Environmental differences can cause reproductive isolation to evolve. Distinct habitats can be particularly important for the evolution of genetically based sexual isolation, which occurs when divergent preferences and mating traits reduce mating between species. Yet, we know little about environmental effects on the potentially plastic expression, and thus the current maintenance, of sexual isolation. This is especially intriguing in the context of reverse speciation, where previously isolated taxa begin hybridizing and merge. Environmental change could weaken reproductive isolation underlain by plastic traits even before any genetic change occurs. Here, we examine how differences in mating habitats affect the expression of both female discrimination between species and male traits that underlie sexual isolation. We used 2 species pairs of threespine stickleback fish (Gasterosteus spp.): an intact species pair and a formerly distinct but now hybridizing species pair, where habitat change presumably triggered reverse speciation. The expression of female discrimination was fairly insensitive to habitat, despite the central importance of habitat differences to the initial evolution of sexual isolation. Only the ecotype being subsumed by hybridization showed habitat sensitivity, suggesting this plasticity may have contributed to reverse speciation either as a cause or consequence of gene flow. Also, we found plasticity in male courtship across habitats that could further erode sexual isolation. Thus, environmental differences may play different roles in the genetic evolution versus plastic maintenance of sexual isolation, with implications for the forward versus reverse processes of speciation.

**Key words:** environmental change, mating habitats, reverse speciation, sexual isolation, speciation.

**INTRODUCTION**

For new species to evolve, environmental differences can be important both in facilitating the evolution of reproductive isolation and in maintaining isolation that has already evolved (Schluter 2001; Rundle and Nosil 2005; Seehausen 2006; Maan and Seehausen 2011). This is the case for multiple components of reproductive isolation, including sexual isolation, where differences in mate preferences and traits used in mate choice reduce mating between species before hybrids are formed (Lande and Kirkpatrick 1988; Panhuis et al. 2001; Turelli et al. 2001). Substantial data suggest that sexual selection that acts on mate preferences and traits involved in sexual isolation is especially important to speciation when it interacts with natural selection via ecology (Ritchie 2007; Maan and Seehausen 2011). As reproductive isolation evolves, distinct environments select for divergent phenotypes, and the evolution of those phenotypes causes reproductive isolation as a by-product (Mayr 1947; Schluter 2001; Rundle and Nosil 2005; Nosil and Harmon 2009). In particular for sexual isolation, female preferences and male mating traits may diverge between environments when natural or sexual selection favor different mating trait values or mating preferences in distinct environments (Lande 1982; Lande and Kirkpatrick 1988; Coyne and Orr 2004; Maan and Seehausen 2011).

Maintaining sexual isolation requires sustained differences in 1) genetic underpinnings of mate choice between species and/or 2) environmental features that affect the expression of divergent mate choice between species in a facultative or plastic manner (Eges et al. 2007; Maan and Seehausen 2011). Environmental properties can influence how females detect and evaluate mating traits (Schluter and Price 1993; Boughman 2002; Myhre et al. 2013). Environments can also affect how much males court and how they signal, potentially altering which signals transmit well (Endler 1992), which are preferred by females (Heuschele et al. 2009), and which help males outcompete rival males (Lackey and Boughman 2013a). Despite the interest in these issues, it remains unclear whether the environment is equally critical to the evolution and maintenance of sexual isolation. We explore these issues here.

Sexual isolation often plays a central role throughout the speciation process. It is likely to evolve early and thus may help initiate speciation (Mendelson 2003; Coyne and Orr 2004). Later in the speciation process, reinforcement can favor increased sexual isolation to avoid costly heterospecific matings (Servadio and Noot 2003).
For both reasons, sexual isolation may contribute substantially to how quickly or completely 2 taxa progress toward becoming distinct species. When the evolution or expression of sexual isolation depends on the environment, changes in environment can either enhance or undermine sexual isolation throughout the speciation process. Environmental changes that weaken sexual isolation could halt the speciation process early or even reverse it after substantial isolation has accumulated. In several cases of reverse speciation for example, anthropogenic environmental change has reduced sexual isolation by undermining the expression of female preference for particular male traits (e.g., Seehausen et al. 1997; Fisher et al. 2006; Ward and Blum 2012). These environmentally induced changes in expression have had evolutionary consequences by fostering hybridization.

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

A key aspect of the environment that can affect mating interactions and the expression of male and female mating traits is the presence of vegetation. The structural complexity of vegetation can provide safety from predators (Murdoch and Oaten 1975) and shield individuals from competitors (Hixon and Menge 1991; Danley 2011), but it can also obscure some mating signals, interfering with the expression of male signals and/or female preferences (Dziwecewski and Rowland 2004; Håbler and Houde 2006; Candolin et al. 2007; Wiley 2009; Myhre et al. 2013). Conversely, a lack of vegetation can facilitate transmission of signals but increase the intensity of male competition and the risk of predation. Therefore, the presence and absence of vegetation can alter how males court, how females evaluate potential mates both within and between species, and how much sexual isolation results.

