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Evolution of reproductive isolation in stickleback fish

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To understand how new species form and what causes their collapse, we examined how reproductive isolation evolves during the speciation process, considering species pairs with little to extensive divergence, including a recently collapsed pair. We estimated many reproductive barriers in each of five sets of stickleback fish species pairs using our own data and decades of previous work. We found that the types of barriers important early in the speciation process differ from those important late. Two premating barriers—habitat and sexual isolation—evolve early in divergence and remain two of the strongest barriers throughout speciation. Premating isolation evolves before postmating isolation, and extrinsic isolation is far stronger than intrinsic. Completing speciation, however, may require postmating intrinsic incompatibilities. Reverse speciation in one species pair was characterized by significant loss of sexual isolation. We present estimates of barrier strengths before and after collapse of a species pair; such detail regarding the loss of isolation has never before been documented. Additionally, despite significant asymmetries in individual barriers, which can limit speciation, total isolation was essentially symmetric between species. Our study provides important insight into the order of barrier evolution and the relative importance of isolating barriers during speciation and tests fundamental predictions of ecological speciation.

KEY WORDS: **Asymmetric isolation, incipient species, postmating isolation, premating isolation, reverse speciation.**

The process of speciation has been studied for decades, but key questions remain. One essential question is how and when various reproductive barriers evolve at different stages of the speciation process (Coyne and Orr 2004; Lowry et al. 2008; Sobel et al. 2009; Schemske 2010). As speciation proceeds, a single population can diverge into partially distinct populations that still exchange genes, which can further differentiate into distinct species with little gene flow or none at all. The speciation process involves the accumulation of reproductive isolation (RI). Yet, the order in which reproductive barriers evolve is still unclear, which limits an understanding of which reproductive barriers characterize initial divergence and which complete speciation. Moreover, partially isolated taxa do not necessarily evolve into distinct species. Taxa can maintain an intermediate extent of isolation and gene flow over time, appearing halted in the speciation process (Nosil et al. 2009b). Gene flow between partially isolated taxa can also increase through hybridization and reverse differentiation. Such loss of isolation can result from environmental changes that alter selective regimes (Seehausen 2006; Seehausen et al. 2008; Behm et al. 2010; Gilman and Behm 2011; Vonlanthen et al. 2012). Little is known about whether the reverse process may (or may not) be different from the forward process or why taxa may halt indefinitely at one stage.

The entire speciation process is typically too long to observe, but studying taxa at different stages of divergence provides

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snapshots of the process that can be used to infer the order of barrier evolution and the types of RI important at early, intermediate, and late stages. A number of studies have comprehensively examined many of the reproductive barriers that contribute to total isolation between a single pair of populations at a single stage of the speciation process, primarily in plants (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 (Matsubayashi and Katakura 2009; Dopman et al. 2010; Sanchez-Guillen et al. 2012). However, findings in one pair of populations cannot be generalized across all stages of the speciation process because the relative importance of individual barriers and the underlying evolutionary forces may differ between early and late stages of divergence (Nosil et al. 2009b; Schemske 2010). Other studies have measured a few barriers in multiple pairs of populations that vary in extent of genetic or phenotypic divergence to determine the order and rate of barrier evolution (Coyne and Orr 1989; Tilley et al. 1990; Coyne and Orr 1997; Presgraves 2002; Mendelson 2003; Christianson et al. 2005). Most work that examines multiple species pairs (e.g., Coyne and Orr 1989, 1997; Mendelson 2003; Baack et al. 2015) has presented two to six individual barriers for each species pair (but see Lowry et al. 2008, which provided two to nine). 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). We estimate six to nine individual barriers for each species pair, and have multiple estimates for many individual barriers. Thus, our data allow a much more detailed examination of the order of individual barrier evolution and contribution of individual barriers to total isolation than has been possible in previous studies.

Premating isolation, which limits gene flow before mating occurs, may evolve early as populations begin to diverge, whereas postmating isolation may evolve later and more slowly, thereby completing speciation and maintaining distinct species (Coyne and Orr 1989, 1997; Mendelson 2003; Rieseberg and Willis 2007; Lowry et al. 2008). However, there is not universal agreement that premating precedes postmating isolation. Comparative, theoretical, and empirical data suggest that pre- and postmating isolation can evolve at similar rates (Coyne and Orr 1989, 1997; Servedio and Saetre 2003; Moyle et al. 2004), or postmating isolation can evolve first (e.g., Kozak et al. 2012). Earlier studies emphasized the relative rates of evolution for sexual isolation and hybrid incompatibilities (e.g., Coyne and Orr 1989, 1997; Mendelson 2003), but we lack similar comparative evidence for other barriers such as habitat and temporal isolation, gametic incompatibilities, and sexual selection against hybrids.

RI can accumulate asymmetrically if evolutionary forces act differently on each species. For instance, asymmetric isolation can result from differences between populations in the extent of mate discrimination (Kaneshiro 1976, 1980; Arnold et al. 1996), hybrid fitness relative to each parental species (Kuwajima et al. 2010), or incompatibilities between mitochondrial and nuclear genomes (Tiffin et al. 2001; Turelli and Moyle 2007). Extensive asymmetric gene flow could limit either the accumulation or maintenance of isolation and thus impede further divergence or even facilitate reversal of speciation (Arnold et al. 1996; Servedio and Kirkpatrick 1997; Chunco et al. 2007). Previous work has emphasized asymmetries in individual barriers (e.g., Tiffin et al. 2001; Lowry et al. 2008). However, individual asymmetries may not predict the extent of asymmetry in total isolation if, for instance, two barriers asymmetric in opposite directions yield symmetric total isolation (Wade et al. 1995; Kitano et al. 2007a; Takami et al. 2007). Thus, fully understanding how asymmetric isolation affects speciation requires measures of asymmetry in both individual barriers and total isolation.

Here, we focus on incipient and recently diverged species pairs that are closely related. Incipient species provide the most relevant data for barriers that evolve early in speciation, even though these taxa may not ultimately diverge into fully distinct species. Strongly isolated species that have diverged relatively recently are ideal for determining which barriers complete speciation and maintain distinct species. In contrast, for species that evolved complete isolation several millions of years ago, the order of barrier evolution and their relative importance is obscured; some of the current isolation may not have been involved during the speciation process per se because additional RI can accumulate after speciation was essentially complete. Comparing isolation across closely related species is necessary for inferring the order of barrier evolution. By studying closely related, incipient, and recently diverged species pairs, we can infer the evolutionary order and relative importance of barriers at different stages of the speciation process and circumvent many of the problems underlying debate about how isolation evolves during speciation.

We estimate the strength of each barrier alone as well as relative to other barriers. 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 and Orr 2004; Martin and Willis 2007; Sobel et al. 2010). A barrier's current strength reflects the outcome of divergence that has occurred to date. We can compare an individual barrier's strength across species pairs at different stages of the speciation process to infer the importance of that barrier for early, intermediate, and late stages of divergence. In comparison, a barrier's relative strength indicates how much that barrier contributes to current total isolation given the strengths of all other barriers. Relative barrier strengths and total isolation are calculated by ordering barriers as they occur during the life cycle, with barriers acting later in the life cycle only restricting gene flow allowed by earlier acting barriers. This approach yields a measure of the extent of RI for each

species pair as well as which barriers contribute to the current strength of isolation at different stages of the speciation process (Coyne and Orr 1989, 1997; Ramsey et al. 2003).

