomal inversions should be symmetrical, whereas incompatibilities between mitochondrial and nuclear genomes or differences in silencing patterns between parents of each sex can cause asymmetrical isolation (Tiffin et al., 2001, Turelli & Moyle, 2007).

Taxa with strong asymmetries and/or many asymmetric barriers may get stuck or move backward along the speciation continuum because asymmetric gene flow may impede forward movement. Indeed, previous work on sexual isolation predicts that asymmetries may be most likely at intermediate levels of divergence (Arnold et al., 1996), but it is unknown if this generalizes to other barriers. Asymmetries in individual barriers may not, however, result in asymmetric total isolation between taxa if, for instance, asymmetries in two barriers act in opposite directions and effectively cancel each other out (e.g., Kitano et al., 2007, Takami et al., 2007). So we should examine asymmetries in individual barriers and total isolation to determine potential impacts of asymmetries on the speciation process.

In this study, we use stickleback fish taxa pairs that span the speciation continuum to address questions about how reproductive isolation evolves. Stickleback fish are an excellent system in which to address these questions for many reasons. First, different taxa pairs of sticklebacks (e.g., Japan Sea-Pacific Ocean, Limnetic-Benthic, Lake-Stream) serve as representatives of stages early and late along the speciation continuum (McPhail, 1994, Hendry

et al., 2009). We often have replication within taxa pairs, as parallel speciation commonly occurs and replicate pairs of the same type have been studied. Extensive previous work exists on reproductive isolation and evolutionary forces generating isolating barriers (reviewed in McPhail 1994, Boughman 2006, see also references in Table 1). Also, there are measures of gene flow and genetic differentiation between taxa across many systems (see references in Table 2), which we can use to evaluate how well our estimates of total isolation reflect actual gene flow. For all stickleback pairs, divergence is relatively rapid and recent. The oldest taxa pair diverged within the past 1.5 million years (Kitano et al., 2007), while most other pairs likely diverged within the past 13,000 years (McPhail, 1994, Bell & Foster, 1994). Lastly, one pair of Limnetic-Benthic species has recently collapsed after environmental change (Gow et al., 2006, Taylor et al., 2006). Thus, sticklebacks provide ideal representatives to study initial divergence as well as completion and maintenance of speciation.

Here, we estimate the strength of many reproductive barriers and total isolation across taxa pairs of stickleback fish that span the speciation continuum. We use sympatric and parapatric populations to examine the barriers sufficient to restrict current gene flow. Our estimates of reproductive isolation will thus reflect outcomes from direct interactions between members of each taxon, divergent natural and sexual selection, and by-products of divergent selection. We cannot, however, distinguish between isolation that results from direct versus by-product mechanisms.

Barriers are important to isolation if, by acting alone, they strongly impede gene flow and if, relative to other barriers, they strongly contribute to total isolation (Coyne & Orr, 2004, Martin & Willis, 2007, Sobel et al., 2010). Thus, we estimate the strength of each barrier alone

as well as relative to other barriers. A barrier that is currently strong was likely important for divergence that has occurred to date. A barrier's relative strength reveals how much that barrier contributes to current total isolation given the strengths of all other barriers. We first compare barrier strengths as well as total pre- and postmating isolation without considering how barriers act sequentially to infer the potential importance of different types of barriers as reproductive isolation evolved. Then we calculate relative barrier strengths and total isolation by ordering barriers as they occur during the life cycle, with later-acting barriers only restricting gene flow allowed by earlier acting barriers. This approach shows how complete isolation is as well as which barriers are important for maintaining the current strength of isolation at different stages of the speciation process (Coyne & Orr, 1989, Coyne & Orr, 1997, Ramsey et al., 2003).

We address a series of questions about how reproductive isolation evolves, which provides insight into the speciation process. (1) How strong is total isolation in each of the systems we examine, and what do the patterns of reproductive isolation in these systems tell us about forward, reverse, and halted movement along the speciation continuum? (2) Do the types of barriers important for speciation differ between early and late stages of the process, and what is the relative importance of types versus sheer numbers of barriers across the speciation continuum? (3) Does premating isolation evolve first or last, and how does this reflect the importance of pre- and postmating barriers early and late during the speciation process? (4) Do the strengths and directions of asymmetries in individual barriers and in total isolation change across the speciation continuum? For all of these questions, we ask how

patterns in reproductive isolation may reveal the underlying evolutionary forces that act across the speciation continuum.

Materials and Methods

Study systems

We compare five pairs of taxa that range from late to early along the speciation continuum: Japan Sea-Pacific Ocean, Limnetic-Benthic, Anadromous-Freshwater, Lake-Stream, and Limnetic-Benthic collapsed. Hereafter we refer to these as ‘species pairs’, but we recognize that some of these pairs have not fully diverged into distinct species or have collapsed and are no longer distinct species. We use ‘species pairs’ for simplicity and to emphasize our focus on the speciation process.

In these systems of stickleback species pairs, fish migrate from feeding habitats to mating habitats in the breeding season. For each system below, we provide additional details on whether one or both species migrates. Day length is a trigger for fish to come into reproductive condition (Guderley, 1994). Males become reproductive first and establish territories and build nests (Ostlund-Nilsson, 2007). Once females develop eggs, they search for mates. After a series of courtship interactions between a male and female, a female may deposit eggs in a male’s nest. The female leaves immediately and the male stays to perform all parental care for eggs and young fry. Females are the choosier sex, and there is little to no evidence of male choice in most systems (Kitano et al., 2007, Kozak et al., 2009, Raeymaekers et al., 2010, but see Hay & McPhail, 1975). After fry hatch, juvenile growth rate is important for avoiding gape-limited trout predators (Reimchen, 1991), and high parasite loads can reduce

growth rate (Whoriskey & FitzGerald, 1994). As adults, body size and condition are important for female fecundity and male courtship and parental care (Ostlund-Nilsson, 2007, Baker et al., 2008). Both males and females can have multiple reproductive events per breeding season. Most threespine stickleback fish live approximately one year in the wild, though fish from some populations may live up to 2 years but rarely longer (Baker, 1994).

The species pair predicted to have the strongest isolation and fall furthest along the speciation continuum is the Japan-Pacific pair. The estimated divergence time is about 1.5 million years (Kitano et al., 2007), and divergence likely began in allopatry because the Sea of Japan has been isolated from the Pacific Ocean more than once in the past 2 million years (Higuchi & Goto, 1996, Kitano et al., 2007). The Japan Sea and Pacific Ocean species are both anadromous and can encounter each other in the breeding season when they migrate upstream to mate. Previous work has shown that the following barriers contribute to reproductive isolation: temporal, sexual, and hybrid sterility (Kitano et al., 2009). Sexual isolation and hybrid sterility are both asymmetric, resulting in stronger isolation for one species over the other, but they act in opposite directions with hybrid sterility blocking the direction of gene flow allowed by sexual isolation (Kitano et al., 2007).

Limnetic-Benthic species pairs recently diverged about 13,000 years ago and occur in multiple lakes, which constitute replicate populations. Previous work has shown that sexual isolation as well as ecological and sexual selection against hybrids are likely important barriers to reproduction between the species. Previous work also suggested that habitat isolation may occur, but gametic and genetic incompatibilities are absent. One pair of limnetic-benthic species in Enos Lake collapsed into a hybrid swarm quickly after a drastic environmental change

about 30 years ago. We measure isolation in three pairs of limnetic-benthic sticklebacks from Paxton, Priest, and Enos lakes. Data for Enos Lake fish come from before (1996 and earlier) and after its collapse (Taylor et al., 2006).

Anadromous-Freshwater pairs are found across the northern hemisphere often as anadromous-stream resident pairs but sometimes as anadromous-lake resident pairs. The most comprehensively studied pair for reproductive barriers exists in the Little Campbell River in British Columbia (Hagen, 1967, McPhail, 1994). Studies in other pairs have typically measured one or two reproductive barriers, with sexual isolation as a primary focus. Previous work has suggested that sexual isolation could contribute significantly to total reproductive isolation (McKinnon et al., 2004). The work by Hagen (1967) showed that premating barriers, especially habitat and temporal, seemed to contribute more to total isolation than any other barriers.

Lake-Stream pairs also occur across the northern hemisphere. Of the many allopatric lake and stream species, just a few lake-stream pairs are parapatric, and species may encounter each other where the stream intersects the lake. Like Anadromous-Freshwater and Limnetic-Benthic pairs, glacial history indicates that divergence time is approximately 13,000 years (Bell & Foster, 1994, McPhail, 1994). Previous work has shown evidence for some potential isolating barriers, but barriers tend to be weak and do not occur across all Lake-Stream pairs or even across all years for the same pair (Hendry et al., 2009). Immigrant inviability may isolate the two species, but again this varies spatially and temporally (Hendry et al., 2002). Gametic and genetic incompatibilities are absent in the Misty Lake system (Lavin & McPhail, 1993). Sexual isolation and hybrid mating are also thought to be absent (Raeymaekers et al., 2010, Rasanen et al., 2012).

Calculating reproductive isolation

We follow the method of Coyne and Orr (1989, 1997) with adjustments from Ramsey et al. (2003), Sobel (2010), and Sobel and Chen (*in review*) to estimate the strengths of individual barriers and their relative contributions to total reproductive isolation. See Table 3 for a list of the barriers and metrics used in this study. The strength of reproductive isolation from an individual barrier is defined as:

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

where H is the frequency of heterospecific events and C is the frequency of conspecific events. Events are defined for each barrier, e.g., matings for sexual isolation or hatched eggs for genetic incompatibilities. For most barriers, RI ranges from -1 to 1, where -1 indicates, for example, disassortative mating or hybrid vigor, 0 indicates random mating or equal fitness of hybrid and parental forms, and 1 indicates assortative mating or parental vigor. For temporal and habitat isolation, however, RI ranges from 0 to 1, where 0 is no isolation due to complete overlap in space or time and 1 is complete isolation due to no overlap in space or time. We can use the same equation for pre- and postzygotic barriers, so estimates of individual barrier strength are directly comparable across all barriers. We can also compare our measures of reproductive isolation to those from other studies; this equation is either equivalent to or easily adjusted to match other measures. For detailed coverage of how this metric compares to previously used metrics see Sobel and Chen (*in review*).

Martin & Willis (2007) argue that it is sometimes necessary to scale measures of RI to reflect different null expectations for each species. We account for differences in expectations

between conspecific and heterospecific events, due to, for example, differences in relative abundances, by expanding equation 1 to:

$$RI = 1 - 2 \left(\frac{\frac{H_{obs}}{H_{exp}}}{\frac{C_{obs}}{C_{exp}} + \frac{H_{obs}}{H_{exp}}} \right), \quad (2)$$

where *obs* is the number of observed events and *exp* is the number of expected events. If expected *H* and expected *C* are equivalent, equation 2 simplifies to equation 1.

We then calculate each barrier's sequential strength, *SS*, (Dopman et al., 2010) (also “absolute contribution” in Ramsey et al., 2003). We ordered isolating barriers by when they occur in the life cycle. The sequential strength of the n^{th} barrier, SS_n , depends on its individual strength, RI_n , and the amount of gene flow allowed by earlier-acting barriers:

$$SS_n = RI_n \left(1 - \sum_{i=1}^{n-1} SS_i \right). \quad (3)$$

Total isolation, *T*, is the sum of all sequential strengths and generally varies from 0 to 1, where 0 indicates 100% gene flow and 1 indicates 0% gene flow or complete isolation. The relative contribution of each barrier, *RC*, is each barrier's sequential strength divided by total isolation:

$$RC_n = \frac{SS_n}{T}. \quad (4)$$

For each barrier, we calculated 95% confidence intervals for *RI* for each sampling location within a study. When we had multiple sampling locations and/or studies for a single barrier, we calculated weighted mean *RI*, weighting each individual *RI* with its inverse variance. We then calculated 95% confidence intervals for each weighted mean *RI*. Generally, calculating a confidence interval involves multiplying the critical z-value by the standard error and adding

or subtracting that product from the sample mean. To calculate 95% confidence intervals for weighted means, we use the standard error of the weighted mean, which is the square root of the sum of the inverse variance weights (Hedges & Olkin, 1985, Lipsey & Wilson, 2001). Using 95% confidence intervals for single estimates or weighted means, we can test whether each barrier estimate is greater than zero and therefore contributes to reproductive isolation. We can also test whether each barrier's strength differs from other barrier strengths by asking if their 95% confidence intervals overlap.

Confidence intervals generate upper and lower estimates of *RI* for each barrier. We used these upper and lower bounds to calculate a strongest and weakest estimate of total isolation, *T*. For the strongest estimate of total isolation, we (1) used all of the upper bounds for *RI* from each barrier to calculate upper estimates of sequential strength, *SS*, for each barrier, and then (2) summed these upper estimates of *SS*. Ideally we would use confidence intervals for weighted means for all barriers, however, for a few barriers, we only had confidence intervals for single estimates. Thus, we calculated the weakest and strongest estimates of total isolation using confidence intervals from single estimates and from weighted means. Confidence intervals of weighted means are typically smaller than those of single estimates. By including confidence intervals for single estimates, our weakest and strongest estimates of total isolation are likely somewhat conservative. We interpret our estimates of total isolation with this in mind.

We tested for homogeneity across studies and sampling locations used to calculate weighted means with the *Q* statistic, which tests whether the effect sizes for each sampling location or study all estimate that sample population effect size (Hedges, 1982, Rosenthal &

Rubin, 1982, Lipsey & Wilson, 2001). The Q statistic is distributed as a chi-square, and degrees of freedom are one minus the number of effect sizes. The Q statistic is calculated as:

$$Q = \sum (w_i ES_i^2) - \frac{(\sum w_i ES_i)^2}{\sum w_i}, \quad (5)$$

where w_i is the individual weight of each effect size, ES_i . Our effect sizes are calculated as

proportions using the $\left(\frac{H}{C+H}\right)$ part of equation 1. We tested for homogeneity among all

sampling locations and studies used to calculate a weighted mean. We performed Q tests for each barrier within each system of sticklebacks: Japan-Pacific, Limnetic-Benthic, Anadromous-Freshwater, Lake-Stream, and Limnetic-Benthic collapsed.