Here, we test how the environment can modulate the expression of female preferences and male courtship traits important for sexual isolation. We explore these questions in limnetic—benthic threespine stickleback species pairs (Gasterosteus spp.), a model system for studying ecological speciation (Schluter 2001; McKinnon and Rundle 2002) and an excellent system to test how the environment affects the expression of sexual isolation. Habitat differences are key to the evolution of many reproductive barriers, including sexual isolation (e.g., Schluter 1995; Boughman 2001; Rundle 2002; reviewed in Boughman 2006), and divergent natural selection arising from environmental differences is essential for speciation (e.g., Rundle et al. 2000). Moreover, sexual selection and sexual isolation are ecologically dependent (Boughman 2001, 2006, Boughman et al. 2005). Divergent natural selection between distinct feeding and mating habitats has generated species differences in color, size, and shape (Bentzen and McPhail 1984; Bentzen et al. 1984; Schluter 1993, 1995; Boughman 2001), each of which females pay attention to during mate choice within and/or between species (Nagel and Schluter 1998; Boughman 2001; Boughman et al. 2005; Head et al. 2009; Kozak et al. 2009; Conte and Schluter 2013; Head et al. 2013). Furthermore, these male traits are at least in part environmentally mediated (male nuptial color: Boughman 2001; Lewandowski and Boughman 2008; body size: Nagel and Schluter 1998; McKinnon et al. 2004; Boughman et al. 2005, body shape: Day et al. 1994; Day and McPhail 1996; Head et al. 2013). Sexual selection via male competition also generates divergent selection on male traits in different habitats (Lackey and Boughman 2013a). How female choice and male competition are jointly affected by habitat has not been explored however.

In the current study, we expand on all this prior work by evaluating whether environmental differences affect how females express divergent preferences that produce sexual isolation, how males court, and how males compete with each other during courtship. We test whether environments affect the expression of sexual isolation (immediate plastic change) in the context of well-known environmental effects on the evolution of reproductive isolation (genetic change) for stickleback species. We measured the strength of sexual isolation in dichotomous choice trials with a single female choosing between a benthic and limnetic male across 3 habitat treatments: native, alternative, and open. Native habitat matched wild nesting patterns (benthics in vegetation and limnetics in the open) and alternative habitat swapped male ecotypes between habitats. Open habitat lacked all vegetation. We asked if habitats affected how strongly sexual isolation was expressed, and if this depended on having both habitats present, or on whether mating took place in native or alternative habitat. We reasoned that the strongest isolation would be expressed in native habitat given that the species evolved there. First, we asked whether habitat affected the extent to which females discriminated between conspecific and heterospecific males and the strength of sexual isolation. We also asked whether this discrimination depended on the same male traits across habitats. Second, we asked if habitat modulated male mating traits by comparing male color, size, courtship, and male-male aggression across habitats. Third, we explored whether male-male aggression affected female discrimination and if this depended on habitat.

Last, we compared fish from 2 lakes that differ currently in the presence of different microhabitats and where they are in the process of speciation. Paxton Lake has 2 distinct mating habitats, and limnetic males nest in the open and benthic males nest in dense plants (McPhail 1994). Sexual isolation was strong historically (Hatfield and Schluter 1996; Rundle et al. 2000) and remains so to the present day (Lackey and Boughman 2013b). Enos Lake historically had these 2 distinct mating habitats (McPhail 1984; Ridgway and McPhail 1984; McPhail 1994), but an invasive crayfish likely destroyed habitats with dense plants, and only open habitat now remains (Taylor et al. 2006). Sexual isolation was strong historically in Enos fish (Ridgway and McPhail 1984) but has been lost since the introduction of the crayfish (Lackey and Boughman 2013b). Using Paxton fish, we test whether habitats help to maintain strong sexual isolation currently. If female preferences and male courtship traits change across habitats, then this reveals which environmental conditions are necessary for the expression of strong sexual isolation. However, if female preferences and male courtship traits are insensitive to habitat, then the expression of strong sexual isolation does not depend on particular environmental conditions, and sexual isolation can be maintained without habitat features that facilitated its initial evolution. Using Paxton fish also provides insight...
into whether the loss of habitat with dense plants was likely an immediate cause of weakened sexual isolation in Enos fish. Using Enos fish, we test whether changes in mating habitats affect the expression of female preferences and male courtship traits, which can suggest how plasticity may contribute to reverse speciation in Enos fish. The expression of sexual isolation across habitats in Enos fish may further reveal potential causes and consequences of hybridization.