In this study, we provide a comprehensive examination of nearly all reproductive barriers across multiple species pairs at different stages of the speciation process in a model vertebrate system for ecological speciation: stickleback fish. With this approach, we can determine which barriers result from divergence that initiates speciation, facilitates continued accumulation of isolation, or completes speciation, as well as which barriers characterize halted and reversed speciation (Coyne and Orr 2004; Schemske 2010). We address a series of questions about how RI evolves to determine what patterns of RI in these systems can tell us about early, intermediate, and late stages in the speciation process. (1) How strong is total isolation in each of the systems we examine, and how does that compare to estimates of ecological differentiation, divergence time, and genetic differentiation? (2) Which barriers evolve first, and do the barriers important early in divergence differ from those important late? (3) What patterns in the presence and strength of individual barriers characterize reversed or halted speciation? (4) What are the patterns of asymmetry in total isolation and individual barriers, and what are the potential consequences for divergence? For all of these questions, we ask how patterns in RI may reveal the underlying evolutionary forces that act during the speciation process.

Methods

STUDY SYSTEMS

We estimated the strength of six to nine reproductive barriers and total isolation in each of five sets of species pairs of different ages: Japan Sea–Pacific Ocean, Limnetic–Benthic, Anadromous–Freshwater, Lake–Stream, and Limnetic–Benthic collapsed (Tables 1, 2, and S1). The Japan–Pacific pair is the oldest species pair, which diverged within the past 1.5 million years (Kitano et al. 2007a). Both species are anadromous with one species originating in the Sea of Japan and the other in the Pacific Ocean, and the species currently overlap along the coast of Japan (Higuchi and Goto 1996; Kitano et al. 2007a). Limnetic–Benthic, Anadromous–Freshwater, and Lake–Stream species pairs have diverged within the past 13,000–15,000 years since the last ice age (McPhail 1994; Table 1). For these three recent systems, multiple species pairs exist (three to eight pairs per system), which constitute replicate populations because they evolved independently of one another (Tables 2 and S1). Limnetic and benthic sticklebacks are sympatric freshwater species that, respectively, inhabit pelagic and littoral zones of a few lakes in British Columbia, Canada (McPhail 1994). Anadromous–Freshwater pairs occur across the northern hemisphere with freshwater species inhabiting streams and lakes (McPhail 1994). Lake–Stream pairs have been

Table 1. Estimates of hybridization and genetic differentiation between taxa.

System	RI	Expected hybrid (%)	Observed hybrid (%)	Ecological differentiation	Divergence time	Genetic differentiation
Japan–Pacific	0.970 (0.938-0.989)	3.0 (1.1–6.2)	1.08–5.71[1–3]	2.64–6.23[2–4]	1.5 million years[2]	$F_{ST} = 0.160$[2]
Limnetic–Benthic	0.870 (0.809–0.985)	13.0 (1.5–19.1)	<1–5.21[5–7]	0.38–5.67[6–9]	11,000–13,000 years[10]	$F_{ST} = 0.209–0.213$[11]
Anadromous–Freshwater	0.895 (0.392–0.998)	10.5 (0.20–60.8)	24.1[12]	0.49–4.85[4,13–14]	22–13,000 years[10–15]	$F_{ST} = 0–0.641$[16]
Lake–Stream	0.716 (0.691–0.750)	28.4 (25.0–30.9)	0.36[17]	0.46–3.20[10,17–19]	11,000–13,000 years[10]	$F_{ST} = 0–0.197$[20–23]
Limnetic–Benthic collapsed	0.499 (0.424–0.583)	50.1 (41.7–57.6)	7.48–24.0[5,24]	1.05–3.42[25–27]	11,000–13,000 years[10]	Allelic diff. = 13.41[28]

For each system, we present our estimates of reproductive isolation (RI) and resulting expected percent of hybrids with the range of RI and expected hybrids calculated from weakest and strongest estimates of RI in parentheses. For comparison, we present previously published data on the extent of observed hybridization measured as the percent of hybrids detected by morphological and/or molecular measures. We also present estimates of ecological differentiation, divergence time, and genetic differentiation, primarily using F_{ST}, which is most widely available metric across systems. For the Limnetic–Benthic collapsed pair, we present the only data available to estimate differentiation: average differences in allelic counts between red and black forms that represent limnetic- and benthic-like forms. Superscript numbers denote the source and method for estimating genetic differentiation: (1) Higuchi and Goto (1996; allozymes), (2) Kitano et al. (2007a; microsatellites), (3) Kitano et al. (2009; microsatellites), (4) Kitano et al. (2007b), (5) Gow et al. (2006; microsatellites), (6) McPhail (1984; morphology), (7) McPhail (1992; morphology), (8) Schluter (1993), (9) Schluter (1995), (10) McPhail (1994), (11) Taylor and McPhail (2000; mtDNA), (12) Hagen (1967; morphology), (13) Karve et al. (2008), (14) Gelmond (2007), (15) Furin et al. (2012), (16) Jones et al. (2012; SNPs), (17) Lavin and McPhail (1993; morphology), (18) Rasanen et al. (2012), (19) Stinson (1983), (20) Hendry et al. (2002; mtDNA, microsatellites), (21) Hendry and Taylor (2004; microsatellites), (22) Berner et al. (2009; microsatellites), (23) Roesti et al. (2012; SNPs, microsatellites), (24) Taylor et al. (2006; microsatellites), (25) Kraak et al. (2001), (26) Lackey and Boughman (2013a), (27) Lackey and Boughman (2014), (28) Malek et al. (2012; microsatellites).

Table 2. Independent estimates of isolation in each system with data sources.

	System (# independent measures[sources])				
Source for each barrier	Japan-Pacific	Limnetic-Benthic	Anadromous-Freshwater	Lake-Stream	Limnetic-Benthic collapsed
Habitat Isolation	$2^{1,2}$	$4^{3,4,5,6}$	$3^{7,8,9}$	1^{10}	$2^{11,12}$
Immigrant Inviability	---	$3^{13,14,15}$	---	$2^{16,17}$	---
Temporal Isolation	1^{18}	$2^{5,6}$	$3^{7,8,9}$	$2^{19,20}$	$2^{11,12}$
Sexual Isolation	1^{21}	$4^{3,22,23,24}$	$6^{25,26,27,28,29,30}$	$2^{31,32}$	$2^{33,34}$
Gametic Incompatibility	*	$2^{35,36}$	*	*	1^{37}
Genetic Incompatibility	$*1^{38}$	$2^{35,36}$	$*1^{7}$	$*1^{10}$	1^{11}
Hybrid Ecological Inviability	1^{21}	$8^{13,14,35,39,40,41,42,43}$	1^{7}	---	1^{44}
Sexual Selection Against Hybrids	---	1^{15}	---	1^{31}	---
Hybrid Sterility	1^{38}	$3^{35,45,46}$	1^{7}	---	---

