

Loss of sexual isolation in a hybridizing stickleback species pair

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Abstract One approach to understand the importance of reproductive barriers to the speciation process is to study the breakdown of barriers between formerly distinct species. One reproductive barrier, sexual isolation, reduces gene flow between species through differences in mate preferences and mating signals and is likely important for species formation and maintenance. We measure sexual isolation in two limnetic-benthic threespine stickleback species pairs (*Gasterosteus* spp.). One species pair maintains strong reproductive isolation while the other species pair has recently collapsed into a hybrid swarm. We compare the strength of sexual isolation in the hybridizing pair to the currently isolated pair. We provide the first evidence that sexual isolation has been lost in the hybridizing 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 considers the role of sexual isolation in speciation [*Current Zoology* 59 (5): 591–603, 2013].

Keywords Sexual isolation, Speciation, Hybridization, Female preferences, Male mating traits, Stickleback species pair

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

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sexual selection (Lande, 1981; Lande and Kirkpatrick, 1988; Butlin and Ritchie, 1994; Mendelson, 2003; McPeck and Gavrilets, 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), and all the com-

ponents 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 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

Table 1 Current and historical spawning proportions

Lake	Female-Male type	Present Study	Previous Studies
		Spawning proportion (N)	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.

were lost, we would expect more hybrid and back-crossed 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 melanic 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), but 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 showed that male redness, length (Boughman et al., 2005, Table 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.

1 Materials and Methods

1.1 Study populations

In March and April 2008, we used minnow traps to collect limnetic and benthic threespine stickleback fish

Table 2 Historical trait differences between male types across multiple lakes

Trait	Difference of Means (L-B)	<i>t</i>	<i>df</i>	<i>P</i>
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.

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.

1.2 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 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.

1.3 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) 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 log 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.

1.4 Shape analyses

To analyze male shape, we placed 19 landmarks on digital images of the left side of each fish (Fig. 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

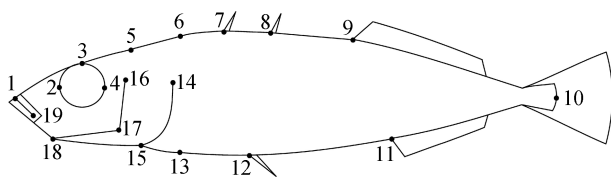


Fig. 1 Morphometric landmarks

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.

analysis to visualize how distinct limnetic(-like) and benthic(-like) fish were from Paxton and Enos Lakes.

1.5 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 Fig. 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.

2 Results

2.1 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 3: male type*male lake and male type terms are not significant, Fig. 2A, C). In contrast, Paxton females had

Table 3 Enos and Paxton female discrimination between male types

Source of Variation	A. Inspection						B. Preference Score					
	df	F	P	df	F	P	df	χ^2	P	df	χ^2	P
female type	95	0.77	0.3829	88	37.22	<0.0001	1	0.18	0.6694	1	26.65	<0.0001
male type	96	0.02	0.8782	89	3.64	0.0595	1	1.48	0.2234	1	1.03	0.3103
male lake	95	0.06	0.8051	88	7.61	0.0071	1	0.46	0.4992	1	3.55	0.0594
female type*male type	96	0.00	0.9595	89	1.78	0.1851	1	1.42	0.2334	1	1.03	0.3101
female type*male lake	95	3.39	0.0688	88	0.21	0.6457	1	2.91	0.0883	1	0.42	0.5179
male type*male lake	96	0.20	0.6537	89	8.45	0.0046	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.

strong sexual isolation; females strongly discriminated between Paxton male types and preferred homotypic males (Table 3: male type*male lake term is significant, Fig. 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 4A). Preference score showed the same pattern, but the difference was not significant after correction for multiple tests (Table 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 3, Fig. 2A, C). Further, Enos female discrimination of Paxton

male types was significantly weaker than Paxton sexual isolation as measured by inspection (Table 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 3, Fig. 2B, D). Also, Paxton females discriminated between Enos male types significantly less than between Paxton male types (Table 4A). These results suggest that Enos males lacked traits that would have allowed females to discriminate between them.