**METHODS**

We collected wild stickleback fish in mid-April 2011 from Paxton Lake, Texada Island and Enos Lake, Vancouver Island in British Columbia. We identified reproductive males and females by the presence of nuptial color and a distended abdomen full of eggs, respectively. We used species-specific characteristics of body shape, size, and color to identify limnetic and benthic fish in Paxton Lake and the most limnetic-like and benthic-like fish in Enos Lake (McPhail 1984, 1992, 1994). Although fish from Enos Lake are collectively classified as a hybrid swarm, with a higher frequency of intermediate than extreme phenotypes (Gow et al. 2006; Taylor et al. 2006), morphologically and genetically limnetic-like and benthic-like fish still exist (Malek et al. 2012). Moreover, categorizing fish by body shape has been successful in another study, where identification by body shape and genetics matched at a 97% success rate (Taylor et al. 2006). We use “ecotype” to refer to both Paxton species and Enos morphs. We use “homotypic” to refer to fish of the same ecotype (e.g., 2 limnetic[-like] fish) and “heterotypic” to refer to fish of different ecotypes (e.g., a limnetic[-like] and a benthic[-like] fish). Fish were transported to our lab and housed in tanks by sex, ecotype, and lake. We maintained fish at summer conditions with 14-h day lengths and 18 °C room temperatures. We fed fish brine shrimp (Artemia spp.) and bloodworms (Chironomus spp.) once per day.

**Mating trials**

We set up 75-gallon tanks for female dichotomous choice trials with 1 female and 2 males. To each tank, we added a 1-inch thick layer of fine-grain sand as nesting substrate. We divided each tank in half with an opaque divider, and each half was either “vegetated” with 16 plastic plants evenly spaced or “open” with no plants. This plant density mimics average percent cover from vegetated habitats in Paxton Lake (Boughman JW and Head ML, unpublished data). Native habitat had a benthic(-like) male in the vegetated half and a limnetic(-like) male in the open half. Alternative habitat swapped the male ecotypes with respect to habitat so that a benthic(-like) male was in the open and a limnetic(-like) male was in the vegetation. In open habitat, both halves of the tank were open with a benthic(-like) male in one-half and a limnetic(-like) male in the other. We selected males that had developed nuptial color and territorial behaviors. We randomized habitat and male placement with respect to the left or right side of the tank as allowed by our design. To each tank half, we added Chara spp., green algae with a plant-like structure, which males use to build nests in the wild. We enticed males to build nests by removing the divider and placing a gravid female in a clear jar in the middle of the tank for 15 min a day. Afterward, we replaced the divider and ensured males were on their assigned sides. We alternated the female ecotype seen each day, so each male saw equal numbers of homo- and heterotypic females. In the wild, males are very likely to encounter both female ecotypes during the breeding season (Boughman 2006). During enticements, males could court the female and engage in competition, which reflects how males in the wild will court females and compete with other males simultaneously (van den Assem 1967). Additionally, males of each species are often territorial neighbors even if they nest in different microhabitats (Ridgway and McPhail 1987). Our experimental setup best replicates male interactions between ecotypes where open and vegetated habitats meet. Habitat patches in the wild are larger than we use here, and some males in the wild may not nest next to heterotypic males. However, females can easily travel between habitat patches (Boughman 2006), so it is highly relevant and appropriate to explore how females evaluate males of each ecotype as potential mates.

Once both males built nests (a prerequisite for spawning), we conducted female choice trials. We removed the divider and placed a female in an opaque holding container in the middle of the tank. After a 5-min acclimation period, we released the female. We started the trial when one of the males interacted with the female, and we recorded courtship and male–male aggressive behaviors for 25 min or until the female entered one of the nests to spawn. For courtship, we recorded the following male behaviors involved in attracting the female: zig-zag, bite, chase, and lead to the nest (Wootton 1976; Ridgway and McPhail 1984; Rowland 1989). For females, we recorded the following courtship behaviors: head-up (indicating receptivity), approach, follow a male’s lead, examine a male’s nest, and enter the nest (Wootton 1976; Rowland 1989; Kozak et al. 2009). If a female entered a nest, we promptly removed her before she could deposit her eggs so that she could be used in a subsequent trial. For male–male aggression, we recorded bites, chases, and charges between males (Jersel 1953). Males defended their assigned territories, and we did not notice any tendencies for males in alternative habitats to move to native habitats. At the end of the trial, we removed the female and replaced the divider in the middle of the tank.