We also evaluated whether species contribute asymmetrically to reproductive isolation. In general, we tested how each species contributed to each reproductive barrier using equation 1, where H is frequency of heterospecific events and C is the frequency of conspecific events for a single species. We wanted to include heterospecific events for both sexes of a species, e.g., when species A was both the mother and father of hybrid offspring. Thus, for the RI estimate from a single study, the value of H is the same for both species. We then used methods described above to calculate weighted mean barrier strengths for each species with 95% confidence intervals. We also tested for homogeneity among estimates used to calculate the weighted mean for each species using the Q statistic. We can test whether species contribute asymmetrically to reproductive isolation by asking whether their 95% confidence intervals overlap.

Habitat isolation

Habitat isolation reduces encounters between potential mates of different species due to use of different mating sites. Habitat isolation is likely very important for and common in the speciation process (Coyne & Orr, 2004, Schemske, 2010). We measure habitat isolation on a ‘microspatial’ scale, where individuals of each species could encounter each other but are less likely to do so because they prefer different habitats within a shared general area.

We measured the number of wild reproductive fish (i.e., colorful males and gravid females) caught at the same site in the field in sympatric regions of species distributions. A ‘site’ included fish caught in a single trap or in multiple traps within a sampling area. Effect sizes from studies using either methods of reporting data were homogeneous. However, estimates from these two methods do differ in the size of their confidence intervals because we use the number of ‘sites’ as the sample size. Thus, studies that report fish caught summed across traps tend to have smaller sample sizes and larger confidence intervals than studies that report fish caught in each trap.

We focused on sympatric regions of species’ distributions because four of the species pair systems are parapatric while the Limnetic-Benthic species pairs are sympatric. If we had included a measure of habitat isolation that extended into zones of allopatry for each species (i.e., ecogeographic isolation), that would have increased isolation for all systems but the Limnetic-Benthic species pairs. We wanted to measure barriers across all five systems as consistently as possible. Thus, our measure of habitat isolation underestimates spatial isolation experienced by the Japan-Pacific, Anadromous-Freshwater, and Lake-Stream pairs.

We account for relative population sizes and potential differences in relative abundance of each species at each sampling point, i , and across all sampling points, $total$, by adjusting equation 2 above to:

$$RI = 1 - 2 \frac{\frac{\sum_i \left(\frac{A_i}{A_{total}} \times \frac{B_i}{A_i + B_i} \right)}{\frac{B_{total}}{A_{total} + B_{total}}}}{\frac{\sum_i \left(\frac{A_i}{A_{total}} \times \frac{A_i}{A_i + B_i} \right)}{\frac{A_{total}}{A_{total} + B_{total}}} + \frac{\sum_i \left(\frac{A_i}{A_{total}} \times \frac{B_i}{A_i + B_i} \right)}{\frac{B_{total}}{A_{total} + B_{total}}}}. \quad (6)$$

Immigrant inviability

Immigrant inviability reduces encounters between potential mates of different species because migrants between habitats suffer reduced fitness relative to fish that stay in their native habitat. For premating isolation, immigrant inviability is analogous to postmating hybrid ecological inviability (Nosil et al., 2005); both forms of isolation result from selection against maladapted individuals. Migration between habitats must happen for immigrant inviability to occur. If habitat isolation is complete, then members of each species always use different habitats and migration should be zero. However, when isolation is less than complete, individuals in foreign habitats can experience selection against migrants. Furthermore, strong immigrant inviability can result in increased habitat isolation because selection against migrants would favor individuals that use native habitat.

We used survival or growth rate for fish in their native habitat versus foreign habitat. Growth rate serves as a proxy for reduced fitness and could predict likelihood of survival. Growth rate could also predict reproductive success for males through courtship and parental

care vigor and for females through fecundity and ability to withstand potential mate search costs. Effect sizes from studies using growth rates versus survival were homogeneous in Limnetic-Benthic fish, where both measures were used. This suggests estimates from survival or growth rates are comparable. We assumed that growth rates and survival were equal between species. For Limnetic-Benthic pairs, this is supported by previous data (Schluter, 1995).

Temporal isolation

Temporal isolation reduces encounters between potential mates of different species due to different mating times. Temporal isolation can result from a number of potential causes including different responses of each species to environmental cues, like day length or lunar cycle, or habitat differences that cause species differences in breeding time as a by-product, like soil or water temperature (Coyne & Orr, 2004). The importance of temporal isolation for speciation may vary among species pairs. Temporal isolation may generally have limited importance for speciation across animals and plants; but for some organisms, such as marine broadcast spawning animals, temporal isolation may be the primary reproductive barrier (Coyne & Orr, 2004).

We measured the number of reproductive fish (i.e., colorful males and gravid females) caught at the same sampling time point. Reproductive fish are most relevant for questions about potential matings, but when these data were unavailable, as for Anadromous-Freshwater fish, we used total fish. A few studies provided data on both reproductive and total fish, and we found that using either of these measures gave very similar isolation estimates, so using one measure or the other would not change our conclusions. A 'time point' was typically a single

day, but a few studies summed traps across a few days (see Table 1). We accounted for differences in relative abundance with equation 6 described above in the habitat section.

Sexual isolation

Sexual isolation reduces mating between species due to different mating preferences and signals. Sexual isolation most likely results from direct or indirect sexual selection, natural selection that affects mating traits as a by-product, or reinforcement. Sexual isolation may be particularly important in the speciation process because it is thought to evolve early and rapidly (Mendelson, 2003, Coyne & Orr, 2004)

We preferentially used the number of spawnings, which occur when a female enters a male's nest. When spawning data were unavailable, we used nest inspection, which occurs when a female pokes her head into a male's nest, but she may not enter the nest. Nest inspection directly proceeds and is often used as a proxy for spawning. Our expected values used in equation 2 assume random mating given differences in the number of trials conducted within and between species. We primarily used no-choice trials (see Table 1). Estimates from choice and no-choice trials have homogeneous effect sizes and so should be comparable (Lake-Stream choice vs. no-choice estimates: $Q = 0.96$, $p = 0.3272$).

Gametic and genetic incompatibilities

For gametic isolation, incompatibilities between sperm and eggs reduce the number of zygotes formed. Gametic isolation could evolve rapidly as suggested by theory and lab work (reviewed in Coyne & Orr, 2004), particularly if this barrier results from sexual selection. This

barrier may be strong in many animal and plant systems even in young species, but there are also cases where gametic isolation is absent (Moyle et al., 2004, Mendelson et al., 2007, Lowry et al., 2008). We measured the proportion of fertilized eggs within a clutch. We assumed equal fertilization rates within and between species, and we accounted for differences in the number of crosses performed within and between species.

Genetic isolation reduces the number of viable zygotes due to genetic incompatibilities that impair development. This form of intrinsic postzygotic isolation may evolve gradually and so play a limited role in the early stages of speciation (Coyne & Orr, 1989, Coyne & Orr, 1997, Presgraves, 2002, Price & Bouvier, 2002, Bolnick & Near, 2005). We used the proportion of hatched eggs out of fertilized eggs within a clutch. We assumed equal hatching rates within and between species, and we accounted for differences in the number of crosses performed within and between species.

For the Japan-Pacific, Anadromous-Freshwater, and Lake-Stream pairs, we did not have data that could distinguish between gametic and genetic incompatibilities (i.e., we had hatching rate out of total eggs instead of out of fertilized eggs). Thus, these combined estimates include isolation due to reduced fertilization and hatching of hybrid eggs.

Hybrid ecological inviability

Hybrid ecological inviability reduces survival or growth rate to adulthood because fish are not well adapted to divergent habitats. This barrier results from ecological selection against hybrids due to the poor fit of intermediate hybrid phenotypes to divergent parental niches and so serves as a unique prediction of ecological speciation compared to other types of speciation

(Rundle & Whitlock, 2001, Schluter, 2001, Rundle, 2002). We measured the growth rate or number of fish surviving to the next life stage. Most studies presented survival data for juvenile to adult stages, but we also included data from intermediate stages, such as survival from juvenile to sub-adult or from sub-adult to adult. We assumed equal growth rates and survival of parental species and hybrids, which is supported by previous data in Limnetic-Benthic pairs (Schluter, 1995). We accounted for differences in number of parental and hybrid fish sampled.

Sexual selection against hybrids

Sexual selection against hybrids occurs when hybrids have reduced mating success. Hybrids may not be able to find or attract mates. This barrier is rarely measured, and though it can be strong (Stratton & Uetz, 1986, Hatfield & Schluter, 1996), variability among systems precludes generalization about the strength and importance of sexual selection against hybrids (Hurt & Hedrick, 2003, Nosil et al., 2005). We used the number of spawnings for hybrid and parental fish. For expected values in equation 2, we assumed random mating between hybrid and parental fish, and we accounted for differences in the number of hybrid and parental mating trials conducted.

Hybrid sterility

Hybrid sterility reduces mating success of hybrids due to inviable or incompatible gametes or poor zygote survival. This form of isolation evolves slowly but probably faster than genetic incompatibilities (reviewed in Coyne & Orr, 2004). Thus, hybrid sterility should be more important late than early in the speciation process. We measured the number of hatched eggs

per clutch. This measure of hybrid sterility encompassed problems with fertilization and/or development. We assumed equal hatching rates within types of second-generation hybrid (i.e., F2 and backcross) clutches, and we accounted for different numbers of second-generation hybrid and parental crosses performed.

Results

Where does each system fall along the speciation continuum?

In the five systems sampled, total reproductive isolation ranged from moderate to strong, and total isolation was surprisingly high even in systems with suspected halted movement and known reverse movement along the continuum (Figure 4.2). Isolation has evolved to nearly one in the Japan-Pacific pair, which has diverged for 1.5 million years (Table 4.2). Even with much shorter predicted divergence times, ~13,000 years, the other pairs we examined still have moderate to strong total isolation. Previous work suggested that total isolation in the Limnetic-Benthic pairs would be greater than that in the Anadromous-Freshwater pairs, so we have ordered these systems along the continuum this way. However, total isolation is essentially equivalent in these systems. Isolation is surprisingly strong, blocking about 70% of gene flow, in the Lake-Stream pair, which is thought to be halted along the speciation continuum. The reverse speciation process has reduced but not erased isolation in the collapsed Limnetic-Benthic pair, which still retains enough isolation to restrict 50% of gene flow between species.

Expected gene flow estimated from our weakest and strongest total isolation estimates (Figure 4.2, Table 4.4) was generally consistent with observed gene flow (Table 4.2). Observed

gene flow is a little higher than expected for the Anadromous-Freshwater pairs, but the measure of observed gene flow comes from a single study, while estimated gene flow from total isolation comes from globally distributed pairs. In the Limnetic-Benthic collapsed pairs, observed gene flow is a bit lower than expected from our calculations perhaps because some forms of isolation were estimated from more recent studies.

How does reproductive isolation build up (and break down) along the speciation continuum?

We first examined whether the types of barriers important for isolation early in the speciation continuum are different from those important late in the process. Habitat isolation is one of the strongest, if not *the* strongest, barrier both individually and relatively across all systems, suggesting substantial habitat isolation evolves early, remains strong during the speciation process, and contributes most to total isolation (Figure 4.3). In contrast, other barriers accumulate later along the continuum. Sexual isolation increases across systems and even transitions from weakening total isolation in the Lake-Stream pairs to strengthening total isolation in the other pairs. Yet once sexual isolation evolves, it is significant and moderately strong and remains so further along the continuum. Intrinsic postmating barriers seem to evolve last and most gradually. In most systems, intrinsic postmating barriers are effectively absent. Only the pair furthest along the speciation continuum, the Japan-Pacific pair, has weak but significant hybrid sterility.

We next examined patterns in the number of barriers early and late during the speciation process. The number of significantly positive barriers increases along the speciation continuum (Figure 4.3). The Lake-Stream system with supposed halted movement along the

continuum has the fewest significant barriers that contribute to total isolation, and most isolation comes from a single, early-acting barrier. Moving forward, Anadromous-Freshwater pairs have three strongly acting barriers, and the Limnetic-Benthic pairs increase this even further to five. The Japan-Pacific pair furthest along the continuum has five significant barriers, four of which are moderate to high in strength.

Overall, both types and number of barriers are important for explaining patterns in total isolation across the speciation continuum. Habitat and sexual isolation seem to be key barriers that are moderately strong in systems with higher total isolation. However, these two barriers cannot solely explain where systems fall along the speciation continuum. Systems with greater total isolation are characterized by having more significantly positive barriers.

Types and numbers of barriers may also be intertwined because some barriers that are controlled by similar sources of selection may evolve together. Divergent sexual selection could result in sexual isolation and sexual selection against hybrid mating, while divergent natural selection could generate immigrant and hybrid ecological inviability. Thus, we might expect these pairs of barriers to evolve at the same time and perhaps at similar strengths. We have data to test whether sexual isolation and sexual selection against hybrids are similar in strength within systems for the Limnetic-Benthic and Lake-Stream pairs. These barriers are both positive and moderate to strong in the Limnetic-Benthic pairs, while negative and moderate to strong in the Lake-Stream pairs. We also have data to test whether immigrant and hybrid ecological inviability evolve to the same strength in the Limnetic-Benthic pairs, where both of these barriers are weak but significant (Figure 4.3). Additionally, we looked for patterns consistent with reinforcement, asking if strong premating isolation accompanies strong extrinsic

postmating isolation within a system. This pattern is consistent with isolation in the Japan-Pacific and Limnetic-Benthic pairs. In contrast, Anadromous-Freshwater pairs have strong premating isolation in the absence of strong extrinsic postmating isolation.

Reverse and halted processes

In the collapsed Limnetic-Benthic pair, overall isolation is weaker primarily due to loss of sexual isolation with little change in other premating barriers (Figure 4.3). Surprisingly, we found no loss of habitat isolation even though Enos Lake currently lacks vegetation that historically comprised one of the two distinct mating habitats. While our measure of postmating isolation in the collapsed Limnetic-Benthic pair is much less than that in the other Limnetic-Benthic pairs, this may be primarily due to the lack of an estimate for sexual selection against hybrids in the collapsed pair (Figure 4.3, 4.4). In the Lake-Stream pairs, a single, early-acting barrier contributes to the majority of isolation, but strong negative effects of sexual isolation and sexual selection against hybrids undermine total isolation, which could explain why this system is thought to be ‘stuck’ along the speciation continuum (Figure 4.3).