For each system, we list the number of independent estimates used to calculate isolation for each barrier with the data sources. Superscript numbers denote the source and the specific species pair studied for each system that had multiple replicate species pairs studied: Limnetic-Benthic, Anadromous-Freshwater, and Lake-Stream. All data from the Limnetic-Benthic collapsed pair come from Enos Lake after the collapse. (1) Kume et al. (2005), (2) Kume et al. (2010), (3) Ridgway and McPhail (1984, Enos), (4) Vamosi and Schluter (1999, Paxton), (5) Head and Boughman (unpub., Paxton), (6) Lackey and Boughman (unpub., Paxton), (7) Hagen (1967, British Columbia), (8) Gelmond (2007, Alaska 1), (9) Karve et al. (2008, Alaska 2), (10) Lavin and McPhail (1993, Misty), (11) Head and Boughman (unpub., Enos), (12) Lackey and Boughman (unpub., Enos) (13) Schluter (1995, Paxton), (14) Rundle (2002, Paxton), (15) Vamosi (2002, Paxton), (16) Hendry et al. (2002, Mackie), (17) Hendry et al. (2002, Misty), (18) Kume (2007), (19) Moore and Hendry (unpub., Misty), (20) Stinson (1983, Drizzle), (21) Kitano et al (2009), (22) Boughman et al. (2005, Paxton), (23) Lackey and Boughman (2013a, Paxton), (24) Boughman et al. (2005, Priest), (25) Hay and McPhail (1975, British Columbia), (26) McKinnon et al. (2004, Alaska 3), (27) McKinnon et al. (2004, British Columbia), (28) McKinnon et al. (2004, Japan), (29) McKinnon et al. (2004, Scotland), (30) Furin et al. (2012, Alaska 4), (31) Raeymaekers et al. (2010, Misty), (32) Rasanen et al. (2012, Misty), (33) Boughman et al. (2005, Enos), (34) Lackey and Boughman (2013a, Enos), (35) Hatfield and Schluter (1999, Paxton), (36) Lackey and Boughman (unpub., Paxton), (37) Lackey and Boughman (unpub., Enos), (38) Yamada and Goto (2003), (39) Gow et al. (2007, Paxton), (40) Behm et al. (2010, Paxton), (41) Taylor et al. (2012, Paxton), (42) Gow et al. (2007, Priest), (43) Taylor et al. (2012, Priest), (44) Behm et al. (2010, Enos), (45) McPhail (1984, Enos), (46) McPhail (1992, Paxton). See Table S1 for information on isolation estimates with 95% CIs, sample location, sample sizes, and methods used in each study for each source. An asterisk indicates when the measures of genetic incompatibility includes both gametic and genetic incompatibility estimates as a result of how data were collected in each source. A dashed line --- indicates when no data were available to estimate that barrier. See Text S3 for further explanation.

studied in British Columbia, Canada (McPhail 1994). One pair of Limnetic–Benthic species in Enos Lake collapsed into a hybrid swarm after a drastic environmental change about 14–19 years ago (Gow et al. 2006; Taylor et al. 2006), revealing the dynamic nature of speciation. The recency of this event and the wealth of data available mean that we can provide estimates of isolation before and after this collapse. Estimates of isolation for the collapsed pair use the most phenotypically and genetically diverged forms, which predicts gene flow under the hypothetical scenario where more intermediate forms are absent. Thus, our estimate is of maximal isolation in the collapsed pair.

We have replication within systems, as parallel speciation commonly occurs and replicate pairs of the same type have been studied (see Table S1). Such replication can demonstrate how repeatable speciation is, and gives power for estimating barrier strength. Estimates of each barrier were calculated using one to eight independent measures obtained from multiple populations of each type of species pair (Table 2). In comparison, other studies of reproductive barriers typically provide only a single estimate of each barrier. Using multiple estimates in our study generates more accurate estimates of isolation between each species pair across time and space, and allows us to calculate average barrier strengths and 95% CIs. We estimated 33 barriers from 69 measures of isolation using our own data and previously published and unpublished data (Table S1). Data generated by our laboratory provided measures of isolation for five of nine barriers estimated for Limnetic–Benthic pairs and six of seven barriers estimated for the Limnetic–Benthic collapsed pair (Tables 2 and S1). Our data were essential for calculating a comprehensive estimate of total isolation for the Limnetic–Benthic collapsed pair and providing insight into reverse speciation. Other stickleback taxa pairs, such as the black and red color morphs in the northwestern United States and the lava-mud pairs in Iceland, were excluded from this study due to lack of isolation measures for multiple pre- and postmating barriers.

We examined naturally occurring sympatric and parapatric species to examine the barriers sufficient to restrict current gene flow. Each of these pairs segregates into different habitats to varying extents. To make estimates of isolation comparable across species pairs, we measured isolation at sympatric sites. As a

result, we have minimal estimates for habitat isolation for our parapatric pairs (Japan–Pacific, Anadromous–Freshwater, and Lake–Stream). Our findings are most relevant to cases of ecological speciation, but may be relevant to other processes of speciation as well. Furthermore, our estimates of RI reflect outcomes from direct interactions between species, divergent natural and sexual selection, and the by-products of divergent selection. We cannot, however, distinguish between isolation that results from direct versus by-product mechanisms. We describe stickleback biology relevant to the barriers we estimated, how we estimated each barrier, and findings from previous studies of isolation in each set of stickleback species pairs in Text S1. When different methods were used to estimate the same individual barrier, we tested whether different methods resulted in homogenous effect sizes (see Supporting Information). For sexual isolation, for example, two measures of mating were used: spawning (female enters a male's nest) and nest inspection (female puts her head into a male's nest, which often directly precedes spawning). Effects sizes for isolation estimates from these two measures were homogenous ($Q = 0.0460$, degrees of freedom [df] $= 1$, $P = 0.8298$).

CALCULATING RI

We followed the method of Coyne and Orr (1989, 1997) with adjustments from Ramsey et al. (2003), Sobel et al. (2010), and Sobel and Chen (2014) to estimate the strengths of individual barriers and their relative contributions to total RI. See Table 3 for a list of the barriers and metrics used in this study. We first calculated each barrier's individual strength (i.e., the strength of each barrier irrespective of other barriers). The strength of RI from an individual barrier is defined as:

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

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). RI linearly ranges from -1 to 1. When there is no gene flow (e.g., strong assortative mating or very low hybrid vigor), RI is 1 (complete isolation). When gene flow is 0.5 (e.g., random mating or equal fitness of hybrid and parental forms), RI is 0 (no isolation). When gene flow is 1 (e.g., disassortative mating or hybrid vigor), RI is -1. We used the same equation for pre- and postzygotic barriers, so estimates of individual barrier strength are directly comparable across all barriers. Our measures of RI are easily comparable to those from other studies; this equation is either equivalent to or easily adjusted to match other measures (Sobel and Chen 2014).

Null expectations for the frequency of events may differ between species if, for example, the species differ in relative abundance or number of trials conducted. We accounted for any differences in null expectations for each species, as suggested by Martin and Willis (2007) and developed by Sobel and Chen (2014), by expanding equation (1) to:

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

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 also tested for homogeneity across studies and sampling locations used to calculate weighted means with the Q statistic (see Supporting Information for details). We provide detailed descriptions of measuring and calculating each individual barrier in Supporting Information, including equation adjustments for habitat and temporal isolation due to unequal population sizes.