2.2 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

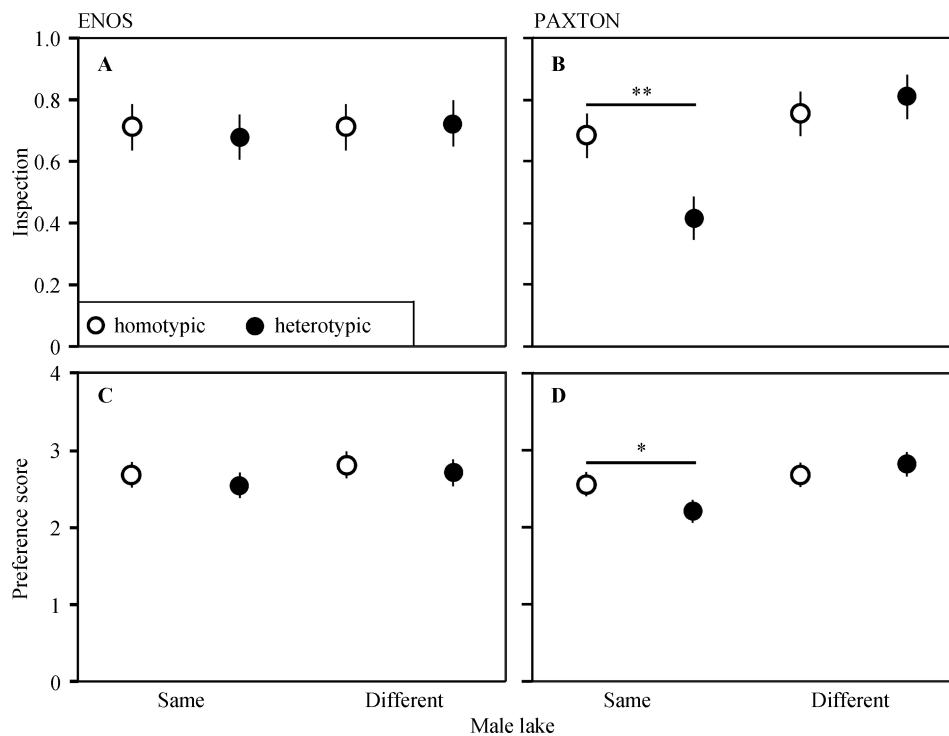


Fig. 2 Female response to males of each type from each lake

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.

both types of Enos females preferred redder limnetic (-like) males but did not prefer redder benthic(-like) males (Table 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 6), which means females

Table 4 Tests for absence of Enos sexual isolation, female mate discrimination, and distinct male traits

Comparison	A. Inspection						
	Difference	SE	df	t	P	P _{FDR}	d
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
Comparison	B. Preference Score						
	Difference	SE	df	t	P	P _{FDR}	d
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
Comparison	C. Spawning						
	Difference	SE	df	t	P	P _{FDR}	d
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 5 Enos female color preference

Female Type	Male Type	Red Slope	SE	df	t	P	P _{FDR}	d
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*.

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 (Fig. 3). The axis of discrimination between limnetic(-like) and benthic(-like) fish in Fig. 3 appears to be very similar between Paxton and Enos Lakes.

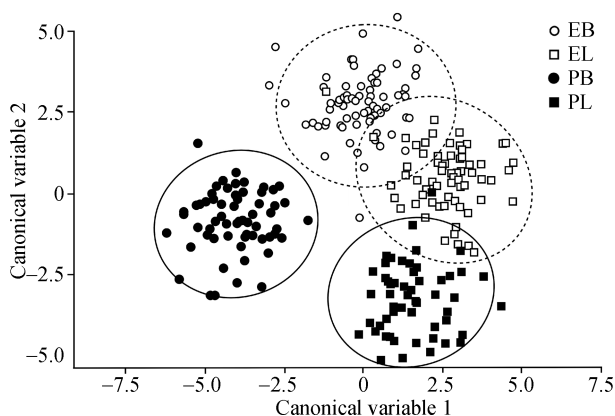


Fig. 3 Canonical shape scores for Paxton and Enos male types

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).

3 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.

3.1 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

Table 6 Trait differences between male types

A. Redness differences						
Lake	<i>n</i>	Mean difference		<i>SE</i>		
Paxton	95	2.04		0.09		
Enos	95	1.21		0.08		
Lake difference	<i>SE</i>	<i>df</i>	Difference	<i>t</i>	<i>P</i>	
Paxton - Enos	0.12	188	0.83	6.94	<0.0001	
B. Length differences						
Lake	<i>n</i>	Mean difference		<i>SE</i>		
Paxton	96	7.15		0.45		
Enos	95	4.08		0.32		
Lake difference	<i>SE</i>	<i>df</i>	Difference	<i>t</i>	<i>P</i>	
Paxton - Enos	0.56	189	3.07	5.52	<0.0001	
C. Shape differences						
Lake	<i>n</i>	Mean difference		<i>SE</i>		
Paxton	92	19.41		0.70		
Enos	95	13.71		0.89		
Lake difference	<i>SE</i>	<i>df</i>	Difference	<i>t</i>	<i>P</i>	
Paxton - Enos	1.14	185	5.71	5.02	<0.0001	

For males from each lake (Paxton and Enos), we show the mean of the absolute trait difference between male 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.

preferences to exclude heterospecific traits could have initiated speciation (McPeck and Gavrilets, 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, in-

creased 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.

3.2 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.

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