Potential variation in selection across time and space

By and large, we found little heterogeneity among estimates that we used to calculate weighted means for each barrier even though all contributing studies varied in time, space, or both. However, for four out of fifteen barriers tested, significant heterogeneity occurs. These cases potentially reveal variation in selection across time and space. In the Limnetic-Benthic pairs, sexual isolation estimates vary across space and are higher in Priest Lake (0.67) than in

Enos and Paxton lakes (0.22-0.25) ($Q = 20.0$, $p = 0.0002$, Table 4.1). For hybrid ecological inviability in the Limnetic-Benthic pairs, estimates vary across time (-0.007-0.44 in Paxton Lake; -0.02-0.29 in Priest Lake) as well as between lakes sampled at the same time (e.g., 0.07 in Paxton Lake and 0.29 in Priest Lake) ($Q = 59.8$, $p < 0.0001$, Table 4.1). In the Anadromous-Freshwater pairs, sexual (0.07 - 0.88) and temporal (0.22 - 0.88) isolation vary considerably (sexual: $Q = 82.0$, $p < 0.0001$, temporal: $Q = 5.59$, $p = 0.056$, Table 4.1).

Does premating isolation evolve first or last?

Premating isolation appears to evolve very early and remains strong along the speciation continuum (Figure 4.4). Across systems, habitat isolation is always present, suggesting it may evolve first (Figure 4.3). Sexual isolation appears to evolve more gradually but may accumulate quickly once it evolves (Figure 4.3). Postmating isolation seems to evolve after premating isolation and builds up slowly; postmating isolation only strengthens total isolation in systems furthest along the continuum (Figure 4.4). Of postmating barriers, extrinsic isolation may evolve first and contribute more to total isolation than intrinsic isolation. Significant individual extrinsic barriers are common across systems, whereas the only significant individual intrinsic barrier occurs in the Japan-Pacific system furthest along the continuum. An even stronger difference between these types of postmating isolation emerges when we examine total intrinsic versus extrinsic isolation (Figure 4.5). Total extrinsic isolation varies from weak to strong, but it is always different than zero considering the weakest and strongest estimates. In contrast, total intrinsic isolation is consistently weak across systems, and the weakest and strongest estimates always encompass zero. Moreover, individual extrinsic barriers are stronger

than intrinsic ones in all systems, except in the Lake-Stream pairs where extrinsic barriers are instead significantly negative (Figure 4.3).

Does the degree of asymmetry between species change along the continuum?

We found different patterns of asymmetries in different types of barriers. We find asymmetries in premating and extrinsic, but not intrinsic, postmating barriers. More than a third of premating (7 out of 17) and all extrinsic postmating (4 out of 4) estimates are asymmetric, which could reflect differences in selection acting on each species. In contrast, none of the 10 intrinsic postmating barrier estimates are asymmetric (Figure 4.6).

Consistent with previous work, we find more asymmetric barriers at intermediate levels of divergence (Figure 4.5). The only system without asymmetries is the most divergent: the Japan-Pacific pair. Asymmetric gene flow may result in reverse or halted progress along the speciation continuum. The Limnetic-Benthic pairs had significant asymmetries in both pre- and postmating isolation that may have allowed reversal (Figure 6). Interestingly, the presence and sometimes the direction of asymmetries differ from before and after the collapse.

Despite significant asymmetries in individual barriers within most systems, none of the systems have strong asymmetries in total isolation (Figure 4.7). Noticeable but non-significant total asymmetries exist for Anadromous-Freshwater and Limnetic-Benthic pairs. We should note that our conservative estimates of strongest and weakest isolation may underplay potential asymmetries between species.

Discussion

By examining reproductive isolation in taxa that span the speciation continuum, we can learn how reproductive isolation accumulates from near zero to the point where no gene flow occurs. This gives us insight into which isolating barriers initiate speciation and which complete it. Because different mechanisms affect the evolution of particular barriers, studying the evolution of isolation along this continuum reveals which selective mechanisms are especially important early in the process, which might depend on the presence of those early mechanisms, and which might be required to complete the speciation process. To gain these insights, we examine patterns along the speciation continuum in the types of barriers, their number, and whether they are symmetrical. Our results confirm some predictions and reveal some surprises. We examine our results in the context of previous findings to identify commonalities and differences across studies, both of which can reveal importance insights into the speciation process. We also explore how patterns in reproductive isolation across the speciation continuum and the nature and strength of individual barriers can reveal insight into the selective and genetic factors that directly or indirectly affected the evolution of isolation.

Patterns of isolation and implications for selection

Divergent natural selection can be a major driver of the speciation process (Dobzhansky, 1937, Mayr, 1947, Schluter, 2001, Schluter, 2009, Sobel et al., 2010). Adaptation to different environments can generate different phenotypes and genotypes between species and result in multiple forms of isolation. Divergent natural selection is most directly related to habitat isolation, immigrant inviability, and hybrid ecological inviability. Adaptation to different

environments should select for individuals that use habitats to which they are best adapted, and select against migrants that move between habitats and intermediate hybrids that are poorly adapted to either habitat. Furthermore, differential adaptation can result in temporal isolation as well as intrinsic postmating isolation. Divergent natural selection may also interact with divergent sexual selection to generate different female preferences and male mating traits between species, resulting in sexual isolation and sexual selection against hybrids (reviewed in Maan & Seehausen, 2011).

Here we find that habitat isolation likely evolves first and acts as one of the primary reproductive barriers throughout the speciation process. Similarly, in other systems, habitat isolation is often the primary barrier contributing to isolation (Matsubayashi & Katakura, 2009, Schemske, 2010). Habitat isolation may be essential for initiating speciation and important for the accumulation of isolation at intermediate and later stages of the speciation process, including, but not limited to, cases of ‘ecological speciation’ as defined by Schluter and colleagues (Schluter, 2001, Rundle & Nosil, 2005). Habitat isolation is likely important throughout the speciation process because the effects of habitat isolation on gene flow are twofold (Rice & Hostert, 1993). The use of different habitats by members of each species first reduces encounter rates between species and second generates divergent selection between species. Such selection should favor increased use of different habitats, which would in turn strengthen divergent selection between species. Thus, habitat may initially be important for restricting encounter rates and remain important because of the feedback between spatial isolation and the divergent selection it generates. The divergent natural selection habitat isolation generates may then result in other forms of isolation.

Previous work has shown that sexual selection can play a key role in speciation whether it acts alone or interacts with natural selection (Lande, 1981, West-Eberhard, 1983, Dieckmann & Doebeli, 1999, Panhuis et al., 2001, Boul et al., 2007, Ritchie 2007, van Doorn et al., 2009, Maan & Seehausen, 2011, Weissing et al., 2011). Theoretical work has demonstrated that sexual selection can easily lead to premating isolation, especially sexual isolation (reviewed in Turelli et al., 2001). Although comparative work finds mixed evidence of a relationship between the strength of sexual selection and speciation (reviewed in Ritchie et al., 2007), measuring the amount of divergent sexual selection is another fruitful way to determine the importance of sexual selection in speciation (Rodriguez et al., 2013).

Here we find that sexual isolation likely evolves relatively early and accumulates quickly once it evolves. This pattern is also found in other work (Coyne & Orr, 1989, 1997, Mendelson, 2003). Moreover, in species with strong sexual selection, sexual isolation may be the primary barrier isolating species (e.g., Elmer et al., 2009). Sexual isolation may evolve rapidly and relatively early in speciation because strong sexual selection within taxa can directly affect mating traits important for mate discrimination between species (Lande, 1981, Panhuis et al., 2001, Mendelson, 2003, Merrill et al., 2011).

Yet, the presence of sexual isolation does not unequivocally implicate sexual selection in speciation. Sexual isolation can alternatively arise from divergent natural selection on traits used in mate choice or selection against hybrids, i.e., reinforcement (Howard, 1993, Servedio, 2004). Although theory and empirical work demonstrate that reinforcement is possible under a range of conditions, the relative importance of reinforcement in speciation is unclear (Servedio & Noor, 2003). To test whether reinforcement commonly occurs, researchers have looked for

stronger premating, but not postmating, isolation to evolve earlier in sympatric than allopatric populations (e.g., Coyne & Orr, 1989, 1997, Tilley et al., 1990). This is a powerful test, but details on the order of barrier evolution within species pairs are obscured. An alternative test that we use here measures pre- and postmating barriers in a series of closely related species pairs that span the speciation continuum to reveal whether reinforcement could be the initial or primary cause of sexual isolation. Our data show that sexual isolation can be strong in the absence of postmating isolation. Thus, reinforcement may have secondarily strengthened sexual isolation, but it is unlikely the first cause of sexual isolation. The patterns of isolation we observe across the speciation continuum also suggest that the current presence of selection against hybrids may not indicate that reinforcement was historically the primary cause of premating isolation as has been suggested (Servedio, 2001, 2004). Other studies that can test for evidence of reinforcement in species pairs across the speciation continuum will further strengthen our understanding of when and how much reinforcement plays a role in speciation. Furthermore, future studies may be able to assess whether sexual isolation was caused primarily by sexual selection or reinforcement using genetic signatures that may differ between these processes (Ortiz-Barrientos et al., 2004).

The same source of divergent selection can also result in more than one reproductive barrier. This could speed the rate at which reproductive isolation accumulates and so facilitate the speciation process. Furthermore, if two or more forms of isolation result from the same source of selection acting on the same traits, this could simplify the requirements for speciation to proceed akin to 'magic traits' (Gavrilets, 2004, Servedio, 2009), multi-effect traits (Smadja &

Butlin, 2011), one-allele mechanisms (Felsenstein, 1981), or multifarious selection (Nosil et al., 2009).

We discuss two pairs of pre- and postmating barriers that may evolve in concert because they result from the same source of selection. Divergent natural selection on traits between environments should result in lower fitness of migrants to a foreign habitat and hybrids with intermediate traits poorly adapted to either parental habitat. Thus, divergent natural selection can result in immigrant and hybrid ecological inviability. Divergent sexual selection, potentially interacting with divergent natural selection, generates differences in mating traits and preferences between species, which can lower the likelihood of mating between species or mating between parental and hybrid forms. Thus, divergent sexual selection can result in sexual isolation and sexual selection against hybrids.

From this, we could predict that forms of isolation under the same source of selection might evolve at similar times and to similar strengths. However, Lowry et al. (2008) in a review of 19 taxa pairs in plants did not find that immigrant and hybrid ecological inviability were similar in strength; immigrant inviability was strong yet hybrid ecological inviability was highly variable and often weak. We similarly found that hybrid ecological inviability was often weak, which could be explained by its variability over time and space within study systems (Levins, 1968, Hoekstra et al., 2001, Siepielski et al., 2009, Siepielski et al., 2013). It makes sense that hybrid ecological inviability may be weaker than immigrant inviability because fitness differences should be greater between members of each parental species in the same habitat, with one species in its native and the other species in a foreign habitat, than between members of one parental species and hybrids with intermediate traits in the same habitat. Despite the

limited evidence for immigrant and hybrid ecological inviability evolving to similar strengths, we did find that sexual isolation and sexual selection against hybrids are of similar strength and act in similar directions. This pattern has also been found in other work in insects (Naisbit et al., 2001, Bridle et al., 2006). If sexual isolation and sexual selection against hybrids were commonly stronger than immigrant and hybrid ecological inviability, this may result from differences in sexual and natural selection. Sexual selection can be stronger than natural selection (Hoekstra et al., 2001, Svensson et al., 2006, Kingsolver & Pfennig, 2007, Safran et al., 2013) and so lead to faster evolutionary change and accumulation of isolation.

Variation in selection over space and time may be common (Hereford, 2009, Siepielski et al., 2009, Siepielski et al., 2013) and affect the likelihood that isolation evolves as well as the strength of particular forms of isolation. Our use of many estimates of a single barrier allowed us to detect that some barriers varied significantly across time and space, namely temporal isolation, sexual isolation, and hybrid ecological inviability. We discuss how variation in selection may explain variation in isolation we observed.

Previous explorations of spatial variation have considered how differences in selection between different environments are important for phenotypic divergence between populations (Cain & Sheppard, 1952, Endler, 1980, Thompson, 2005) and speciation (Schluter, 2001, McKinnon & Rundle, 2002). Our discussion of spatial variation is at a different scale: spatial variation in isolation between species pairs, not between species within a pair. Spatial variation at this scale suggests that although much divergence between species that occurs in different locations has occurred in parallel (Schluter & McPhail, 1992, Boughman et al., 2005, Berner et al., 2008, Berner et al., 2009, Jones et al., 2012), differences in selection between locations may

yield different strengths of particular barriers across species pairs. Across Anadromous-Freshwater pairs, for example, spatial variation in selection could be due to environmental factors that may vary across this system's global distribution, such as salinity, competition, parasites, prey, predators, and water color, which are important for divergence and/or isolation (Marchinko & Schluter, 2007, MacColl, 2009). Spatial variation in sexual isolation in the Limnetic-Benthic pairs may result from differences in female preferences and male traits (Boughman et al., 2005, Kozak et al., 2009) as well as density and operational sex ratio (Tinghitella et al., 2013) across lakes.

In contrast to spatial variation, temporal variation in selection might limit divergence because variability across time favors a broad instead of a narrow niche (Levins, 1968, p. 45). In the Limnetic-Benthic pairs, previous work suggests ecological selection against hybrids is stronger in some years than others (Gow et al., 2007, Taylor et al., 2012), and the temporal variation among estimates generated a weak average estimate of hybrid ecological inviability that we report here. Thus, variation in hybrid ecological inviability may limit its role in total isolation in Limnetic-Benthic pairs across years. Temporal variation in hybrid ecological inviability has even more dramatic effect on isolation between species in Darwin's finches (Grant & Grant, 1993).

Having multiple estimates of a single barrier is rare but can provide insight into which reproductive barriers vary over time or space and reveal variation in the evolutionary forces that underlie isolation. Future work examining variation in individual barriers as well as total isolation could determine when variation in genetic or selective mechanisms helps or hinders

speciation. Furthermore, we need to consider the potential for spatial and temporal variation in isolation estimates when we draw conclusions from a single estimate of isolation.

The relative importance of types of barriers during speciation

We found that premating isolation likely evolves before and is stronger than postmating isolation at early stages of the speciation process. Thus, premating isolation can initiate the speciation process. Stronger pre- versus postmating isolation is common across plants and animals as well as sympatric and allopatric species (Coyne & Orr, 1989, 1997, Kay, 2006, Martin & Willis, 2007, Lowry et al., 2008, Dopman et al., 2009, Schemske, 2010). The earlier evolution of premating isolation, specifically sexual isolation, is supported in sympatric (Coyne & Orr, 1989, 1997) and allopatric (Mendelson, 2003) animal populations. For plants, however, it is not yet clear whether premating consistently evolves before or after postmating isolation (Moyle et al., 2004, Scopece et al., 2007, Widmer et al., 2009), and more studies that examine many premating barriers in addition to postmating barriers are needed.