We then calculated each barrier's sequential strength (Dopman et al. 2010; also "absolute contribution" in Ramsey et al. 2003). We ordered isolating barriers by occurrence during the life cycle. The sequential strength of the nth barrier, SS_n, depends on its individual strength, RI_n, and the amount of gene flow allowed by earlier acting barriers:

$$SS_n = \mathrm{RI}_n\left(1 - \sum_{i=1}^{n-1} SS_i\right). \tag{3}$$

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. We calculated two measures of total isolation. The first includes all barriers measured for each system, which ranges from six to nine barriers in each system. To control for differences in the number and type of barriers measured in each system, we also calculated total isolation using only the four "shared" barriers measured across all systems: habitat isolation, temporal isolation, sexual isolation, and a combined measure of gametic and genetic incompatibilities. The relative contribution of each barrier, RC, measures a barrier's contribution to current total isolation relative to the strengths of earlier acting barriers. Thus, the relative contribution is each barrier's sequential strength divided by total isolation:

$$RC_n = \frac{SS_n}{T}. \tag{4}$$

For each barrier, we calculated 95% CIs for RI for each population within a study. When we had multiple estimates of RI for a single barrier, we calculated weighted mean RI, weighting each individual RI with its inverse variance. We calculated a 95% CI for each weighted mean RI using the SE of the weighted mean, which is the square root of the sum of the inverse variance weights (Hedges and Olkin 1985; Lipsey and Wilson 2001).

Table 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 reduce 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 reduce zygote formation	Number of fertilized out of total eggs per clutch
Genetic incompatibility	Nonenvironmental 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, for example, 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 and Supporting Information.

A barrier contributes significantly to RI when the 95% CI for its individual strength does not encompass zero. The individual strengths of two barriers differ from each other when 95% CIs do not overlap. We calculated a strongest and weakest estimate of total isolation in each system using the highest and lowest single estimates, respectively, for each individual barrier and then calculated sequential strengths and total isolation as described above (Sobel and Chen 2014).

We also calculated measures for different types of isolation (i.e., premating, postmating, intrinsic postmating, and extrinsic postmating) separately. We used individual barrier strengths to calculate sequential barrier strengths within each type of isolation. For postmating isolation, for example, we calculated the sequential strengths of gametic and genetic incompatibilities, hybrid ecological inviability, sexual selection against hybrids, and hybrid sterility based on the life cycle order of these four barriers and ignoring the strengths of premating barriers. Thus, this generates a measure of postmating isolation independent of premating isolation.

We also evaluated whether species contribute asymmetrically to RI. We used equation (1), where C is the frequency of conspecific events for a single species and H is the frequency of heterospecific events. Our calculations of H combine contributions from each sex. Thus, we measure asymmetries between species and not the sexes. We calculated weighted means, 95% CIs, and strongest and weakest estimates of isolation for each species as described above. Two species contribute asymmetrically to an individual barrier if the 95% CIs do not overlap.

MEASURES OF ECOLOGICAL DIFFERENTIATION, DIVERGENCE TIME, AND GENETIC DIFFERENTIATION

To compare the extent of total isolation in each system to other axes of divergence, we calculated an estimate of ecological differentiation between species in each system, and we used estimates of divergence time and genetic differentiation from previously published work. To estimate ecological differentiation, we selected three morphological traits: standard body length, body depth, and number of gill rakers, which are known to differ between species across all systems due to differences in ecological selection between habitats used by each species (McPhail 1994, see references in Table S2). We also selected these three traits because data were readily available from the published literature for all five species pair systems. For each system and each trait, we used means, SDs or SEs, and sample sizes to calculate an effect size of the standardized trait difference between species (Table S2). We calculated 55 effect sizes (nine for Japan–Pacific, 10 for Limnetic–Benthic, 24

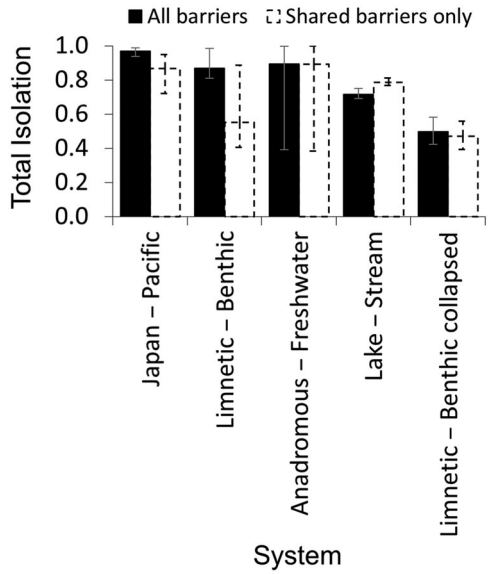

Figure 1. Reproductive isolation across systems. For each system, we plot total isolation, which is the sum of all barriers' sequential strengths ordered across the life cycle (Table S2). Error bars represent the strongest and weakest estimate of total isolation in each system calculated using the highest and lowest single estimate for each individual barrier, respectively. We calculated two estimates of total isolation: one from all barriers (solid black bars) and a second from the four barriers measured in all systems ("shared barriers only" in dashed white bars).

for Anadromous–Freshwater, six for Lake–Stream, and six for the Limnetic–Benthic collapsed pair) from 16 published studies. For each system, we calculated an average effect size and the 95% CIs. For divergence time and genetic differentiation, we gathered estimates from published literature (Table 1), and then tested for a correlation between total isolation and these three measures of differentiation. We log-transformed ecological differentiation and divergence time to improve linearity. Analyses were done in R (version 3.1.1; R Core Team 2014). Lastly, we compared total isolation to estimates of hybrids detected in the field (Fig. S1).

Results

STRENGTH OF ISOLATION VARIED SUBSTANTIALLY AT EARLY STAGES OF DIVERGENCE

In the five systems sampled, total RI estimated from all measured barriers ranged from moderate (0.499) to strong (0.970), and total isolation was surprisingly high even in systems with suspected halted and known reversed differentiation (Fig. 1A, Tables 1 and S3). Total isolation was strongest in the Japan–Pacific pair. Total isolation was also strong in the Limnetic–Benthic and Anadromous–Freshwater pairs; the upper estimates of total isolation in these pairs were as strong as those in the Japan–Pacific pair. Isolation in the Lake–Stream pairs was distinctly weaker than in the Japan–Pacific and Limnetic–Benthic pairs, and isolation in the Limnetic–Benthic collapsed pair was weakest of all. Anadromous–Freshwater pairs had a wide range between the strongest and weakest estimates of total isolation due to variation in individual barrier estimates from these globally distributed pairs (Tables 1, S1, and S3). Total isolation measured from shared barriers was very similar to that from all barriers (Fig. 1A). As gametic and genetic incompatibilities are equivalent to zero in all species pairs, total isolation from shared barriers is effectively a measure of premating isolation. However, in the Lake–Stream pairs, total isolation from shared barriers was slightly higher than from all barriers because significant negative postmating sexual selection against hybrids reduces total isolation from all barriers.