Most females had 2 trials, each with a different pair of males. There was a 2-h resting period between a female’s trials. For male, each pair of males had up to 2 trials: 1 with a limnetic(-like) and 1 with a benthic(-like) female, with at least 2 h between trials. For each male ecotype, we reused an average of 20% of males as part of a new pair because the breeding season is short, about 10 weeks, and we were limited by the number of males that would build a nest. Males took 6 days on average to build a new nest, but males whose nests had been moved fixed them typically in less than a day. If we reused males, we moved the male and his nest into a new tank with a new male partner. We kept the habitat half where he built his nest (open or vegetated) the same. Before using a male in female choice trials, we ensured that the male’s nest had a visible entry hole and that the male was guarding and tending his nest. See the Statistical Analyses for details on how we accounted for multiple trials with each male and female in our models. We ran a total of 231 female choice trials with 156 unique pairs of males. For Enos females, we ran 46 native, 46 alternative, and 45 open habitat trials. For Paxton females, we ran 32 native, 30 alternative, and 32 open habitat trials.

For male morphological traits, we recorded red throat color and body length. We measured each male’s standard length before and after all of his trials using Vernier calipers accurate to 0.2 mm. We averaged these 2 measurements to determine a male’s average standard length. Before and after each trial, we recorded a male’s nuptial throat color using a standardized color scoring method developed in our lab group (Boughman 2001, 2007; Lewandowski and Boughman 2000) that closely matches reflectance data (Albert
et al. 2007; Boughman 2007). We measured male red throat color area and intensity each on a scale of 0–5, where 0 indicates no color and 5 indicates maximum color area or intensity. We summed area and intensity scores to get a red index that ranged from 0 to 10. We averaged the red index before and after each trial to determine the male’s average red index for that trial.

For each trial, we quantified female preference, male courtship, and male–male aggression. First, we calculated the strength of a female’s preference using preference score, which uses the level of progression in courtship on a scale from 0 to 4 to measure the female’s extent of interest in a particular male. A male received a score of 0 if the female did not respond to any of the male’s courtship behaviors, 1 if she approached the male (indicating initial interest), 2 if she followed the male (indicating sustained interest), 3 if she examined the nest (the last step before actual mating), and 4 if she entered the nest (final acceptance of the male for mating). Then, we calculated the difference between a female’s preference scores for homo- and heterotypic males (homo minus hetero). This preference score difference ranged from −4 to 4, and positive values indicate that she showed more interest and proceeded further in courtship with the homotypic than the heterotypic male. This difference in preference score is our measure of female discrimination; hereafter, we refer to it as such. For each male, we calculated the rate per minute of 3 types of courtship by summing the relevant courtship behaviors directed toward the female divided by total trial time. We calculated courtship vigor and its 2 components: aggressive courtship and display courtship. Previous work has shown that males perform more aggressive or display oriented courtship depending on the female ecotype he is courting (Kozak et al. 2009). Calculations for courtship vigor include all male courtship behaviors: zig-zags, leads, bites, and chases (Kozak et al. 2009). Aggressive courtship includes just bites and chases, and display courtship includes just zig-zags and leads (Kozak et al. 2009). Next, we calculated the difference in courtship vigor, aggressive courtship, and display courtship between male ecotypes (homo minus hetero).

Positive values indicate that the homotypic male courted the female more vigorously, aggressively, or with more display than the heterotypic male. We also calculated the rate of male–male aggression for each male by summing the number of bites, chases, and charges directed toward the other male and dividing this sum by the total trial time. We calculated the difference in male–male aggression between homo- and heterotypic males, where positive values indicate that the homotypic male was more aggressive to his rival than vice versa.

Statistical analyses

We analyzed our response variables of female discrimination, courtship difference (vigor, aggressive, or display), and male–male aggression difference using mixed models. All differences were homo- minus heterotypic. We expected that patterns of female discrimination might vary between females from each lake, so we tested whether female discrimination was explained by female ecotype (benthic[-like] or limnetic[-like]), lake (Paxton or Enos), habitat (native, alternative, or open), or their interactions. We found a significant interaction between lake and female ecotype (Figure 1), so we ran all subsequent analyses by lake. All of these “by lake” models included female ecotype and habitat as categorical factors. Each female had up to 2 trials, so we used repeated measures with a compound symmetry covariance structure that assumes each female’s trials were correlated. We also included a random effect of male pair. For female discrimination, we included all of the following continuous covariates: red difference, length difference, courtship difference (vigor, aggressive, or display), and male–male aggression difference. Our analyses are likely unencumbered by collinearity between our continuous covariates because we found a low variance inflation factor of 1.9, and strong collinearity produces variance inflation factor values greater than 10. We included all possible interactions between categorical variables. For continuous covariates, we included all 2-way interactions.
with continuous and categorical variables. We reduced models by removing nonsignificant terms.

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

We next estimated sexual isolation using $I_{PSI}$ (Rolán-Alvarez and Caballero 2000) in the JMATING program that accommodates data from different choice designs, including the female choice design we used here (Carvajal-Rodríguez and Rolan-Alvarez 2006). This program uses the number of mating interactions out of total trials between males and females of different ecotypes. We used whether a female examined a nest to estimate $I_{PSI}$ because entering the nest to spawn happened too infrequently to run statistical tests by both lake and habitat. We used bootstrapping to estimate $I_{PSI}$, its standard deviation (SD), and significance. $P$-values derived from bootstrapping are conservative (Carvajal-Rodríguez and Rolan-Alvarez 2006), so we do not control these $P$-values for multiple comparisons.