Of postmating barriers, extrinsic isolation seems to evolve before intrinsic isolation, yet intrinsic isolation is likely necessary to complete speciation. Only the oldest Japan-Pacific pair has significant intrinsic isolation, and the Limnetic-Benthic pairs that experienced a collapse lack intrinsic isolation. Other studies also find little to no intrinsic postmating isolation in young species (Anurans: Blair, 1964, *Drosophila*: Coyne & Orr, 1989, 1997, Lepidoptera: Presgraves, 2002, centrarchids: Bolnick & Near, 2005). Indeed, intrinsic postmating isolation may evolve after speciation is essentially complete (birds: Price & Bouvier, 2002).

The difference in the evolutionary rate of extrinsic and intrinsic postmating isolation could be due to different underlying evolutionary forces. Extrinsic postmating isolation can evolve as a direct result of divergent adaptation (Hatfield & Schluter, 1999, Schluter, 2000). Divergent natural selection can generate rapid and extensive divergence that results in ecological and sexual selection against hybrids. Intrinsic postmating isolation that results from genetic drift will most certainly be very slow to evolve (e.g., Bolnick & Near, 2005). However, intrinsic postmating isolation can also evolve as a consequence of divergent adaptation (Gavrilets, 2004, reviewed in Nosil, 2012). In fact, incompatibilities can arise more easily when populations adapt to different rather than similar environments because genetic differences will be more common (e.g., Unkless & Orr, 2009). It is unclear how commonly extrinsic and intrinsic isolation are underlain by similar evolutionary forces or facilitate each other to evolve. This gap in our understanding primarily results from studies that measure only intrinsic or extrinsic postmating isolation or combine these two types. Future work that examines the causal mechanisms and resulting genetic signatures of intrinsic and extrinsic isolation can help to clarify why these two forms of postmating isolation may evolve at different rates.

Although intrinsic postmating isolation evolves relatively late in the speciation process, it is likely essential for completing speciation and keeping taxa from reversing along the speciation continuum. In addition to the collapse in the Limnetic-Benthic pairs, other cases of reverse speciation have occurred in species pairs that lacked intrinsic postmating isolation (Seehausen et al., 1997, Vonlanthen et al., 2012). Intrinsic isolation is probably necessary to complete speciation because this form of isolation results from low fitness in hybrids in any

environment, while other forms of isolation commonly depend on particular environmental conditions for their expression (reviewed in Coyne & Orr, 2004).

In addition to the specific roles that particular barriers play in the initiation or completion of speciation, we also find that additional barriers of various types likely contribute to continued accumulation of isolation. Specific types of barriers might be needed early to initially reduce gene flow sufficiently such that diverging phenotypes do not immediately homogenize and late to reduce nearly all gene flow and restrict future mixing between genomes of each species, but any form of reproductive isolation should reduce gene flow and could lead to more divergence. Thus, there is not an exact formula for the barriers needed at intermediate stages of divergence that contribute to total isolation and facilitate forward movement along the speciation continuum.

The reverse and halted processes of speciation

Cases of reverse speciation show that the reverse process can be particularly rapid and dramatic, with significant reduction of genetic and phenotypic divergence as well as the suspected loss of one or more reproductive barriers (Seehausen et al., 1997, Taylor et al., 2006, Vonlanthen et al., 2012). The loss of sexual isolation is common among current examples of reversal (Seehausen et al., 1997, Fisher et al., 2006, Richmond & Jockusch, 2007, Ward & Blum, 2012, Lackey & Boughman, 2013), and theory shows how quickly and permanently the loss of sexual isolation can cause reversal (Gilman & Behm, 2011). Here, we similarly find that sexual isolation was significant lost in the collapsed pair. Additionally, significantly and strongly negative sexual isolation may explain the halted status of the Lake-Stream pairs. However,

more examples are needed, especially in taxa other than fishes, to determine the generality of patterns of reverse speciation. One commonality is that human-induced environmental change is the crux of almost all cases of reverse speciation (Seehausen et al., 1997, Fisher et al., 2006, Taylor et al., 2006, Vonlanthen et al., 2012, Ward & Blum, 2012, but see Richmond & Jockusch, 2007) as well as other instances of extensive hybridization (Rhymer & Simberloff, 1996, Grant & Grant, 2008, Heath et al., 2010).

Previous work suggested that environmental change could be the immediate cause of the loss of sexual isolation in the Limnetic-Benthic collapsed pair (Taylor et al., 2006), but, surprisingly, this does not seem to be the case. A recent study found that females discriminated strongly between species even in the absence of environmental differences (Lackey & Boughman, *in review*) that were important for the evolution of sexual isolation (Boughman et al., 2005). Male competition, however, is environmentally-dependent and could have undermined sexual isolation after environmental change (Lackey & Boughman, 2013).

Reversals documented thus far have provided evidence of loss of genetic and/or phenotypic differentiation, but no study has quantified the strength of many reproductive barriers before and after a collapse because this data is typically unavailable. Although we found the loss of sexual isolation, we were surprised that no other barriers showed significant loss. The maintained strength of habitat isolation was particularly unexpected given the loss of plants, which comprised one of the primary differences between the two historical mating habitats (Ridgway & McPhail, 1987). Limnetic-like and benthic-like forms may still spatially segregate by nesting at different depths (Bentzen et al., 1984) or through competitive interactions between males of each species to establish territories (Lackey & Boughman, 2013).

Thus, perhaps habitat isolation is stable once it evolves. One way that habitat isolation could remain strong is if juveniles learn environmental features of their natal habitat and return there to mate as adults (e.g., Beltman & Haccou, 2005). We also did not detect a pattern of loss of hybrid ecological inviability in the Limnetic-Benthic collapsed pair in contrast to previous work (Behm et al., 2010), which likely results from the temporal and spatial variation in this estimate across studies (see references in Supplemental Table 1). These surprises emphasize the importance of measuring multiple barriers after collapse to determine what forms of isolation have actually degraded. This will also strengthen our understanding of how environmental change, hybridization, and introgression can affect reverse speciation.

We still know little about the nature of reverse speciation. A single case of reversal cannot reveal the order in which barriers evolved in the forward direction. However, we could gain insight into the extent to which the reverse process may or may not mirror the forward process from studies like ours that estimate multiple barriers across the speciation continuum including representatives of reversal. More generally, studying cases of reversal caused by environmental change can reveal the importance of divergent ecological selection for total isolation and individual barriers. If divergent ecological selection is disrupted and species move backward along the speciation continuum, this underscores the important and pervasive role of divergent ecological selection in the speciation process.

Patterns of asymmetrical isolation and potential causes and consequences

Asymmetries in isolation may be common and, though potentially due to chance, can provide insight into how reproductive isolation evolved (Coyne & Orr, 2004, Turelli & Moyle,

2007, Lowry et al., 2008). In this study, we find significant asymmetries in premating and extrinsic, but not intrinsic, postmating isolation. The most frequently asymmetric barriers in this study are immigrant and hybrid ecological inviability, though habitat and sexual isolation were also significantly asymmetric. Although we cannot pinpoint causes for these asymmetries, we discuss below potential contributing factors.

For premating isolation, most work to date has focused on measuring asymmetries in sexual isolation (Kaneshiro, 1976, Watanabe & Kawanishi, 1979, Arnold et al., 1996, Bordenstein et al., 2000, Shine et al., 2002, Hardwick et al., 2013, Oh et al., 2013). These studies have shown asymmetrical sexual isolation may be common and occur by chance or due to differences in sexual selection on each species. Differences in sexual selection between species may result from differences in population size, where the rarer species hybridizes more (Wirtz, 1999), or differences in mating system, where the more polyandrous species hybridize more (Veen et al., 2011). Additionally, evolutionary history could explain differences in sexual selection between species, although it is currently unclear whether sexual isolation is commonly stronger in the ancestral (Kaneshiro, 1976, Tinghitella & Zuk, 2009, Oh et al., 2013) or derived species (Watanabe & Kawanishi, 1979, Hardwick et al., 2013), or whether asymmetry in sexual isolation cannot predict the direction of evolution at all (Arnold et al., 1996).

For postmating isolation, studies of asymmetries have focused on intrinsic postmating isolation and identified the potential underlying molecular mechanisms as cytonuclear interactions in addition to other factors inherited from one parent (Tiffin et al., 2001, Turelli & Moyle, 2007). We focus here on asymmetries between species, but intrinsic postmating isolation asymmetries between the sexes are thought to be common due to Haldane's rule that

when only one sex shows intrinsic inviability or sterility, it is the heterogametic sex, perhaps most commonly due to negative recessive alleles expressed in heterogametic hybrids (Haldane, 1992, Coyne & Orr, 2004). Little attention has been given to asymmetries in extrinsic postmating isolation, although differences in natural selection between habitats could result in asymmetric hybrid ecological inviability (e.g., Kuwajima et al., 2010), and differences in sexual selection between species could similarly result in asymmetric sexual selection against hybrids.

Despite significant asymmetries in individual barriers within most systems, we find no significant asymmetries in total isolation. This may occur because barriers act in reciprocal directions with gene flow allowed by one barrier being blocked by a second barrier (Wade et al., 1995, Takami et al., 2007). Recent work suggests that when postmating isolation is asymmetric, premating isolation that evolves due to reinforcement should be asymmetric in the same direction due to the direction of selection against hybrids (Yukilevich, 2012). Thus, a pattern of reciprocal asymmetries across pre- and postmating barriers suggests that evolutionary forces other than reinforcement are acting. Total isolation may also be more symmetric than the contributing individual barriers because late-acting asymmetric barriers may have little impact on total isolation if an earlier-acting barrier symmetrically blocks the majority of gene flow between species (e.g., Kuwajima et al., 2010). Thus, while asymmetries in individual barriers may reveal patterns of gene flow and perhaps introgression, we should also examine asymmetries in total isolation to understand how asymmetrical isolation might underlie past and future movement along the speciation continuum.

The direction of asymmetrical introgression may give insights into how reproductive isolation evolved. We suggest that the direction of introgression between species will match

that of asymmetrical isolation when asymmetries in multiple barriers act in the same direction or when asymmetry is primarily from a single, strong, and early-acting barrier that has a relatively large impact on total isolation. The former pattern fits the Limnetic-Benthic collapsed pair, where genetic patterns of introgression of limnetic alleles into the benthic genome (Gow et al., 2006, Taylor et al., 2006) are consistent with the direction of asymmetries in sexual isolation as well as immigrant and habitat ecological inviability. For instance, hybrid ecological inviability is more likely to maintain benthic over limnetic alleles in the population because benthic fish outperform hybrids significantly, although weakly, whereas limnetics and hybrids perform equally.

The extent of asymmetries may change along the speciation continuum. Previous theoretical and empirical work in sexual isolation predicts that asymmetries may be highest at intermediate stages of the speciation process (Arnold et al., 1996). Early stages of divergence may be strongly limited by very high levels of asymmetry, while intermediate or low levels of asymmetries can allow polymorphisms to arise (Chunco et al., 2007). At later stages of the speciation process, it is possible that strong asymmetries could halt further divergence. For instance, asymmetric gene flow has been theoretically shown to limit reinforcement (Servedio & Kirkpatrick, 1997). We can further imagine that strong or numerous asymmetries could reverse or halt movement along the speciation continuum if gene flow in one direction overwhelms divergence between species. It is unclear how long it might take for asymmetries to disappear completely once they evolve (Coyne & Orr, 1998), but isolation could become more symmetric as additional incompatibilities arise (Turelli & Moyle, 2007) or if selection pressures on each species equalize. Future work exploring how selection and other

evolutionary forces may result in asymmetric isolation, and what outcomes this has for movement along the speciation, would be fruitful.

Conclusions

Many findings in this study are broadly applicable to cases of speciation driven primarily by divergent natural and sexual selection for both sympatric and allopatric species. Examining patterns of reproductive isolation in species pairs that span the speciation continuum can reveal how reproductive isolation evolves and what evolutionary forces impart isolation early and late in the speciation process. From this study, we can infer the importance of particular barriers at different stages of the speciation process as well as the order of evolution of these barriers.

The types of barriers that contribute most to isolation differ along the speciation continuum, thus the primary barriers that initiate speciation differ from those that complete it. Premating isolation likely plays an especially important role in initiating speciation. Of premating barriers, habitat isolation seems to be essential for initial divergence. Sexual isolation also seems to contribute to divergence relatively early in the speciation process and evolves to be strong quite quickly. Moreover, its loss in reverse speciation and absence in halted movement along the speciation continuum highlights its potential importance for general movement or lack thereof along the speciation continuum. Other pre- and postmating barriers are also important for completing and maintaining speciation, with pre- and postmating isolation playing equal roles in the late stages of the speciation process. Intrinsic postmating isolation is likely necessary to complete and maintain speciation. Asymmetrical

barriers may reveal selection that acts differently on each taxon and could predict the likelihood of forward, halted, or reverse movement along the continuum as well as the direction of introgression if reversal does occur. This study, and others that look at most or all potential reproductive barriers in systems that span the speciation continuum, can generate important insights into how new species evolve, what maintains them, and when and how they might collapse.

APPENDIX

APPENDIX: Chapter 4 tables and figures

Table 4.1 **Individual barrier estimates for each source with sampling details.**

(a) Japan-Pacific

Barrier	Source	Sample location	Sample size	RI	95%CI
Habitat					
	Kume et al., 2005 ^a	Lake Akkeshi system	10 samples ¹	0.2301	0.2609
	Kume et al., 2010 ^a	Lake Akkeshi system	12 samples ²	0.8606	0.1960
Temporal					
	Kume, 2007	Lake Akkeshi system	3 time periods ³	0.4046	0.5554
Sexual					
	Kitano et al., 2009 ^a	Lake Akkeshi system	59 trials ^b	0.4195	0.1259
Gametic & Genetic incompatibilities					
	Yamada & Goto, 2003	Tokachi, Kushiro Riv.	14 clutches	-0.0435	0.1069
Hybrid ecological inviability					
	Kitano et al., 2009 ^a	Lake Akkeshi system	969 fish	0.7354	0.0278
Hybrid sterility					
	Yamada & Goto 2003	Tokachi, Kushiro Riv.	33 clutches	0.1510	0.1222

For each data source used in this study, we provide details on location, sample size, and barrier strength. We provide 95% confidence intervals and bold any estimate with a confidence interval that does not encompass zero. For barriers with multiple contributing studies, we calculated weighted means, which we present in the main text of the paper.