We compared estimates of total isolation to expectations from ecological differentiation, divergence time, and genetic differentiation (Fig. 2, Table 1). As expected, species pairs with greater ecological differentiation tended to have higher total isolation (Fig. 2A). However, the correlation only nears significance ($R^2 = 0.6629$, df $= 4$, $P = 0.0934$) in part because we have only five systems to compare. Japan–Pacific pairs have the greatest ecological differentiation averaged across body length, body depth, and gill raker number. Effect sizes for ecological differentiation in the other systems overlap. Divergence time was not significantly correlated with total isolation (Fig. 2B; $R^2 = 0.2956$, df $= 4$, $P = 0.3436$). Although the Japan–Pacific pair with the longest divergence time had the strongest total isolation, isolation estimates overlapped with those of more recently diverged pairs. Genetic divergence also did not correlate with total isolation (Fig. 2C; $R^2 = 0.2351$, df $= 3$, $P = 0.5151$); F_{ST} range estimates overlapped considerably for the four systems where these data were available.

PREMATING ISOLATION WAS STRONGER AND EVOLVED EARLIER THAN POSTMATING ISOLATION

Premating isolation evolved very early and remained strong across species pairs at intermediate and late stages of the speciation process (Fig. 3). Postmating isolation appeared to evolve after premating isolation and built up slowly; it was moderate to strong only in the more diverged systems (Fig. 3). Of postmating barriers, extrinsic isolation likely evolved first and contributed more to total isolation than intrinsic isolation. Total extrinsic isolation varied from weak to strong but was always greater than zero. In contrast, total intrinsic isolation was consistently weak to nonexistent for all systems (Fig. 3).

We next examined whether the individual barriers important for isolation differed between early and late stages of the speciation process. We found that habitat and sexual isolation evolved

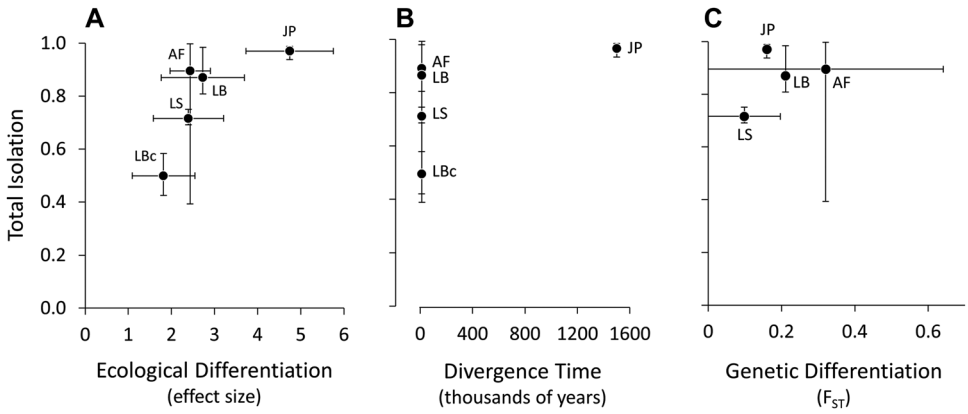

Figure 2. Strength of total isolation plotted against ecological differentiation, divergence time, and genetic differentiation. We plot total reproductive isolation on the *y*-axis with error bars showing the strongest and weakest estimates of total isolation. On the *x*-axes, we plot (A) ecological differentiation, (B) divergence time, and (C) genetic divergence measured as F_{ST}. For ecological differentiation, we plot the average effect size (Cohen's *d*) of morphological differences between species. We use standard body length, body depth, and gill raker number, which are known to vary significantly between species in each system. Measures come from previously published work (number of measures per system: JP = 9, LB = 10, AF = 24, LS = 6, LBc = 6). The horizontal error bars show the range of effect sizes. For divergence time and genetic divergence, we plot the mid-point of the range, and the horizontal error bars show the range of estimates. See Table 1 for ranges of ecological differentiation, divergence time, and F_{ST} as well as data sources.

early, while intrinsic postmating isolation evolved late. Habitat isolation was one of the strongest, if not *the* strongest, barrier both individually and relatively across all systems (Figs. 4 and S2). In contrast, other barriers accumulated later. As divergence increased, sexual isolation increased and even transitioned from weakening total isolation in the Lake–Stream pairs to strengthening total isolation in the other pairs. Yet once sexual isolation evolved, it was significant and moderately strong and remained so in more diverged systems. Extrinsic postmating barriers were common, and were stronger than intrinsic barriers in all systems, except the Lake–Stream system where the single estimated extrinsic barrier was significantly negative (Fig. 4). Intrinsic postmating barriers evolved last and most gradually; weak but significant hybrid sterility was only present in the most isolated and oldest Japan–Pacific pair.

WEAK SEXUAL ISOLATION CHARACTERIZED BOTH REVERSED AND HALTED DIVERGENCE

In the collapsed Limnetic–Benthic pair, overall isolation was weaker primarily due to loss of sexual isolation with little change in other premating barriers (Fig. 4). Habitat isolation maintained its strength despite the loss of vegetation that historically comprised one of the two distinct mating habitats. Although patterns in our data depicted a loss of postmating (total and extrinsic) isolation in the collapsed pair compared to the other Limnetic–Benthic pairs (Fig. 3), we lacked data for the collapsed pair to estimate sexual selection against hybrids, an extrinsic postzygotic barrier (Fig. 4). In the Lake–Stream pairs, a single, early acting barrier contributed to the majority of isolation, but strongly negative sexual isolation and sexual selection against hybrids weakened total isolation (Fig. 4).

TOTAL ISOLATION WAS SYMMETRIC DESPITE ASYMMETRIC INDIVIDUAL BARRIERS

We found asymmetries in premating and extrinsic, but not intrinsic, postmating barriers (Fig. 5). More than a third of premating (seven out of 17) and all extrinsic postmating (four out of four) estimates were asymmetric. In contrast, none of the 10 intrinsic postmating barrier estimates were asymmetric, though most of these estimates were equivalent to zero and thus unlikely to have detectable asymmetries. All systems had asymmetries in individual barriers except the oldest and most isolated: the Japan–Pacific pair. The Limnetic–Benthic and Lake–Stream pairs had significant asymmetries in both pre- and postmating barriers. Interestingly, for the Limnetic–Benthic pairs, the presence and sometimes the direction of asymmetries differed from before and after the collapse. Despite significant asymmetries in individual barriers within most systems, none of the systems had strong asymmetries in total isolation (Fig. 6).