We used path analysis to estimate the relative contributions of red color, body length, courtship vigor, and male–male aggression differences between male ecotypes to female discrimination. We wanted to understand how these traits affected female discrimination and explore interactions between male–male aggression and female discrimination as measured in this study. We tested 3 models. In all models, we allowed a correlation between 1) red color and length differences and 2) courtship and aggression differences. Prior work suggests that red color and length are negatively correlated between limnetic and benthic species as limnetic males are smaller but redder than benthic males (Boughman et al. 2003), however, we did not know if color differences between male ecotypes would correlate with size differences. For courtship and aggression, previous work found a positive correlation between these traits in other populations (Rowland 1984). We also reasoned that a male’s relative competitive ability could affect how much he courted the female. In all models, we predicted that 1) males that differed more in red or length would also differ more in courtship and male–male aggression and 2) females would discriminate more between males with greater differences in any of these traits. Previous work has shown that male traits of color, size, and aggression are interrelated (Bakker and Milinski 1993) and that color, size, and courtship influence female mate choice (Nagel and Schluter 1998; Boughman 2001; Kunzler and Bakker 2001; Boughman et al. 2005; Conte and Schluter 2013).

The essential difference between the 3 models is whether we included paths between 1) female discrimination and courtship differences, 2) female discrimination and aggression differences, or 3) both 1 and 2. The first model predicted that larger courtship vigor differences between male ecotypes strengthened female discrimination, while larger male–male aggression differences did not. The second model predicted the alternative; larger male–male aggression differences between male ecotypes strengthened female discrimination, while larger courtship vigor differences did not. The third model, our baseline model, predicted that both courtship and aggression differences directly increased female discrimination.

We tested the fit of each of these models to our data to determine the relative importance of courtship vigor and male–male aggression differences between male ecotypes for female discrimination. Both models 1 and 2 above fit the data significantly better than the baseline model (model 3 above; differences in chi-square fitting criterion test: both $\chi^2_{10} > 273$, and $P < 0.0001$). Furthermore, model 1 was a relatively better fit for the data than model 2 [model 1: Akaikes Information Criterion (AIC) = 126.51, goodness of fit index = 0.9991; model 2: AIC = 123.38, goodness of fit index = 0.9829]. The model with the best fit (model 1) predicted that greater differences in male–male aggression but not courtship vigor would increase female discrimination. We then used this model to explore habitat differences in the relationship among variables. We pooled males from Paxton and Enos lakes because path diagrams for each lake were nearly identical, except that the relationship between differences in red and male–male aggression was marginally significant in Enos ($P = 0.07$) but not Paxton ($P = 0.22$) fish. We performed these analyses in SAS 9.2 using PROC CALIS.

**RESULTS**

First, we examined female discrimination and sexual isolation pooled across habitats. Both female ecotypes from Paxton Lake discriminated strongly between male ecotypes and preferred homotypic males (Figure 1A), which resulted in strong sexual isolation (Figure 1B). In contrast, only benthic-like females in Enos Lake discriminated between male ecotypes and preferred homotypic males, while Enos limnetic-like females did not (Figure 1A), which yielded no sexual isolation (Figure 1B). The significant interaction of female ecotype and lake ($F_{1,106} = 4.01$, $P = 0.0477$, Figure 1A) warranted running subsequent analyses separately by lake.

Next, we tested the strength of female discrimination and sexual isolation across habitats to determine whether habitats modulate the expression of discrimination and isolation. Habitat had only minor effects for both Paxton female ecotypes and Enos benthic-like females. In contrast, the strength of discrimination and direction of preference changed across habitats for Enos limnetic-like females (Figure 2). Sexual isolation in Paxton fish neared significance in native habitat, where benthic males nested in the plants and limnetic males nested in the open, but not in alternative or open habitats (Figure 2B), perhaps because only 1 species preferred homotypic males in each of these habitats (Figure 2A). Habitat altered how Enos limnetic-like, but not benthic-like, females expressed discrimination (Figure 2C). Enos benthic-like females tended to prefer homotypic males in all habitats, with the strongest effects in native habitat. Unexpectedly, Enos limnetic-like females slightly preferred heterotypic males in both the native and open habitats, with a nonsignificant trend to prefer homotypic males in alternative habitat. Sexual isolation was absent in Enos females across all habitats (Figure 2D).