(a) includes multiple years of sampling

(b) used choice trials instead of no-choice trials

(c) excludes some study sites because both ecotypes do not co-occur at all sites

(1) each of four sites sampled across one to three years

(2) each of three sites sampled once a year for four years

Table 4.1 (cont'd)

- (3) each sampling time period spans 10 consecutive days
- (4) males only
- (5) number of traps for each site: 22 and 24
- (6) females only
- (7) number of traps for each site: 17, 8, 15, and 3
- (8) sampled early in breeding season only
- (9) growth rates
- (10) survival
- (11) limnetic and hybrid fish only
- (12) three lakes and two intertidal zones
- (13) samples reported for May and July
- (14) see McKinnon et al. 2004 supplement for more detail on sampling locations
- (15) two stream sites, five lake sites
- (16) sample size not reported, so set equal to other estimate for same barrier

(b) Limnetic-benthic

Barrier	Source	Sample location	Sample size	RI	95%CI
Habitat					
	Ridgway & McPhail, 1984 ⁴	Enos Lake	2 sites ⁵	0.6234	0.6715
	Vamosi & Schluter, 1999 ^{6, a}	Paxton Lake	4 sites ⁷		0.2095 0.3988
	Head & Boughman, unpub	Paxton Lake	114 traps	0.2592	0.0804
	Lackey & Boughman, unpub ⁸	Paxton Lake	40 traps	0.5068	0.1549
Immigrant inviability					
	Schluter, 1995 ^{9, a}	Paxton Lake	56 fish	0.2717	0.1165
	Rundle, 2002 ⁹	Paxton Lake	48 fish	0.3355	0.1336
	Vamosi, 2002 ^{10, a}	Paxton Lake	1304 fish	0.1451	0.0191

Table 4.1 (cont'd)

(b) Limnetic-benthic (continued)

Barrier	Source	Sample location	Sample size	RI	95%CI
Temporal					
	Head & Boughman, unpub	Paxton Lake	11 days	0.0627	0.1433
	Lackey & Boughman, unpub ⁸	Paxton Lake	3 days	0.0490	0.2444
Sexual					
	Ridgway & McPhail, 1984 ^a	Enos Lake	38 trials	0.2308	0.1340
	Boughman et al., 2005 ^a	Paxton Lake	229 trials	0.2544	0.0564
	Lackey & Boughman, 2013	Paxton Lake	96 trials	0.2174	0.0825
	Boughman et al., 2005 ^a	Priest Lake	80 trials	0.6745	0.1027
Gametic incompatibility					
	Hatfield & Schluter, 1999	Paxton Lake	57 clutches	-0.0015	0.0101
	Lackey & Boughman, unpub	Paxton Lake	49 clutches	-0.0186	0.0379
Genetic incompatibility					
	Hatfield & Schluter, 1999	Paxton Lake	56 clutches	-0.0108	0.0270
	Lackey & Boughman, unpub	Paxton Lake	46 clutches	0.0012	0.0102
Hybrid ecological inviability					
	Schluter, 1995 ^{9, a}	Paxton Lake	39 fish	0.1463	0.1109
	Hatfield & Schluter, 1999 ⁹	Paxton Lake	78 fish	0.1546	0.0802
	Rundle, 2002 ⁹	Paxton Lake	72 fish	0.0450	0.0479
	Gow et al., 2007 ^a	Paxton Lake	1573 fish	0.0709	0.0127
	Behm et al., 2010	Paxton Lake	100 fish	0.4363	0.0972
	Taylor et al., 2012 ^{9, a}	Paxton Lake	181 fish	-0.0068	0.0120
	Gow et al., 2007 ^a	Priest Lake	1331 fish	0.2939	0.0245
	Taylor et al., 2012 ^{9, a}	Priest Lake	183 fish	-0.0182	0.0194

Table 4.1 (cont'd)

(b) Limnetic-benthic (continued)

Barrier	Source	Sample location	Sample size	RI	95%CI
Sexual selection against hybrids					
	Vamosi & Schluter, 1999 ^{11, a}	Paxton Lake	22 trials ^b	0.6364	0.2020
Hybrid sterility					
	McPhail, 1984	Enos Lake	15 clutches	0.0029	0.0273
	McPhail, 1992	Paxton Lake	30 clutches	0.0048	0.0246
	Hatfield & Schluter, 1999	Paxton Lake	83 clutches	0.0222	0.0317

(c) Anadromous-freshwater

Barrier	Source	Sample location	Sample size	RI	95%CI
Habitat					
	Hagen, 1967 ^{a, c}	BC, Little Camp. Riv.	5 sites	0.8326	0.3272
	Gelmond, 2007 ¹²	Alaska, Middleton Is.	5 sites	0.2034	0.3528
	Karve et al., 2008	Alaska, Mud Lake	10 sites	0.1500	0.2213
Temporal					
	Hagen, 1967	BC, Little Camp. Riv.	43 days	0.8800	0.0971
	Gelmond, 2007	Alaska, Middleton Is.	2 time periods ¹³	0.3166	0.6446
	Karve et al., 2008	Alaska, Mud Lake	12 days	0.2178	0.2335
Sexual					
	Hay & McPhail, 1975	BC, Little Camp. Riv.	268 trials ^b	0.2877	0.0542
	McKinnon et al., 2004	Alaska ¹⁴	10 trials	0.3333	0.2922
	McKinnon et al., 2004	BC ¹⁴	54 trials	0.4319	0.1321
	McKinnon et al., 2004	Japan ¹⁴	92 trials	0.8755	0.0670
	McKinnon et al., 2004	Scotland ¹⁴	62 trials	0.0684	0.0628
	Furin et al., 2012 ^a	Alaska, Loberg Lake	86 trials	0.3725	0.1022

Table 4.1 (cont'd)

(c) Anadromous-freshwater

Barrier	Source	Sample location	Sample size	RI	95%CI
Gametic & Genetic incompatibilities	Hagen, 1967 ^a	BC, Little Camp. Riv.	72 clutches	0.0059	0.0177
Hybrid ecological inviability	Hagen, 1967 ^a	BC, Little Camp. Riv.	644 fish	0.0363	0.0144
Hybrid sterility	Hagen, 1967 ^a	BC, Little Camp. Riv.	66 clutches	-0.0241	0.0370

(d) Lake-stream

Barrier	Source	Sample location	Sample size	RI	95%CI
Habitat	Lavin & McPhail, 1993 ^c	Misty system	8 sites ¹⁵	0.8362	0.2564
Immigrant inviability	Hendry et al., 2002	Mackie system	144 fish	0.1459	0.0577
	Hendry et al., 2002 ^a	Misty system ^a	288 fish	0.1526	0.0415
Temporal	Moore & Hendry, unpub.	Misty system ^a	289 fish	0.0380	0.0221
	Stinson, 1983	Drizzle system ^a	289 fish ¹⁶	0.1130	0.0365
Sexual	Raeymaekers et al., 2010	Misty system	70 trials ^b	-0.4476	0.1165
	Rasanen et al., 2012	Misty system	51 trials	-0.2793	0.1231
Gametic & Genetic incompatibilities	Lavin & McPhail, 1993	Misty system	15 clutches	-0.0155	0.0626
Hybrid mating	Raeymaekers et al, 2010	Misty system	130 trials ^b	-0.5616	0.0853

Table 4.1 (cont'd)

(e) Limnetic-benthic collapsed

Barrier	Source	Sample location	Sample size	RI	95%CI
Habitat					
	Head & Boughman, unpub	Enos Lake	169 traps	0.3111	0.0698
	Lackey & Boughman, unpub ⁸	Enos Lake	8 sites	0.2630	0.3050
Temporal					
	Head & Boughman, unpub	Enos Lake	11 days	0.0808	0.1620
	Lackey & Boughman, unpub ⁸	Enos Lake	2 days	0.1704	0.5211
Sexual					
	Boughman et al., 2005 ^a	Enos Lake	141 trials	0.2443	0.0709
	Lackey & Boughman, 2013	Enos Lake	100 trials	0.1206	0.0638
Gametic incompatibility					
	Lackey & Boughman, unpub	Enos Lake	72 clutches	-0.0273	0.0376
Genetic incompatibility					
	Lackey & Boughman, unpub	Enos Lake	69 clutches	-0.0182	0.0315
Hybrid ecological inviability					
	Behm et al., 2010	Enos Lake	138 fish	0.0761	0.0443

Table 4.2 Estimates of hybridization and genetic differentiation between taxa.

System	Hybrid (%)	Genetic differentiation	Divergence time
Japan-Pacific	1.08-5.71 ¹⁻³	$F_{ST} = 0.160$ ²	1.5 my ²
Limnetic-Benthic	<1-5.21 ⁴⁻⁶	$F_{ST} = 0.209-0.213$ ⁷	13,000y ⁸
Anadromous-Freshwater	24.1 ⁹	$F_{ST} = 0-0.641$ ¹⁰	25-15,000y ^{8,11}
Lake-Stream	0.36 ¹²	$F_{ST} = 0-0.197$ ¹³⁻¹⁶	13,000y ⁸
Limnetic-Benthic collapsed	7.48-24.0 ^{6,17}	allelic diff = 13.41 ¹⁸	13,000y ⁸

For each system, we present published data on the extent of hybridization measured as the proportion of hybrids detected by morphological and molecular measures. See footnotes for methods used in each study. We also present estimates of genetic differentiation, primarily using F_{ST} , which is most widely available across systems. For the limnetic-benthic collapsed pair, we provide the only data available to estimate differentiation: average differences in allelic counts between red and black forms that represent limnetic-like and benthic-like forms.

- (1) Higuchi et al., 1996 (allozymes)
- (2) Kitano et al., 2007 (microsatellites)
- (3) Kitano et al., 2009 (microsatellites)
- (4) McPhail, 1984 (morphology)
- (5) McPhail, 1992 (morphology)
- (6) Gow et al., 2006 (microsatellites)
- (7) Taylor & McPhail, 2000 (mtDNA)
- (8) McPhail, 1994
- (9) Hagen, 1967 (morphology)
- (10) Jones et al., 2012 (SNPs)
- (11) Furin et al., 2012
- (12) Lavin & McPhail, 1993 (morphology)
- (13) Hendry et al., 2002 (mtDNA, microsatellites)
- (14) Hendry & Taylor, 2004 (microsatellites)
- (15) Berner et al., 2009 (microsatellites)
- (16) Roesti et al., 2012 (SNPs, microsatellites)
- (17) Taylor et al., 2006 (microsatellites)
- (18) Malek et al., 2012 (microsatellites)

Table 4.3 **Reproductive barriers.**

Barrier	Description	Metric
Habitat	use of different habitats reduces encounter rates between potential mates	number of fish caught at the same site in sympatric region of distributions during breeding season
Immigrant inviability	mortality of poorly adapted immigrants to foreign habitats reduces encounter rates between potential mates	survival or growth rate, as a proxy for survival, for fish in their native versus foreign habitat
Temporal	different reproductive periods reduce encounter rates between potential mates	number of reproductive or total fish caught at the same time during breeding season
Sexual	different mating preferences and signals reduces mating between potential mates	number of spawnings or nest inspections, a proxy for spawning, out of total no-choice or choice trials
Gametic incompatibility	incompatibilities between sperm and eggs reduces zygote formation	number of fertilized out of total eggs per clutch
Genetic incompatibility	non-environmental incompatibilities reduce zygote survival	number of hatched out of fertilized eggs per clutch
Hybrid ecological inviability	environmentally-dependent survival of hybrid offspring	survival or growth rate, as a proxy for survival, of fish to the next life stage, e.g., juvenile to adult
Sexual selection against hybrids	reduced mating success of hybrids due to behavior or environment	number of spawnings or nest inspections, a proxy for spawning, out of total no-choice or choice trials
Hybrid sterility	reduced mating success of hybrids due to inviable or incompatible gametes or low zygote survival	number of hatched out of total eggs per clutch

We list all barriers with definitions and metrics as used in this study. For further detail, see Methods section.

Table 4.4 **Sequential barriers strengths across systems.**

(a) Japan-Pacific

Barrier	Barrier strength		
	Sequential	Strongest	Weakest
Habitat	0.6330	0.7899	0.4765
Temporal	0.1486	0.2017	-0.0789
Sexual	0.0917	0.0046	0.2022
Gametic & Genetic incompatibilities	-0.0056	0.0002	-0.0732
Hybrid ecological inviability	0.0972	0.0027	0.3960
Hybrid sterility	0.1009	0.2160	0.0204
	Total Isolation	Strongest	Weakest
	1.0659	1.2151	0.8747

(b) Limnetic-benthic

Barrier	Barrier strength		
	Sequential	Strongest	Weakest
Habitat	0.3120	0.3823	0.2417
Immigrant inviability	0.1046	0.1055	0.1011
Temporal	0.0346	0.0936	-0.0423
Sexual	0.1693	0.1461	0.1875
Gametic incompatibility	-0.0010	0.0019	-0.0063
Genetic incompatibility	-0.0001	0.0025	-0.0051
Hybrid ecological inviability	0.0195	0.0157	0.0230
Sexual selection against hybrids	0.2298	0.2113	0.2178
Hybrid sterility	0.0038	0.0103	-0.0039
	Total Isolation	Strongest	Weakest
	0.8725	0.9693	0.7136

Sequential barrier strengths are calculated by ordering individual barrier strengths by occurrence in the life cycle. Later-acting barriers can only reduce gene flow not restricted by earlier-acting barriers. The strongest estimates come from using all of the 95% confidence interval upper bounds for individual barrier strengths to calculate sequential barrier strengths. We sum all sequential barrier strengths to get total strength. For some barriers, the “strongest” estimate is not higher than the “weaker” estimate. This is due to the nature of equations 3 and 4 (see methods), where the sequential strength of a barrier depends on the strength of the preceding barrier. We bold estimates when the strongest and weakest estimate do not encompass zero.