Discussion

THE STRENGTH OF TOTAL ISOLATION VARIED EARLY IN THE SPECIATION PROCESS

Ecological differentiation resulting from divergent selection predicted the extent of total isolation between species better than divergence time or genetic differentiation (Fig. 2). Early in speciation, isolation appeared to accumulate independently of time,

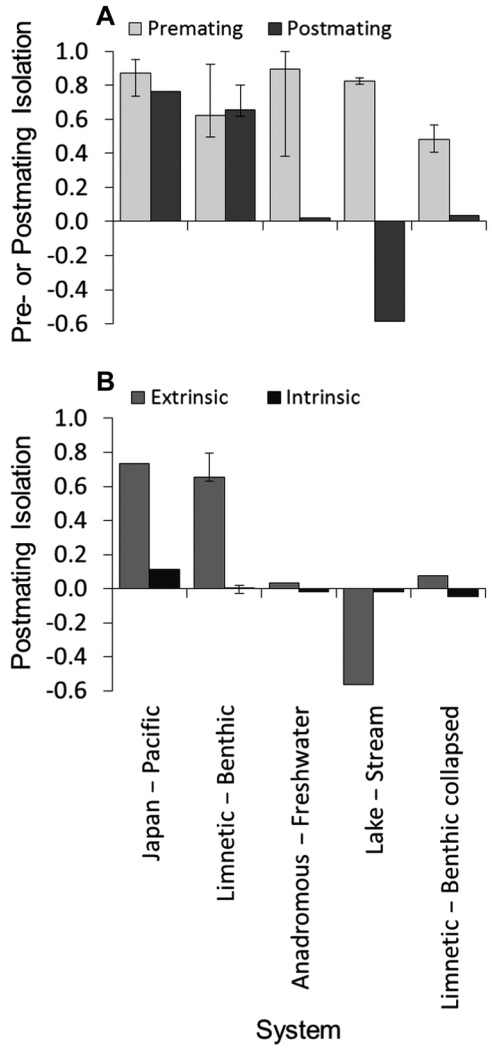

Figure 3. Types of isolation. For each system, we plot (A) pre- and postmating isolation and (B) extrinsic and intrinsic postmating isolation separately. Each type of isolation is estimated from the sum of sequential barrier strengths ordered across the life cycle and calculated within each type of isolation. Error bars represent the strongest and weakest estimate of total isolation calculated using the highest and lowest single estimate for each individual barrier, respectively. When error bars are absent, isolation was calculated from single estimates for each barrier of that type of isolation.

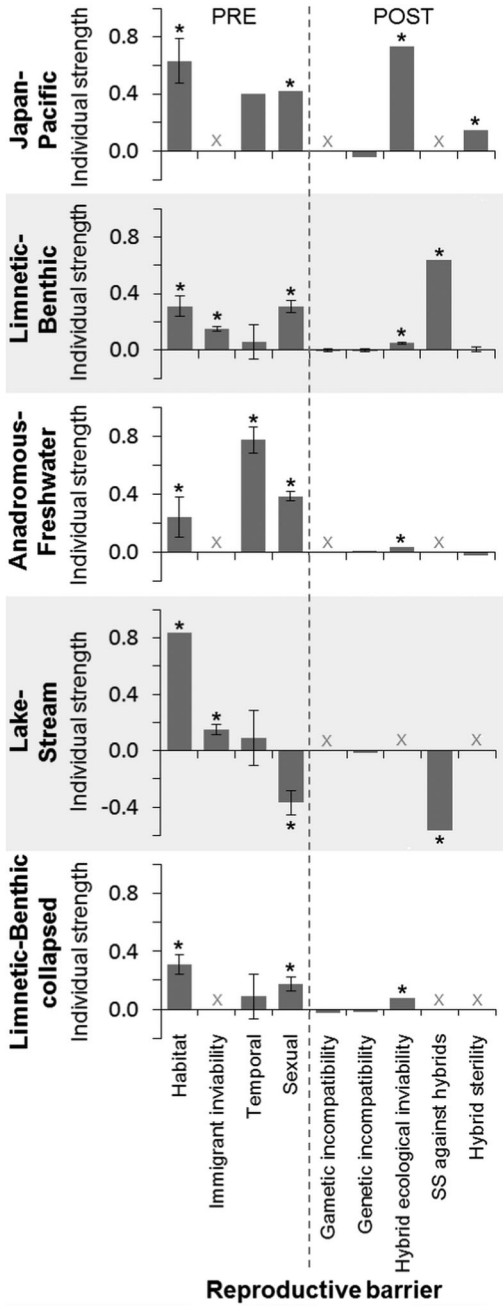

Figure 4. Individual barrier strengths. For each system, we show the individual strengths for each barrier. Individual strengths come directly from study estimates. Asterisks denote weighted mean and single barrier estimates with 95% CIs that do not encompass zero. We show 95% CIs around weighted means for every barrier with multiple estimates with error bars. Barriers estimated from a single study have no error bars. We present 95% CIs for all single study estimates in Table S1. Each "X" indicates where we did not have data to estimate a barrier. For Japan–Pacific, Anadromous–Freshwater, and Lake–Stream pairs, the bar for genetic incompatibility shows isolation due to both gametic and genetic incompatibilities, whereas these forms of isolation are estimated separately in the Limnetic–Benthic and Limnetic–Benthic collapsed pairs.

given that total isolation varied from weak to nearly as strong as the oldest pair. Similar variation in total isolation at early divergence times has been found in comparative work on other taxa (e.g., Coyne and Orr 1997). Thus, we emphasize that divergence time likely poorly predicts the strength of total isolation early in divergence. Genetic differentiation measured as F_{ST} also poorly predicted total isolation. Patterns of genetic divergence are expected to be highly variable across pairs of populations that experience gene flow (Michel et al. 2010; Martin et al. 2013; Seehausen et al. 2014). These findings provide multiple insights.

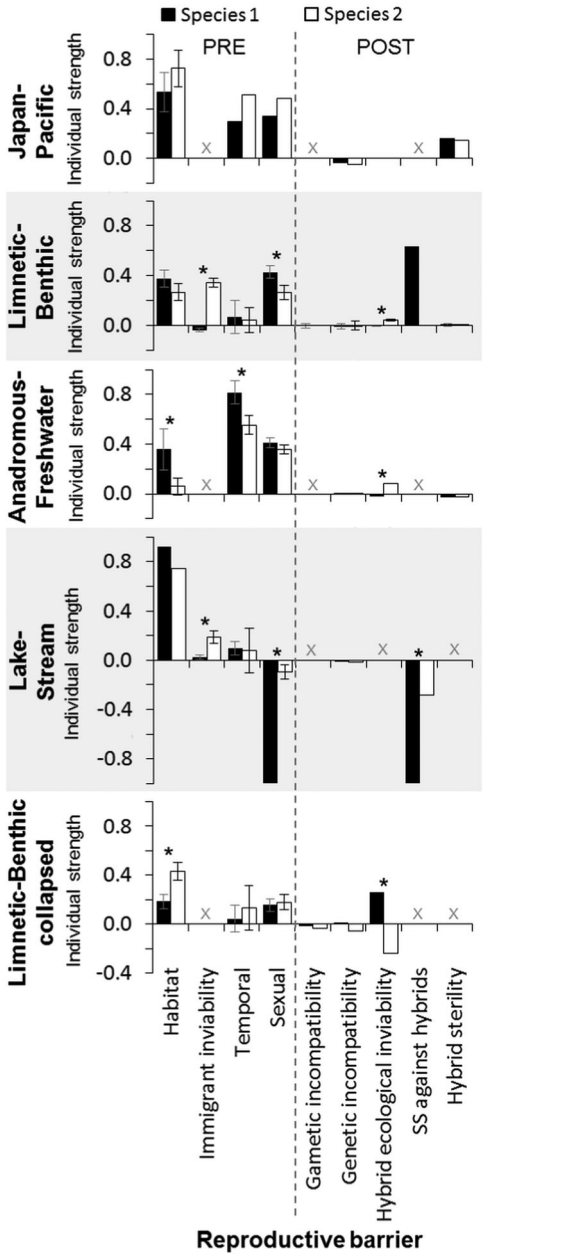

Figure 5. Asymmetry between taxa for individual barrier strengths. For each system, we show the individual strengths for each barrier due to each species. Species 1 refers to the first species listed in each system name (e.g., Japan in the Japan–Pacific pair). We present weighted means with 95% CIs for every barrier with multiple estimates. Barriers estimated from a single study have no error bars. Asterisks denote significant asymmetries between species, where 95% CIs of weighted means or single estimates for each species do not overlap.