We then tested which trait differences influenced female discrimination between homo- and heterotypic males. We considered differences between male ecotypes in red color, body size, courtship vigor, and male–male aggression (Table 1). Of these differences in male traits, the difference in courtship vigor between males was the best predictor of female discrimination. Females discriminated between male ecotypes more strongly with large differences in vigor (Table 1). Only in Enos females did this effect depend on habitat, and the effects were strongest for Enos limnetic-like
females (Table 2; Figure 2C). Enos males changed how aggressively they courted in alternate habitat, especially so when courting limnetic-like females (interaction of habitat with female ecotype: Enos: $F_{2,63} = 3.90, P = 0.0254$; Paxton: $F_{2,41} = 2.62, P = 0.0846$).

These differences parallel the pattern of discrimination for Enos limnetic-like females (Figure 2C). In native and open habitats, Enos benthic-like males courted limnetic-like females more aggressively than did limnetic-like male rivals. In alternative habitat, Enos male ecotypes courted females with equal aggression. Enos females discriminated more strongly when male ecotypes differed more in aggressive courtship (Enos: $F_{1,54} = 8.20, P = 0.0059$; Paxton: $F_{1,32} = 1.96, P = 0.1716$).

Finally, we used path analysis to examine how interactions between female choice and male competition might influence female discrimination. We predicted that larger differences in red color and body length would increase female discrimination and also lead to larger differences in courtship vigor (a target of female choice) and in male–male aggression (a target of male competition). These differences in courtship and male–male aggression in turn would yield stronger discrimination in females. However, testing the best fit model (Figure 4) across habitats revealed that the only significant predictor of female discrimination was the difference in courtship vigor (Figure 5). Surprisingly, females did not discriminate between male ecotypes based directly on male differences in red color or body length. Instead, males that differed more in red and/or length differed more in courtship vigor, and those differences in courtship led to stronger female discrimination. These findings apply to fish from Paxton and Enos lakes, which were pooled in this analysis.

It is also possible that males might court more vigorously when females are responsive, which would obscure whether courtship

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<th>Table 1</th>
<th>Effects of trait differences between male ecotypes on female discrimination and preference for homo- and heterotypic males</th>
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<td>Model effects for female discrimination (homo-hetero)</td>
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Separately by lake (Paxton and Enos), we ran models to test for effects of continuous covariates on female discrimination between male ecotypes. We show model effects for continuous covariates and their interactions with categorical variables of habitat (native, alternative, or open), female type (limnetic-like) or benthic-like). The models also included main effects of habitat and female type as well as their interaction. Significant $P$ values are indicated in bold.

for Enos limnetic-like females (Figure 2C). In native and open habitats, Enos benthic-like males courted limnetic-like females more aggressively than did limnetic-like male rivals. In alternative habitat, Enos male ecotypes courted females with equal aggression. Enos females discriminated more strongly when male ecotypes differed more in aggressive courtship (Enos: $F_{1,54} = 8.20, P = 0.0059$; Paxton: $F_{1,32} = 1.96, P = 0.1716$).

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It is also possible that males might court more vigorously when females are responsive, which would obscure whether courtship
was the cause or consequence of differences in discrimination. If males courted more vigorously when females were more responsive, then we would expect preferred males to court more. However, our data do not support this expectation in Paxton or Enos benthic-like females. Paxton females preferred homotypic males, but heterotypic males courted more vigorously \((t_{141} = 2.54, P = 0.0122)\). Enos benthic-like females also preferred homotypic males, but homo- and heterotypic males courted these females with equal vigor \((t_{91} = 1.58, P > 0.11)\). Enos limnetic-like females generally had no preference but weakly preferred heterotypic males in native and open habitat, and heterotypic males courted these females more than homotypic males \((t_{105} = 2.16, P = 0.0332)\). Thus, it is unlikely that the extent of female response is the primary explanation for patterns in male courtship.

Overall, habitat had very little effect on the relationships between male courtship traits, male–male aggression, and female discrimination. The single difference between habitats was that red color had no direct effects on vigor or aggression in open habitat, leaving body length as the sole determinant. We also found that male–male aggression had no direct effects on female discrimination (see model fitting description in Methods).
DISCUSSION

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

Differences in environment figure prominently in ecological speciation because contrasting environments generate divergent selection. As phenotypic traits diverge, various isolating barriers can arise as a pleiotropic consequence, and speciation proceeds. Given this central role of environment in the evolution of reproductive isolation, we asked whether contrasting environments affect the plastic expression and thus, the maintenance of reproductive isolation, focusing specifically on sexual isolation. Our results are surprising. In contrast to our expectations, habitat had little effect on the expression of female discrimination, and these effects were confined to limnetic-like females from Enos Lake. Moreover, some effects of habitat that we observed were opposite from our expectations. We expected the strongest female discrimination and preference for homotypic males in native habitat compared with alternative and open habitat. Unexpectedly, Enos limnetic-like females slightly preferred heterotypic over homotypic males in both native and open habitat. Because we found no immediate change in female discrimination and sexual isolation in Paxton fish across different habitats, this strongly suggests that environmental differences do not maintain sexual isolation via immediate plastic responses.