Table 4.4 (cont'd)

(c) Anadromous-Freshwater

Barrier	Barrier strength		
	Sequential	Strongest	Weakest
Habitat	0.2384	0.3771	0.0996
Temporal	0.5889	0.5369	0.6162
Sexual	0.0661	0.0356	0.0996
Gametic & Genetic incompatibilities	0.0006	0.0012	-0.0022
Hybrid ecological inviability	0.0039	0.0025	0.0041
Hybrid sterility	-0.0089	0.0055	-0.0172
	Total Isolation	Strongest	Weakest
	0.8895	0.9588	0.8000

(d) Lake-stream

Barrier	Barrier strength		
	Sequential	Strongest	Weakest
Habitat	0.8362	1.0927	0.5798
Immigrant inviability	0.0246	-0.0171	0.0490
Temporal	0.0081	-0.0058	0.0146
Sexual	-0.0482	0.0198	-0.1615
Gametic & Genetic incompatibilities	-0.0028	-0.0042	-0.0405
Sexual selection against hybrids	-0.1023	0.0407	-0.3613
	Total Isolation	Strongest	Weakest
	0.7157	1.1260	0.0801

(e) Limnetic-benthic collapsed

Barrier	Barrier strength		
	Sequential	Strongest	Weakest
Habitat	0.3087	0.3768	0.2407
Temporal	0.0620	0.1523	-0.0494
Sexual	0.1107	0.1052	0.1039
Gametic incompatibility	-0.0142	0.0038	-0.0458
Genetic incompatibility	-0.0097	0.0048	-0.0373
Hybrid ecological inviability	0.0413	0.0430	0.0251
	Total Isolation	Strongest	Weakest
	0.4989	0.6858	0.2373

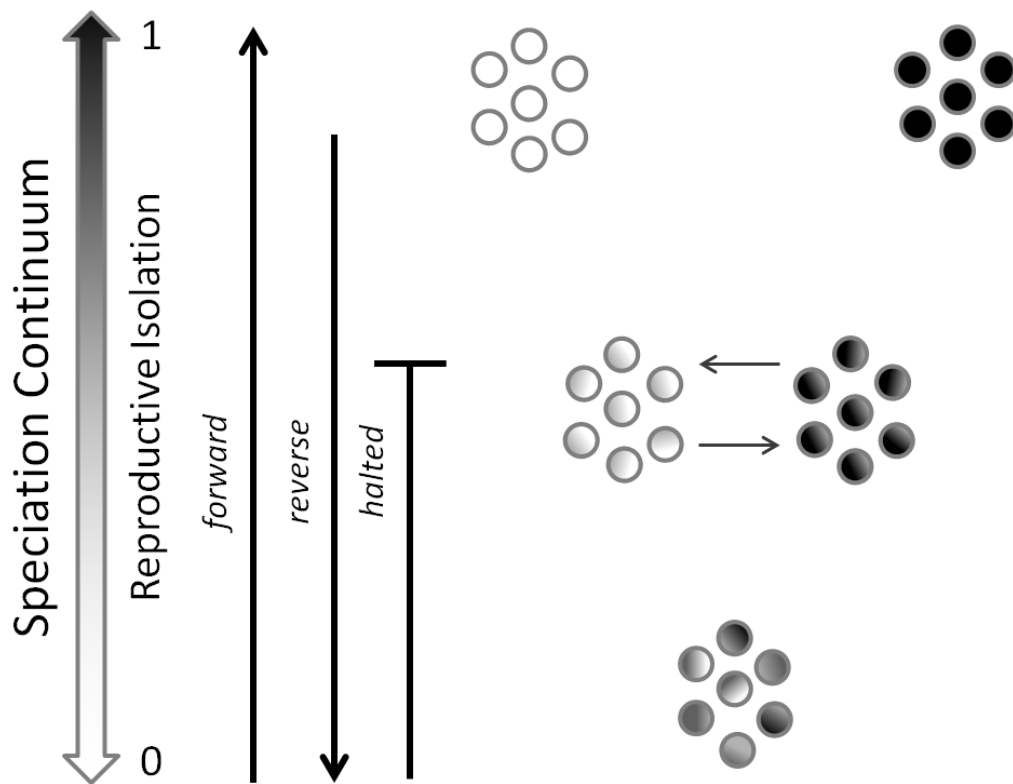


Figure 4.1 **The speciation continuum.**

We depict the speciation continuum with reproductive isolation ranging from 0 (no isolation) to 1 (complete isolation). We show how taxa might move forward or in reverse along the continuum or even become stalled (halted). We illustrate the extent of differentiation and gene exchange between populations as taxa move along the continuum.

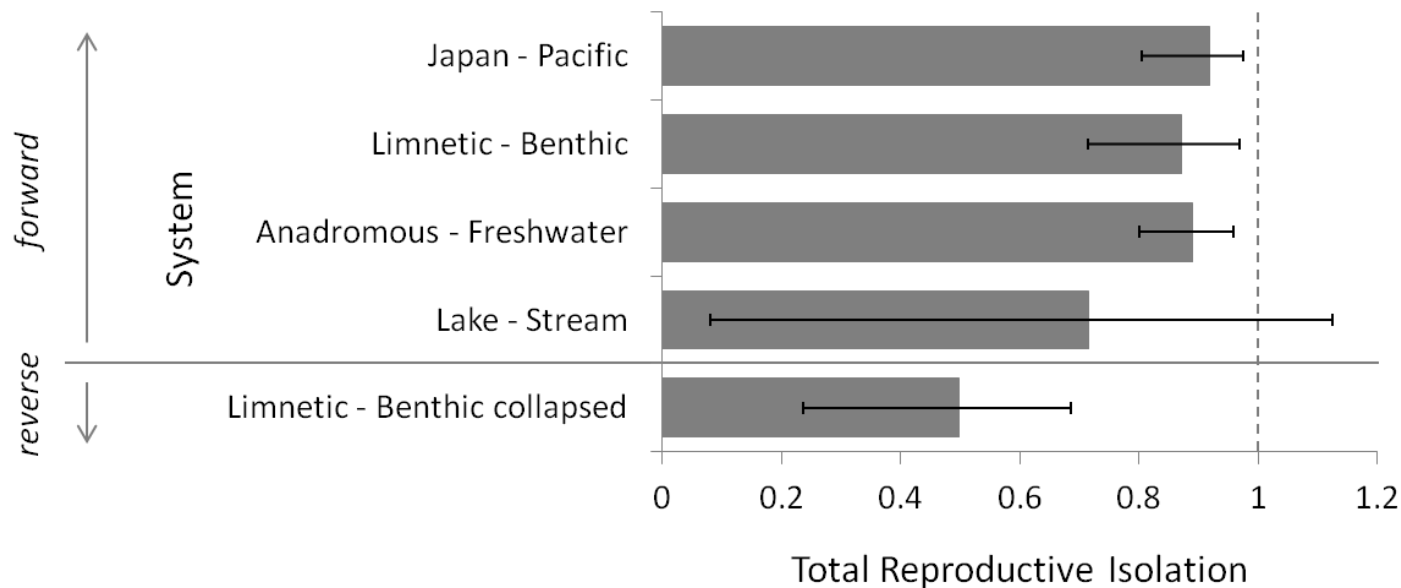


Figure 4.2 Total reproductive isolation across systems.

We depict the speciation continuum along the y-axis with forward and reverse movement. For each system, we show the total reproductive isolation, which is the sum of all barriers' sequential strengths ordered across the life cycle (Table 4). Error bars represent the strongest and weakest estimate of total isolation calculated just like total isolation but using the upper or lower bounds of each barrier's 95% confidence interval. The dashed line shows total isolation of 1, where no gene flow occurs between taxa.

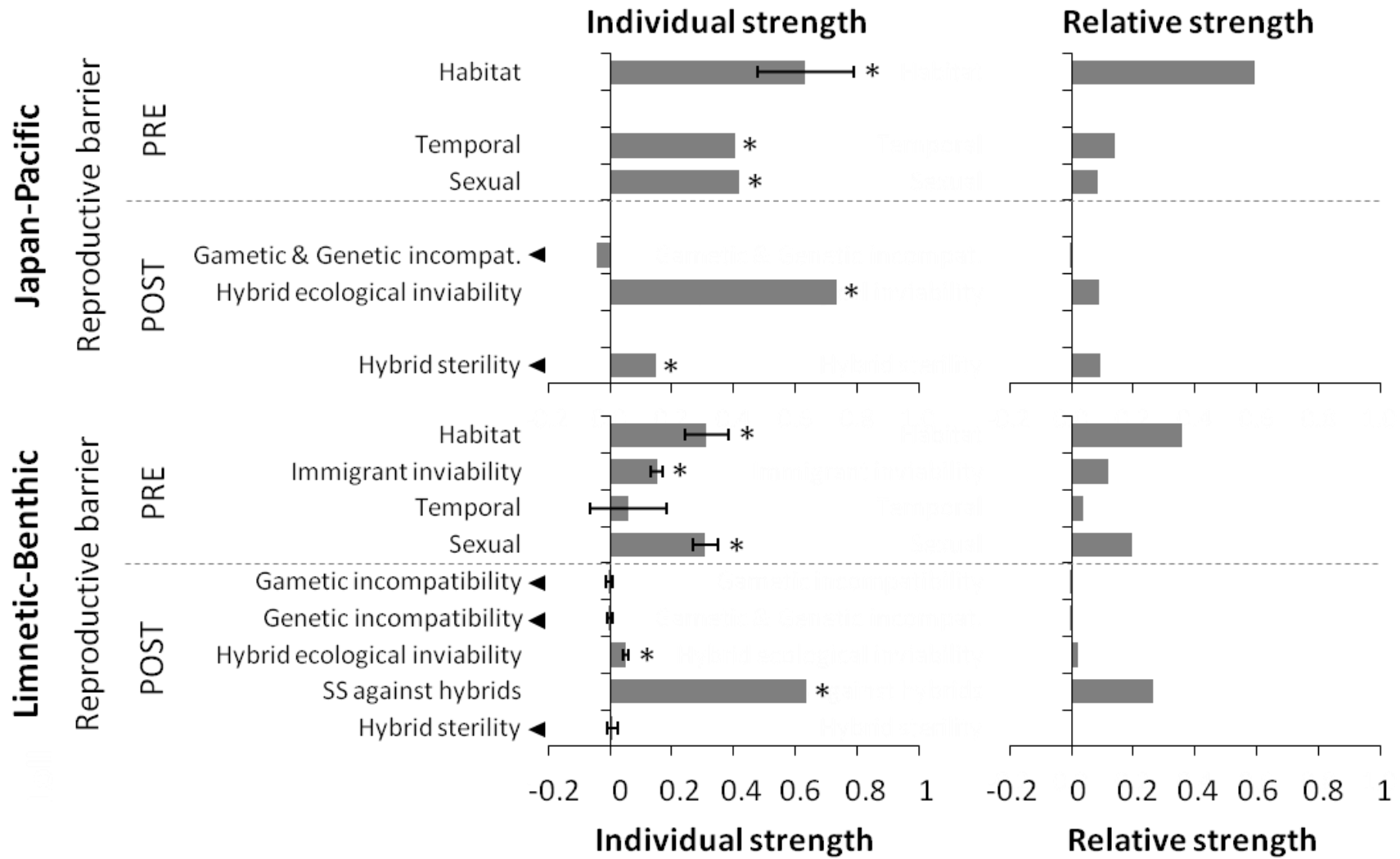


Figure 4.3 **Individual and relative barrier strengths.**

Figure 4.3 (cont'd)