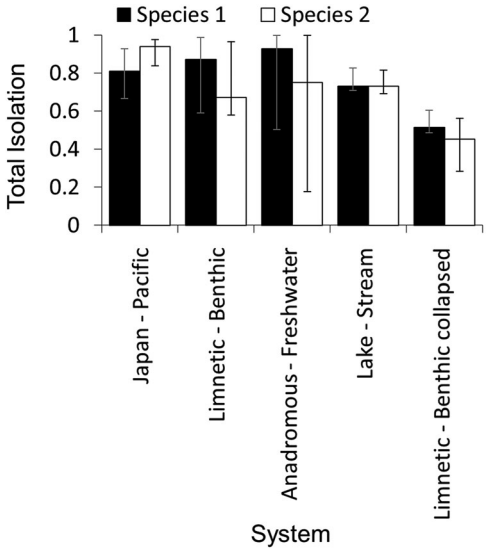

Figure 6. Asymmetry in total reproductive isolation between taxa. Separately for each species in each system, we show the total reproductive isolation, which is the sum of all barriers' sequential strengths ordered across the life cycle. Species 1 refers to the first species listed in each system name on the *y*-axis. Error bars represent the strongest and weakest estimate of total isolation in each system calculated using the highest and lowest single estimate for each individual barrier, respectively.

First, a single measure of divergence (e.g., time or genetic differentiation) cannot reliably predict total isolation (Hey 2009), especially in young species pairs. Second, isolation does not necessarily accumulate incrementally in early stages of divergence, which implicates evolutionary forces that could cause "jumps" in accumulating isolation, such as strong selection on one or a few traits (Nosil et al. 2009b), rapid changes in selection (Siepielski et al. 2009, 2013), or very few loci of large effect contributing to isolation (Schemske and Bradshaw 1999; Wu 2001; Bradshaw and Schemske 2003; Turner et al. 2005; Nosil et al. 2009a). Early stages of divergence can also result in widespread genomic differentiation (e.g., Michel et al. 2010), though the conditions under which this occurs are more limited (Barrett and Schluter 2007; Feder and Nosil 2009).

Of the recently diverged species pairs, the more strongly isolated pairs had multiple moderate to strong individual barriers rather than a single strong barrier. This is true of other plant and animal systems as well (Coyne and Orr 1989, 1997; Lowry et al. 2008; Schemske 2010). Generally, a single barrier or cause of isolation (e.g., chromosomal rearrangement, niche dimension, selection on one trait) allows too much gene flow to generate distinct species (reviewed in Rieseberg 2001; Coyne and Orr 2004; Nosil and Harmon 2009; Nosil et al. 2009b); instead, it appears that multiple barriers evolve over time.

Differences in total isolation markedly affect the number of hybrids produced, and high levels of gene flow can limit local adaptation and genetic differentiation. In the Lake–Stream pairs, for which isolation no longer appears to be accumulating, gene flow was more than twice that in more strongly isolated pairs

(Fig. 1). This should limit additional differentiation. The reverse speciation process has strongly reduced, but not fully erased, isolation in the collapsed Limnetic–Benthic pair; current isolation still restricted 50% of gene flow. However, our approach gives a maximal estimate of remaining isolation in the collapsed pair by using the most phenotypically and genetically divergent forms. Given that this pair used to be as strongly isolated as other Limnetic–Benthic pairs, our estimate represents a substantial loss of isolation.

PREMATING EVOLVED BEFORE POSTMATING ISOLATION

We found that premating isolation evolved before and was stronger than postmating isolation at early stages (Fig. 3). This suggests that divergent selection first favored premating isolation, while any reinforcement (e.g., Rundle and Schluter 1998) may have secondarily strengthened premating isolation. Previous studies largely support the primacy of premating isolation in plants and animals for both sympatric and allopatric species (Coyne and Orr 1989, 1997; Mendelson 2003; Kay 2006; Martin and Willis 2007; Lowry et al. 2008; Dopman et al. 2010; Schemske 2010; Baack et al. 2015). Yet, whether pre- or postmating isolation consistently evolves first is still unclear, especially in plants (Moyle et al. 2004; Scopece et al. 2007; Widmer et al. 2009). Previous work suggested that premating isolation would evolve faster than postmating isolation because premating isolation can result from direct selection, whereas postmating isolation often results from drift or indirect selection (Coyne and Orr 1997; Gleason and Ritchie 1998; Mendelson 2003). However, these studies focus on intrinsic postmating isolation only.

Intrinsic postmating isolation can evolve slowly (Barton 2001; Bolnick and Near 2005; Unckless and Orr 2009; Nosil 2012), and may be very weak to nonexistent in young species (anurans: Blair 1964; *Drosophila*: Coyne and Orr 1989, 1997; lepidoptera: Presgraves 2002; birds: Price and Bouvier 2002; centrarchids: Bolnick and Near 2005, but see a few exceptions reviewed in Schluter and Conte 2009), which is consistent with our findings. However, extrinsic postmating isolation could evolve quickly when it results from divergent adaptation to different environments (Hatfield and Schluter 1999; Schluter 2000; Coyne and Orr 2004; Nosil 2012). Thus, in early stages of divergence, extrinsic isolation can be stronger and more important than intrinsic isolation (Rundle 2002; Egan and Funk 2009; Schluter 2009; Schluter and Conte 2009; Kuwajima et al. 2010; Seehausen et al. 2014). Extrinsic postmating isolation could evolve potentially at similar or even faster rates than premating isolation (e.g., Kozak et al. 2012). More studies that examine many premating barriers as well as both intrinsic and extrinsic postmating barriers in recent and more diverged species are needed to identify common patterns in the relative rates of evolution of different types of barriers (Baack et al. 2015).

Although intrinsic postmating isolation evolved relatively late in the speciation process, it was likely essential for completing speciation and limiting reversal; many cases of reverse speciation have occurred in species pairs that lacked intrinsic isolation (Seehausen et al. 1997; Vonlanthen et al. 2012). Intrinsic isolation may limit reversal by reducing hybrid fitness in all environments, and so would persist after an environmental change that might weaken other forms of isolation (reviewed in Coyne and Orr 2004).

HABITAT AND SEXUAL ISOLATION EVOLVED EARLY IN DIVERGENCE

Theory predicts that speciation will occur most readily when disruptive selection acts on both ecological and sexual traits (van Doorn et al. 2009, Weissing et al 2011). In sticklebacks, we found that habitat isolation likely evolved first and contributed to restricting gene flow throughout the speciation process (Fig. 4), similar to findings in plant and other animal systems (Matsubayashi and Katakura 2009; reviewed in Schemske 2010). Habitat isolation may be essential early and throughout the speciation process because its effects on gene flow are multiple (Rice and Hostert 1993). When species use different habitats, this reduces encounter rates between species and also generates divergent selection. This divergent selection and subsequent local adaptation can facilitate speciation (Doebeli and Dieckmann 2004) and potentially cause immigrant and hybrid ecological inviability, result in stronger habitat isolation, and even alter responses to environmental cues that could then yield temporal or sexual isolation.