The small effects we found of habitat on the expression of female discrimination and maintenance of sexual isolation contrast markedly with substantial effects of habitat on the evolutionary change of these traits. Different environments can generate divergent selection on male signaling traits and female preferences, leading to their evolutionary divergence and enhanced female discrimination between con- and heterospecific males. These evolutionary changes in male and female traits may enhance sexual isolation in multiple groups of fishes, including sticklebacks (Boughman 2001; Craig and Foote 2001; Boughman et al. 2005; Maan et al. 2006; MacColl 2009) as well as other taxa (reviewed in Maan and Seehausen 2011). Many environmental factors can affect male signal transmission and courtship as well as female detection and discrimination, including those that alter underwater light spectra, such as vegetation and turbidity (Boughman 2001; Fuller 2002; Carleton et al. 2005; Seehausen et al. 2008, Heuschele et al. 2009). Our findings suggest that these environmental differences may be more critical for the evolution of female discrimination than for its maintenance or plastic expression. Indeed, the absence of plasticity in female discrimination may facilitate continued divergence and/or maintain distinct species because females would consistently select homotypic mates even across different habitats. However, given that environmental change was hypothesized to be a primary cause of the loss of premating isolation in Enos Lake (Taylor et al. 2006), we were surprised to find little plasticity across habitats in female discrimination. Such plasticity, if present, could have immediately eroded sexual isolation and allowed hybridization. Whether limited behavioral plasticity generally acts as an accelerator or brake on speciation deserves further study, as it fits into the ongoing debate about whether plasticity accelerates or retards evolutionary change generally (reviewed in Pfennig et al. 2010).

Although habitat does not immediately affect the plastic expression of sexual isolation in this study, habitat should be essential for the expression of other important isolating barriers, especially immigrant inviability and ecologically dependent postmating isolation. Immigrant inviability results from selection against migrants that leave their native habitat (generally: Nosil et al. 2005; in sticklebacks: Schluter 1993, 1994, 1995; Rundle 2002; Vamosi 2002). Ecologically dependent postmating isolation is expressed when hybrids suffer reduced fitness in distinct parental habitats because their intermediate phenotypes are poorly adapted to either environment (generally: Schluter 2000, 2001; Nosil et al. 2005; in sticklebacks: Schluter 1995; Hatfield and Schluter 1999; Rundle 2002; Gow et al. 2007; but see Taylor et al. 2012). In the Enos sticklebacks, ecologically dependent postmating isolation is weaker after environmental change, and hybrids that are formed no longer experience low fitness (Behm et al. 2010). These findings suggest that environmental differences are critical both to the expression and evolution of these isolating barriers.

Habitat-sensitive expression of male courtship

Despite weak effects of habitat on female discrimination, we found that habitat affects the expression of courtship behavior in males, primarily by altering how aggressively they court females. The presence of vegetation could change how male ecotypes interact with
females and with each other during courtship, potentially reducing simultaneous and competitive courtship (Myhre et al. 2013). The pattern of plasticity in response to habitat in aggressive courtship mirrors changes in Enos limnetic-like female discrimination across habitats. This makes sense in light of our finding from multiple regression and path analyses that the difference in courtship between male ecotypes directly predicted female discrimination, whereas differences in red, length, and male–male aggression influenced female discrimination indirectly, acting only through their effects on courtship. Our path analyses also reveal that both body size and red color affect male courtship and male–male aggression in the presence of vegetation, yet in open habitat, only size mattered; we found the same patterns in an earlier study, where we measured male–male aggression as well as territory and nesting behaviors across habitats (Lackey and Boughman 2013a). Given that female discrimination depends strongly on courtship vigor in all habitats, this suggests that female discrimination may be responding to variation in male courtship, rather than habitat per se.

Our findings suggest that the expression of male courtship and aggressive behavior may be modulated by environment to a greater extent than the expression of female discrimination behavior. Males may use environmental cues to adjust when and how they court females and compete with other males because they can benefit from avoiding predation, increasing the likelihood of mating, and decreasing the costs of fighting (Ryan et al. 1982; Enquist et al. 1990; Kozak et al. 2009; Sullivan-Beckers and Hebets 2011; Palaoro et al. 2013). Mating with heterospecifics can be costly (Arnold 1997; Coyne and Orr 2004), potentially more so for females than males (Andersson 1994; Parker and Partridge 1998), so female discrimination may be less plastic when costs of hybridizing are high. However, the environment may still mediate female discrimination through learning or imprinting. In sticklebacks, discrimination is shaped by imprinting on father color and odor (Kozak et al. 2011) and learning from close contact with other juveniles (Kozak and Boughman 2009).