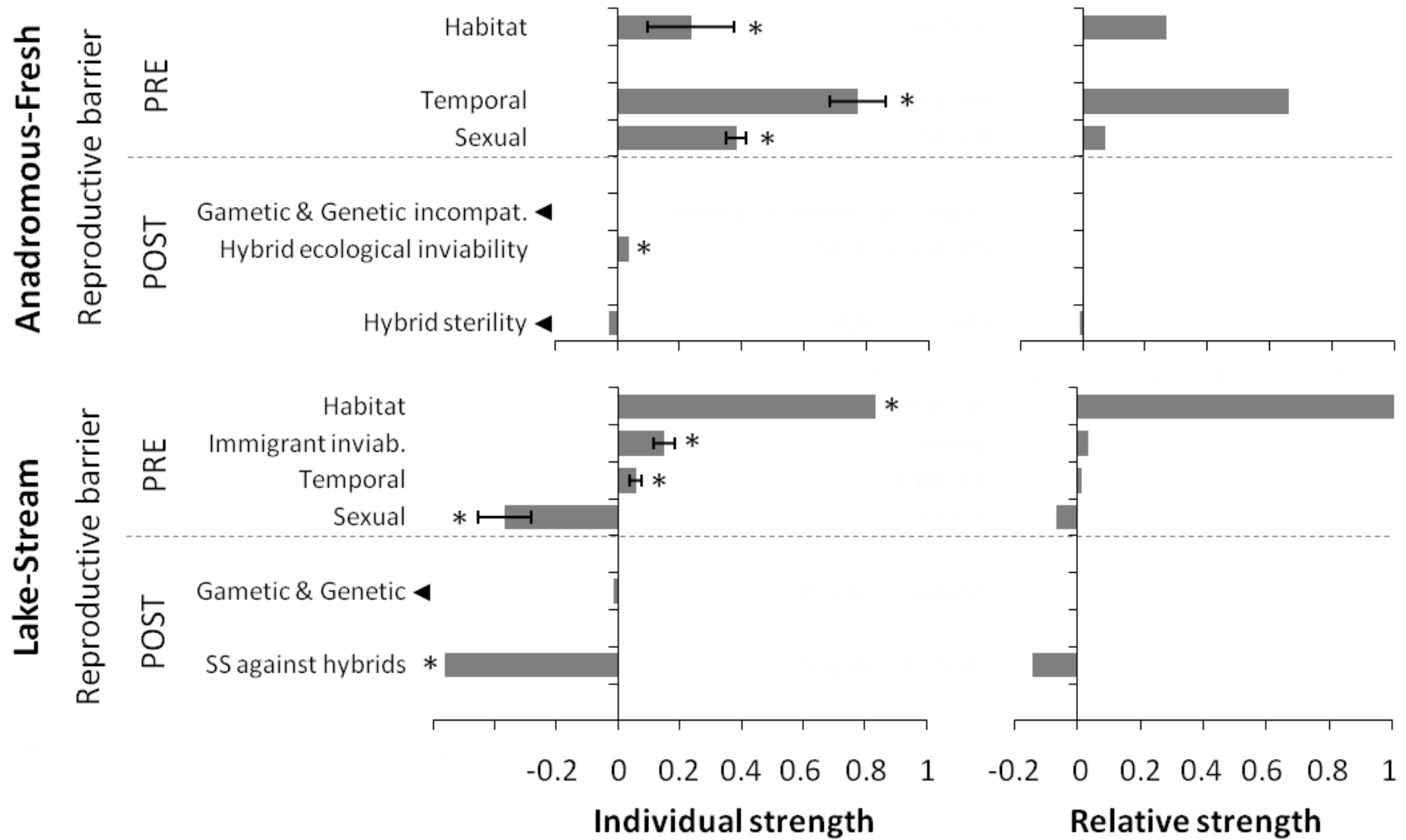
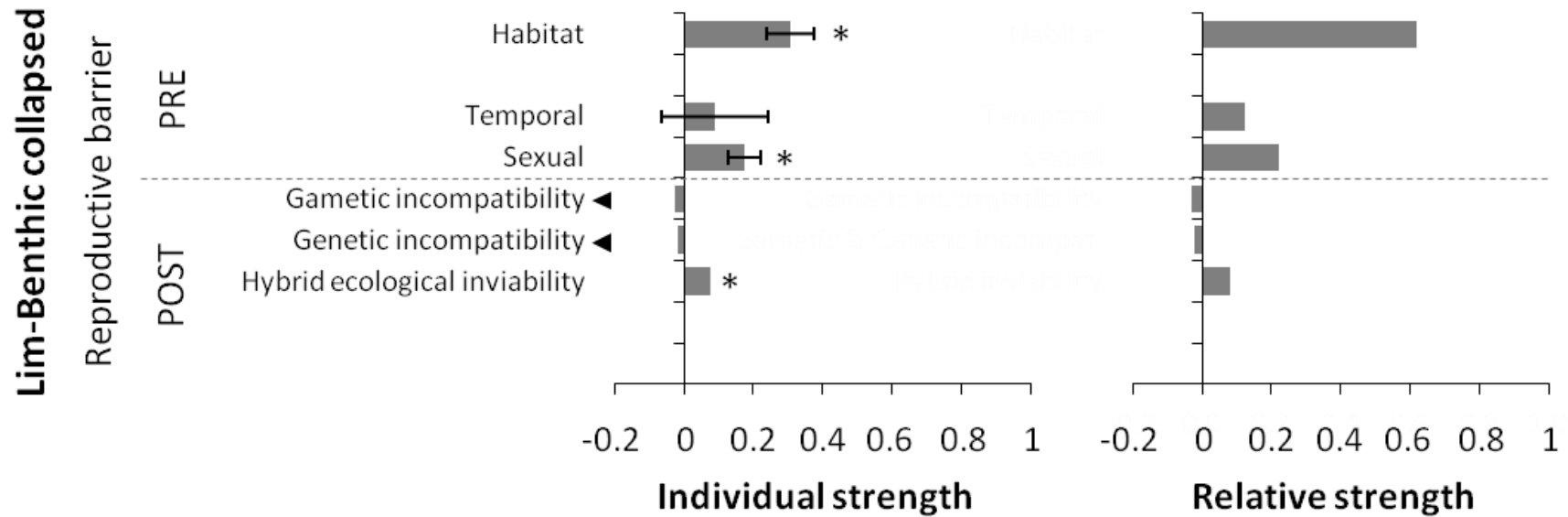


Figure 4.3 (cont'd)



For each system, we show the individual (left column) and relative (right column) strengths for each barrier. Individual strengths come directly from study estimates. We present weighted means with 95% confidence intervals for every barrier with multiple estimates. Barriers estimated from a single study have no error bars. Asterisks denote weighted mean and single barrier estimates with 95% confidence intervals that do not encompass zero. We present 95% confidence intervals for all single study estimates in Table 1. Relative strengths are calculated as the proportion of total isolation each barrier contributes using sequential barrier strengths. See methods for more detail. Black triangles signify intrinsic postmating barriers.

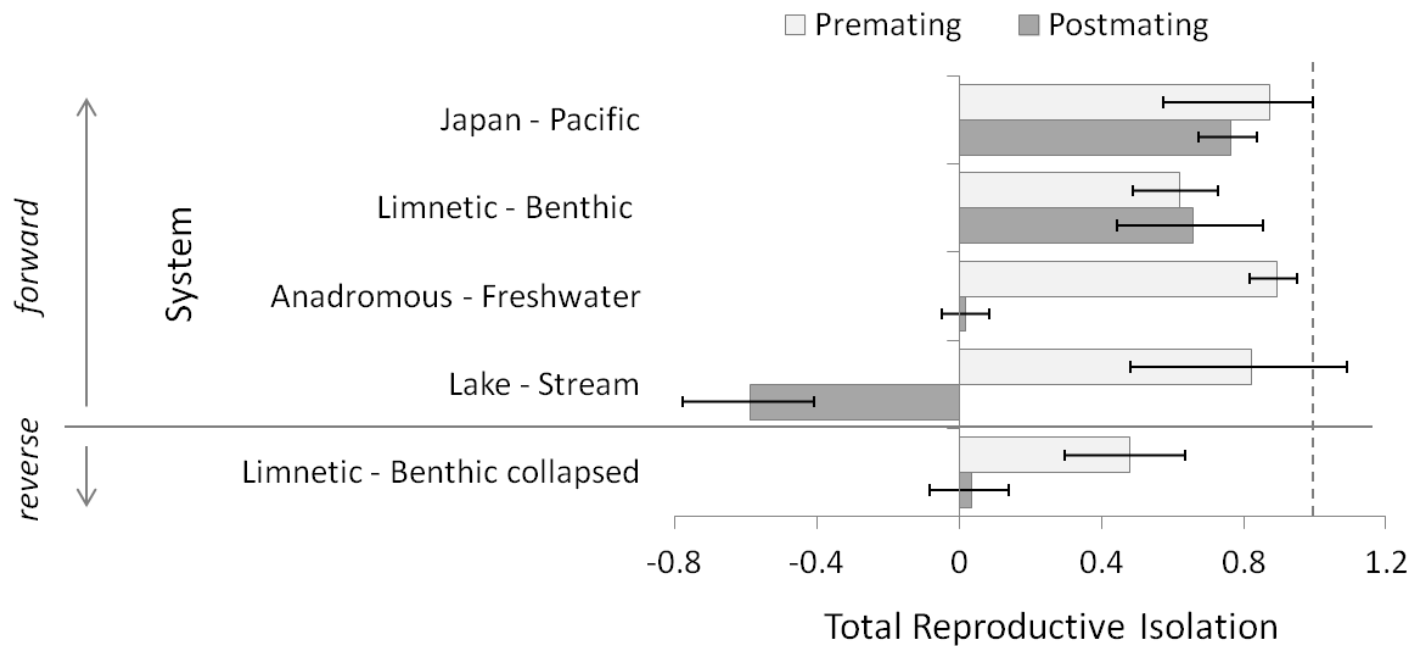


Figure 4.4 **Pre- and postmating isolation.**

For each system, we show the total reproductive isolation due to pre- or postmating isolation alone, which each come from the sum of pre- or postmating barriers' sequential strengths ordered across the life cycle. Error bars represent the strongest and weakest estimate of total pre- or postmating isolation calculated with the upper or lower bounds of each barrier's 95% confidence interval. The dashed line shows total isolation of 1, where no gene flow occurs between taxa. We also depict the speciation continuum with forward and reverse progress along the y-axis.

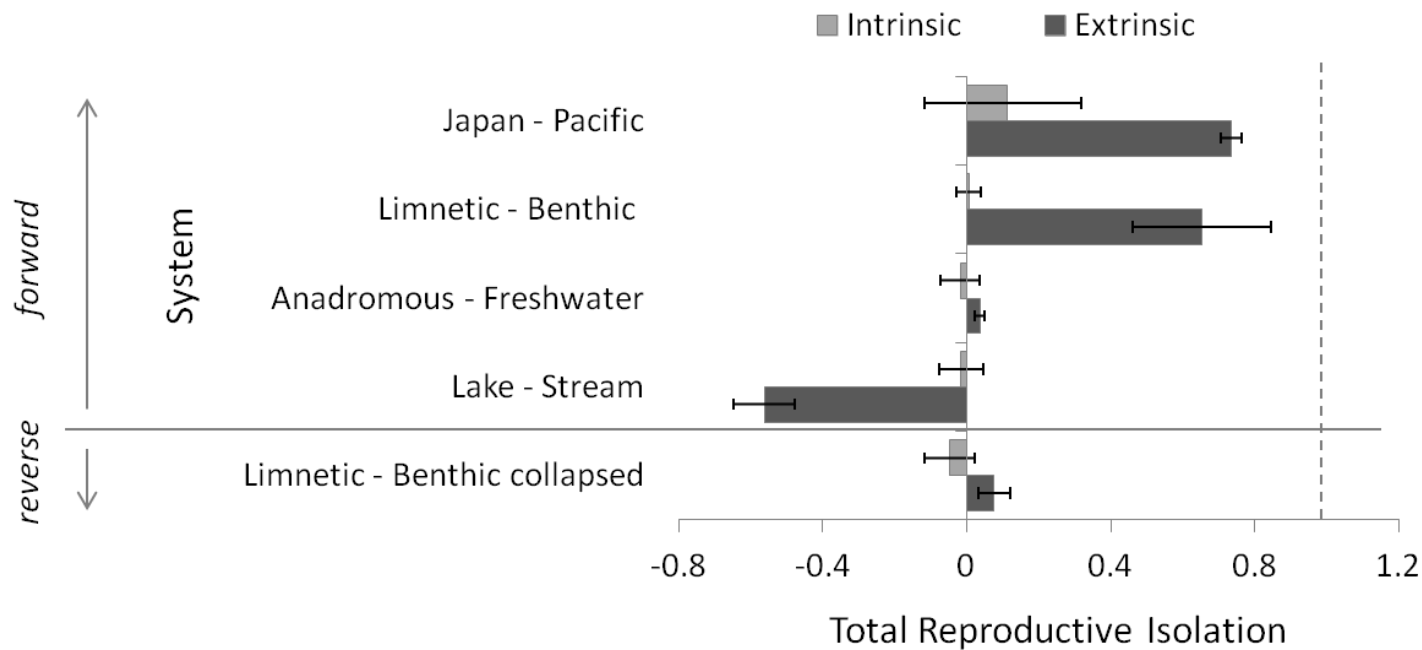


Figure 4.5 **Intrinsic and extrinsic postmating isolation.**

For each system, we show the total reproductive isolation due to intrinsic and extrinsic postmating isolation. Each isolation estimate comes from the sum of intrinsic or extrinsic postmating barriers' sequential strengths ordered across the life cycle. Error bars represent the strongest and weakest estimate of total intrinsic or extrinsic postmating isolation calculated with the upper or lower bounds of each barrier's 95% confidence interval. The dashed line shows total isolation of 1, where no gene flow occurs between taxa. We also depict the speciation continuum with forward and reverse progress along the y-axis.

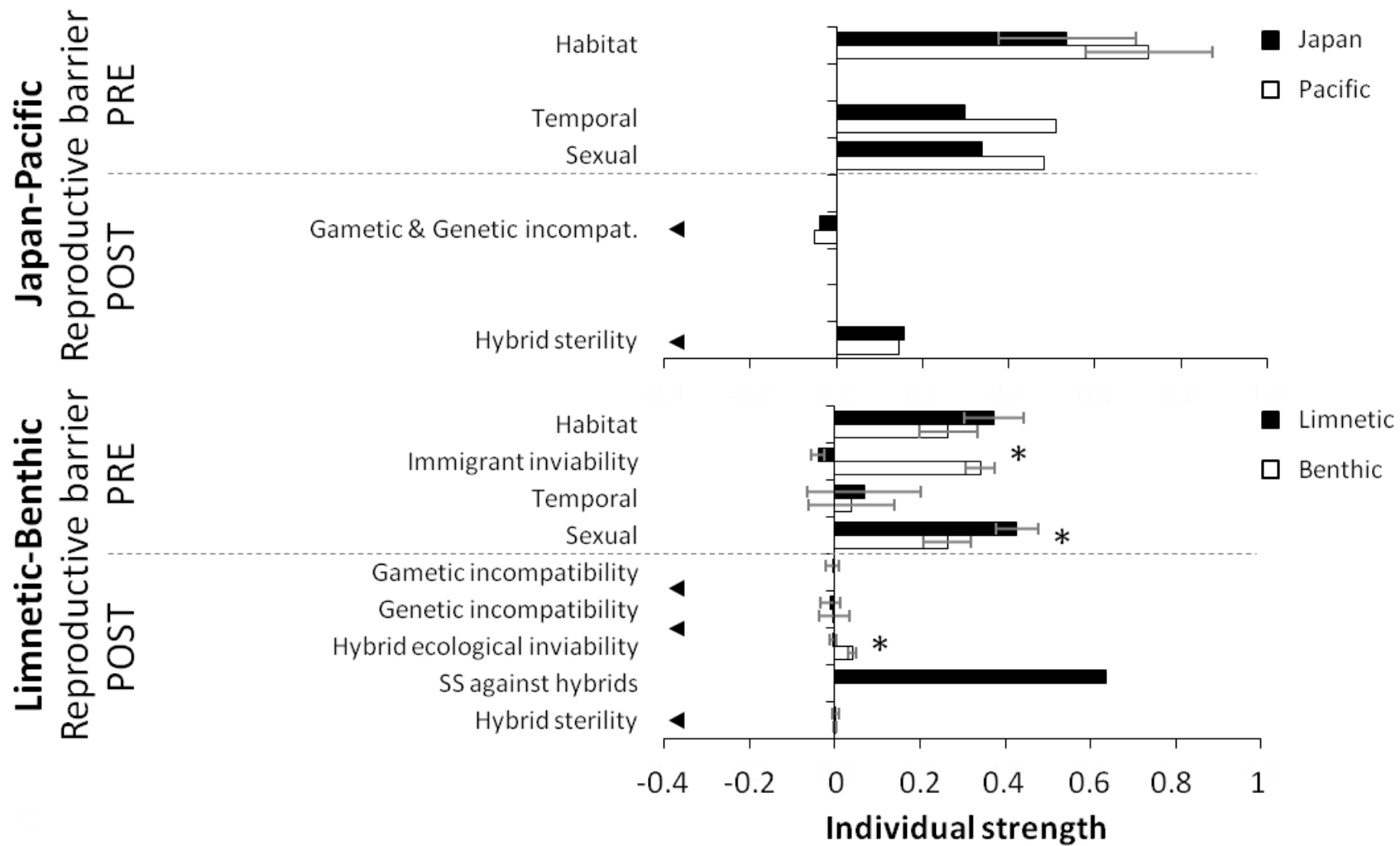


Figure 4.6. Asymmetry between taxa for individual barrier strengths.