Sexual isolation likely evolved relatively early and accumulated quickly once it appeared, consistent with work in other animal systems (Coyne and Orr 1998, 1997; Mendelson 2003) and akin to pollinator isolation in plants (e.g., Baack et al. 2015). Strongly divergent sexual selection within taxa that directly affects mating preferences and traits important for mate discrimination between species could underlie the early and rapid evolution of sexual isolation (Lande 1981; Panhuis et al. 2001; Turelli et al. 2001; Mendelson 2003; Merrill et al. 2011). In fact, the extent of *divergence* in sexual selection between taxa is more important than the *strength* of sexual selection, (Rodriguez et al. 2013), which may explain why comparative work finds mixed evidence for a relationship between speciation and the *strength* of sexual selection (Ritchie 2007). Sexual selection can play a key role in speciation whether it acts alone or interacts with natural selection (Lande 1981; West-Eberhard 1983; Lande and Kirkpatrick 1988; Dieckmann and Doebeli 1999; Panhuis et al. 2001; Boul et al. 2007; Ritchie 2007; van Doorn et al. 2009; Maan and Seehausen 2011; Weissing et al. 2011).

IN THE REVERSE AND HALTED PROCESSES OF SPECIATION, WEAK OR NEGATIVE SEXUAL ISOLATION FACILITATED GENE FLOW

Although sexual isolation may evolve early in divergence, it may also be lost rapidly. Sexual isolation was significantly weaker in the collapsed Limnetic–Benthic pair compared to the intact pairs, and was also strongly negative in Lake–Stream pairs with halted divergence. This may be a general finding because the loss of sexual isolation is common (Seehausen et al. 1997; Fisher et al. 2006; Richmond and Jockusch 2007; Ward and Blum 2012; Lackey and Boughman 2013a). Theory shows how quickly and permanently its loss can further facilitate reversal, especially when gene flow occurs for longer durations (Gilman and Behm 2011).

Cases of reverse speciation show that reversal can be 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). Human-induced environmental change is the crux of almost all known cases of reverse speciation (Seehausen et al. 1997; Fisher et al. 2006; Taylor et al. 2006; Vonlanthen et al. 2012; Ward and Blum 2012, but see Richmond and Jockusch 2007) as well as other instances of extensive hybridization (Rhymer and Simberloff 1996; Grant and Grant 2008; Heath et al. 2010). In the Limnetic–Benthic collapsed pair, environmental change is associated with the rapid increase of hybrids (Taylor et al. 2006). Recent work in this collapsed stickleback pair posits that environmental change may affect male competition and courtship more dramatically than female mate choice (Lackey and Boughman 2013b, 2014), and hybrid males do well in male competition (Keagy et al. 2016). Environmental change may have also diminished natural selection against hybrids (Behm et al. 2010).

Our data from before and after collapse suggest that reversal does not mirror the forward process. Instead, some barriers are more evolutionarily labile; they may evolve first, but also be lost first (e.g., sexual isolation in the Limnetic–Benthic collapsed pair). Although habitat isolation also evolved early, it surprisingly remained strong after the collapse despite the loss of vegetation, which comprised one of the primary differences between the two historical mating habitats (Ridgway and McPhail 1987). Habitat isolation should be susceptible to environmental change but may be stable once it evolves, potentially if habitat preference is based on multiple components, as it may be in limnetic and benthic sticklebacks that mate at different depths (Bentzen et al. 1984), in different plant densities (Ridgway and McPhail 1987), and in different light environments (Boughman 2001). We found no loss of hybrid ecological inviability, likely because even though some point estimates were moderately strong (Behm et al. 2010), on average this barrier was weak even before the collapse (Table S1), limiting our ability to detect change. These surprises emphasize the importance of measuring multiple barriers after collapse to determine what forms of isolation have actually degraded.

TOTAL ISOLATION WAS SYMMETRIC DESPITE ASYMMETRY IN INDIVIDUAL BARRIERS EARLY IN DIVERGENCE

Despite significant asymmetries between species in individual barriers within most systems, we found that total isolation was fairly symmetric (Figs. 5 and 6). Asymmetries acting in different directions were most pronounced for Limnetic–Benthic pairs and the Limnetic–Benthic collapsed pair. Reciprocally acting pre- and postmating barriers suggest that reinforcement cannot be the primary cause of premating isolation, because any asymmetry in the direction of selection against hybrids would yield the same direction of asymmetry in pre- and postmating isolation (Yukilevich 2012). Previous work in the Japan–Pacific pair that considered contributions of each sex separately found asymmetric sexual isolation and hybrid infertility (Kitano et al. 2007a); similar to our overall results, these asymmetries acted in different directions to yield more symmetric total isolation between species. Total isolation may also be more symmetric than the contributing individual barriers if a symmetric early acting barrier blocks the majority of gene flow, restricting the impact of asymmetric late acting barriers on total isolation (Kuwajima et al. 2010). Individual asymmetries in the Lake–Stream pairs were consistent with this pattern; very strong habitat isolation dampened the effects of extreme asymmetries in sexual isolation and sexual selection against hybrids.

We found greater individual asymmetries at earlier compared to later stages of the speciation process. The occurrence of asymmetries as isolation initially accumulates could allow polymorphisms to arise (Chunco et al. 2007) that may facilitate speciation. Asymmetries might diminish over time as additional incompatibilities arise (Turelli and Moyle 2007) or if selection on each species becomes more symmetric. However, strong asymmetries that persist later in the speciation process could halt further divergence (Servedio and Kirkpatrick 1997) or even reverse speciation if gene flow in one direction overwhelms diversifying forces. Consistent with this, individual asymmetries in the Limnetic–Benthic pairs may have allowed reversal to occur in one pair. Future work needs to determine whether the pattern of greater asymmetry in individual barriers early versus late in the speciation process is a common one.

Conclusion

We use our data and decades of previous work in stickleback fish to provide a wealth of information on the evolution of RI in this

system. Overall, patterns of isolation that characterized early divergence differed from patterns late in divergence. Our findings emphasize that multiple, strong barriers underlie rapid, early increases in isolation. Premating isolation, especially habitat and sexual isolation, is essential early in the speciation process, and intrinsic postmating isolation is necessary for completing speciation. The loss of sexual isolation may be common in reverse speciation, especially when collapse is triggered by environmental change. Interestingly, individual barrier asymmetries were more common early in divergence, but they may not limit further divergence when total isolation is fairly symmetric between species. Although our findings speak most directly to cases of ecological speciation, future work will determine how generalizable these results are to other processes of speciation. We hope this study motivates research that examines multiple pre- and postmating barriers concurrently across species pairs that range from little to extensive divergence, including cases of reverse speciation. This will expose what is necessary for new species to evolve and what causes species to collapse.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Individual barrier estimates for each source with sampling details.
Table S2. Ecological differentiation effect sizes for each study.
Table S3. Sequential barriers strengths across systems.
Figure S1. Strength of total isolation compared to percent hybrids.
Figure S2. Relative barrier strengths.