Figure 5
Tested relationships among female discrimination and male morphological and behavioral traits across habitats. We show significant paths for Paxton and Enos fish combined in native (A), alternative (B), and open (C) habitats. Line thickness shows the strength of the standardized path coefficient. Straight lines depict causal relationships, while curved lines show correlations. Solid lines indicate positive relationships between variables, while dashed lines indicate negative relationships. Paths are estimated from the best fit model shown in Figure 4. See Methods text for other models tested.
Environmental change and reverse speciation

As seen in this and previous work, several factors appear to cause increased and asymmetric hybridization leading to reverse speciation in Enos sticklebacks. Overall, sexual isolation is weak in the Enos pair, shown both here and in our earlier work (Lackey and Boughman 2013b), even though historically sexual isolation was strong (Ridgway and McPhail 1984). Here, we show that only limnetic-like females have plastic preferences, and they appear to favor heterotypic males except when homotypic males nest in vegetation. We do not know whether the plasticity expressed by Enos limnetic-like females in this experiment was a potential cause or consequence of species collapse. Plasticity in ecotype preference may be influenced by variation in whether homotypic males are common (Rodriguez et al. 2013). Additionally, because limnetic-like fish are now relatively rare in Enos Lake (Gow et al. 2006; Taylor et al., 2006), Enos limnetic-like females that are more willing to mate with heterotypic males can ensure mating. Thus, the current extent of plasticity and preference for heterotypic males could be a response to new environmental conditions. It is also possible that gene flow has eroded genetic differences in preferences between ecotypes. Moreover, plasticity in male courtship could further enhance the likelihood that Enos limnetic-like females will hybridize. Previous work has shown that Enos limnetic-like females were missing 1 dimension of discrimination, which could weaken the contribution of this female ecotype to sexual isolation. Females of limnetic and benthic ecotypes generally reject heterospecific males that differ in size relative to the female (Nagel and Schluter 1998; Boughman et al. 2005); this was true of Enos benthic-like but not Enos limnetic-like females (Boughman et al. 2005). Our finding that Enos limnetic-like and benthic-like females contributed asymmetrically to sexual isolation is also consistent with patterns of asymmetric introgression of limnetic alleles into the benthic genome reported in earlier work (Gow et al. 2006).

Our results reveal important insights into how changes to mating habitats influence traits that underlie sexual isolation. We found that size alone affects male courtship vigor differences in open habitat, which in turn influence female discrimination; thus, the historically strong effects of differences in male color and female color preference in generating sexual isolation (Boughman 2001) do not appear to be in force any longer, mediated in part by the loss of vegetation in the lake and consequent changes in light environment. Earlier work found that size alone influenced success in male competition in the absence of vegetation, with larger males winning (Lackey and Boughman 2013a). Given that benthics are larger than limnetics, benthic males would be more likely to establish territories, build nests, and court females in the open habitat remaining in the lake. This could increase encounter rates between species as well as limit opportunities for limnetic-like females to choose limnetic-like males as mates.

The invasion of the lake by signal crayfish (Pacificus leniusculus) and subsequent loss of vegetation is thought to have triggered reverse speciation in Enos sticklebacks (Taylor et al. 2006). Yet, our finding of little immediate plastic behavioral changes in female discrimination across habitats suggests that some other environmentally sensitive mechanism likely initiated the loss of reproductive isolation. Environmental change could affect the development and expression of ecologically dependent traits that are important for imprinting in juveniles (Kozak et al. 2011) and for mate discrimination in females (Nagel and Schluter 1998; Boughman 2001; Boughman et al. 2005; Raifferty and Boughman 2006; Vines and Schluter 2006; Head et al. 2013). Indeed, positive assortative mating based on environment was found in allopatric stickleback populations (Vines and Schluter 2006). Additionally, the loss of a fitness disadvantage of hybrids means more hybrids would survive to reproduce. And lastly, habitat changes can alter selection on male traits via male competition (Lackey and Boughman 2013a), which could change the distribution of trait values observed by females, and thus the basis for discrimination.

CONCLUSIONS

A key conclusion from our results is that environmental differences play only a supporting role in the current expression and maintenance of sexual isolation, which was surprising considering the central role of environmental differences in the evolution of sexual isolation shown in previous work (Boughman 2001; Boughman et al. 2005) and the suspected role of environmental change in increasing hybridization (Taylor et al. 2006). Thus, even though divergent female preferences and male mating traits that cause sexual isolation evolved due to differences in environment, the current expression of sexual isolation does not appear to depend on habitats being different. This pattern contrasts with other components of reproductive isolation, which depend on environmental differences for both their evolution and maintenance. Our work highlights how studying the interactions between environments, mate choice, and plasticity can yield important and potentially surprising insights into the speciation process.

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