Figure 4.6 (cont'd)

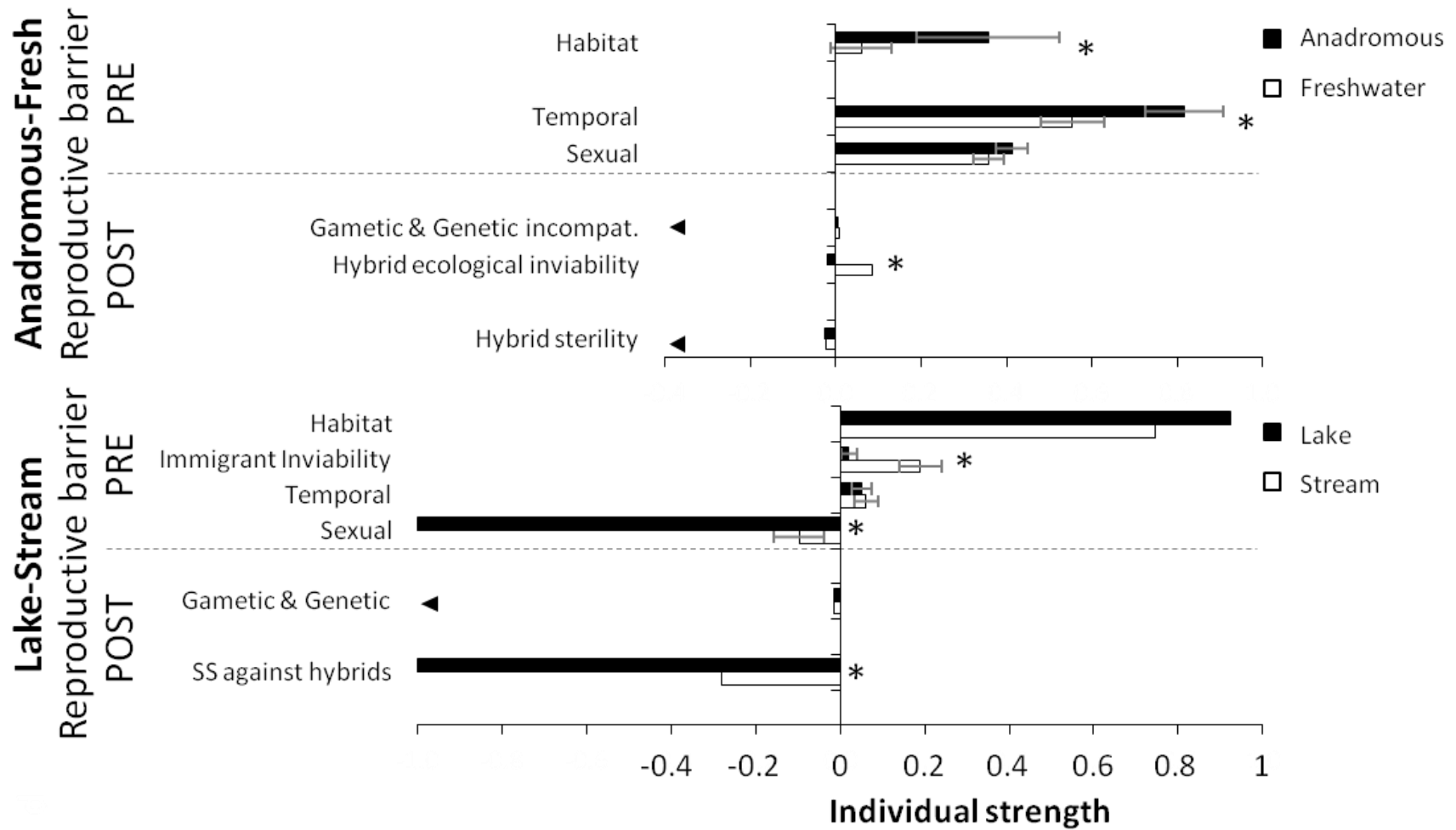
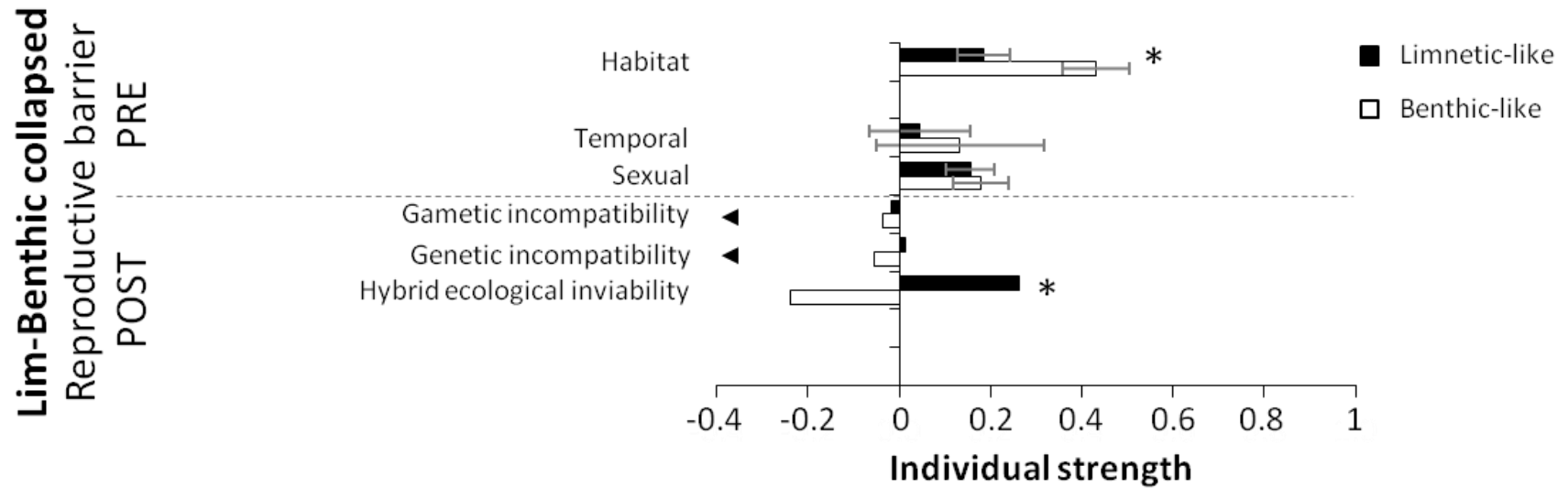


Figure 4.6 (cont'd)



For each system, we show the individual strengths for each barrier due to each taxon. Individual strengths come directly from study estimates. We present weighted means with 95% confidence intervals for every barrier with multiple estimates. Barriers estimated from a single study have no error bars. Asterisks denote significant asymmetries between taxa, where 95% confidence intervals of weighted means or single estimates for each taxon do no overlap.

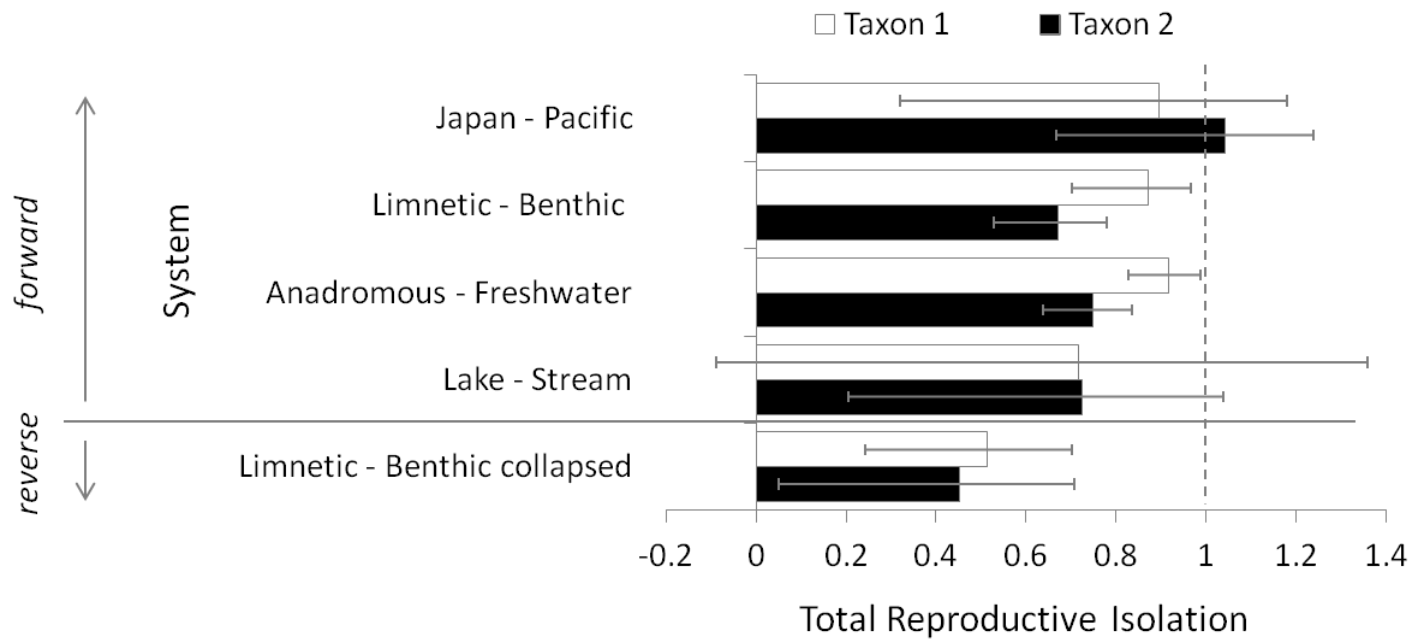


Figure 4.7. Asymmetry in total reproductive isolation between taxa.

Separately for each taxon in each system, we show the total reproductive isolation, which is the sum of all barriers' sequential strengths ordered across the life cycle. Taxon 1 refers to the first taxon listed in each system name on the y-axis. Error bars represent the strongest and weakest estimate of total isolation calculated with the upper or lower bounds of each barrier's 95% confidence interval. The dashed line shows total isolation of 1, where no gene flow occurs between taxa. We also depict the speciation continuum with forward and reverse progress along the y-axis.

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CONCLUSION

In this series of studies, we have shown the important and sometimes surprising roles of sexual selection and ecology in speciation. We have also examined the complexities of the speciation process as it proceeds and reverses. This work strengthens our understanding of how speciation occurs from the perspectives of evolution, ecology, and behavior.

In Chapter 1, we provide the first evidence that sexual isolation has been lost in the collapsed limnetic-benthic species pair and show furthermore that preferences females have for conspecific mates and the traits they use to distinguish conspecific and heterospecific males contribute to this loss. This work highlights the fragility of reproductive isolation between young species pairs and, along with results from Chapter 4, points to the importance of sexual isolation as species both evolve and dissolve.

In Chapter 2, we showed that male competition can favor divergence and maintain species differences but only when environmental differences are present. In the absence of environmental differences, male competition could hinder divergence and homogenize species differences. Intriguingly, the outcomes of selection solely from male competition reflect species differences observed in nature. This begs the question of how much of a role male competition may play in speciation. Is male competition ever the primary cause of speciation or does male competition always act alongside other evolutionary forces, such as divergent sexual selection from female mate choice and divergent natural selection from environmental differences? We also know little about how much the role of male competition in speciation might rely on environmental differences. How commonly are behavioral mechanisms, such as “like competes with like”, sufficient to favor divergence in the absence of environmental differences? If we are

to better understand the role of sexual selection in speciation, we need to further study both female mate choice and male competition as potential diversifying forces.

In Chapter 3, we tested how differences in mating habitats affect the expression of both female discrimination between species and male traits that underlie sexual isolation. Surprisingly, we found that the expression of female discrimination was fairly insensitive to habitat, despite the significance of habitat differences for sexual isolation to evolve. Female sensitivity to habitat was only shown by the ecotype being subsumed by hybridization, suggesting this plasticity may have contributed to reverse speciation. We also found habitat sensitivity in the expression of male courtship that would further erode sexual isolation. Thus, environmental differences may play very different roles in the evolution versus maintenance of sexual isolation and the forward versus reverse process of speciation. Future work could determine whether sexual isolation is unique among forms of isolation in that environmental differences required to evolve isolation are not required to maintain it.

In Chapter 4, we confirm some predictions about the speciation process and also reveal some surprises. We find that premating isolation, especially habitat and sexual isolation, seem to evolve early in the speciation process. Additional barriers help contribute to accumulating isolation in the stages between initiation and completion of speciation. Indeed, some pre- and postmating barriers may evolve together because they are underlain by the same source of selection. Completing speciation likely requires intrinsic postmating isolation that is insensitive to environmental change. We were surprised to find how variable some barriers were over time and space, which suggests that selection associated with these barriers is also variable. The most variation seems to occur in ecological selection against hybrids and sexual selection that

limits hybridization. Variation in these sources of selection could result in strong divergence when conditions are right or, in contrast, could limit divergence if variation is large and recurring. We were also surprised that more total isolation and particular barriers were not lost in the collapsed limnetic-benthic species pair. Indeed, habitat isolation remained strong despite the absence of plants that historically comprised one of two distinct mating habitats, thus other environmental or spatial factors must underlie this form of isolation. Future studies that can combine approaches of looking at potentially all contributing reproductive barriers and using representatives that span the speciation continuum will further add to our understanding of how new species evolve and whether these species are maintained long-term.

We are currently in an exciting time for research on speciation. Data are accumulating across taxonomic systems and modes of speciation (e.g., allopatry and sympatry; selection and drift) such that we can identify pervasive patterns and processes. The work here contributes to the depth of our understanding of how sexual selection and ecology interact to result in isolation as well as the breadth of our understanding of how reproductive isolation evolves and what evolutionary forces underlie it.