

Sexual isolation promotes divergence between parapatric lake and stream stickleback

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Abstract

Speciation can be initiated by adaptive divergence between populations in ecologically different habitats, but how sexually based reproductive barriers contribute to this process is less well understood. We here test for sexual isolation between ecotypes of threespine stickleback fish residing in adjacent lake and stream habitats in the Lake Constance basin, Central Europe. Mating trials exposing females to pairings of territorial lake and stream males in outdoor mesocosms allowing for natural reproductive behaviour reveal that mating occurs preferentially between partners of the same ecotype. Compared to random mating, this sexual barrier reduces gene flow between the ecotypes by some 36%. This relatively modest strength of sexual isolation is surprising because comparing the males between the two ecotypes shows striking differentiation in traits generally considered relevant to reproductive behaviour (body size, breeding coloration, nest size). Analysing size differences among the individuals in the mating trials further indicates that assortative mating is not related to ecotype differences in body size. Overall, we demonstrate that sexually based reproductive isolation promotes divergence in lake–stream stickleback along with other known reproductive barriers, but we also caution against inferring strong sexual isolation from the observation of strong population divergence in sexually relevant traits.

Introduction

Understanding speciation implies identifying the nature and strength of the reproductive barriers restricting gene flow between diverging populations. Studying the emergence of reproductive barriers is particularly rewarding in young diverging populations exhibiting incomplete reproductive isolation. In such systems, observed reproductive barriers are likely to be instrumental to divergence and are not yet obscured by evolutionary divergence occurring after the achievement of full reproductive isolation (Coyne & Orr, 2004; Sobel *et al.*, 2010). Because gene flow between diverging populations might be constrained by multiple barriers acting concurrently (Tauber & Tauber, 1977; Rice & Hostert, 1993; Coyne & Orr, 2004; Lowry *et al.*, 2008a;

Sobel *et al.*, 2010; Nosil, 2012), a comprehensive characterization of reproductive isolation remains a major challenge in studying speciation and has been attempted in only a few systems (e.g. Ramsey *et al.*, 2003; Nosil, 2007; Lowry *et al.*, 2008b). However, such information across taxa is needed to recognize generalities in the relative importance of different reproductive barriers and in the timescale and chronological order at which they emerge (Coyne & Orr, 2004; Ritchie, 2007; Sobel *et al.*, 2010; Nosil, 2012).

In the present study, we pursue the characterization of reproductive barriers in a powerful system for studying the early stages of speciation: populations of threespine stickleback fish (*Gasterosteus aculeatus* L.) residing in adjacent, ecologically different lake and stream habitats. Importantly, lake stickleback typically exploit primarily zooplankton in the open water, whereas stream populations forage on benthic (substrate-dwelling) macro-invertebrates (Gross & Anderson, 1984; Berner *et al.*, 2008; Lucek *et al.*, 2012; Moser *et al.*, 2012; Ravinet *et al.*, 2013). This ecological divergence is associated

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with phenotypic divergence in foraging traits, often occurring on a short geographic scale despite the absence of physical dispersal barriers (Moore & Hendry, 2005; Berner *et al.*, 2009, 2010; Bolnick *et al.*, 2009; Deagle *et al.*, 2012; Ravinet *et al.*, 2013) and being at least partly genetically based (Lavin & McPhail, 1993; Sharpe *et al.*, 2008; Berner *et al.*, 2011, 2014; Lucek *et al.*, 2014). Moreover, phenotypic differentiation across lake–stream habitat transitions often coincides with substantial differentiation at neutral genetic markers (Hendry & Taylor, 2004; Berner *et al.*, 2009; Bolnick *et al.*, 2009; Deagle *et al.*, 2012; Ravinet *et al.*, 2013), indicating the presence of at least partial reproductive isolation between the ecotypes.

Our understanding of the specific barriers causing reproductive isolation between lake and stream stickleback, however, is fragmentary (Bolnick *et al.*, 2009; Hendry *et al.*, 2009; Eizaguirre *et al.*, 2012; Hanson *et al.*, 2016; Moser *et al.*, 2016). For instance, divergent selection between ecologically distinct habitats can drive divergence between populations in traits mediating reproductive interactions within or between the sexes, thereby causing sexually based reproductive isolation between the populations (Templeton, 1981; Coyne & Orr, 2004; Ritchie, 2007; Maan & Seehausen, 2011; Thibert-Plante & Gavrilets, 2013). However, if and how strongly sexual barriers promote reproductive isolation between lake and stream stickleback remains poorly understood. A study in a European lake–stream system found that females preferred the odour of males from the same ecotype to the odour of males from the opposed ecotype in a laboratory setting (Eizaguirre *et al.*, 2011), but the relevance of this result to reproductive behaviour and sexual isolation in nature is unclear. Studying a Canadian lake–stream pair, Raeymaekers *et al.* (2010) found no evidence that mate choice contributes to reproductive isolation, but since only laboratory-raised individuals were tested, this study might have overlooked sexual isolation contingent on trait divergence arising from phenotypic plasticity in the wild. To gain stronger insights into sexual isolation between lake and stream stickleback, we here present a mating experiment performed with field-caught individuals in outdoor mesocosms allowing for reproductive interactions within and between sexes under natural conditions. This reveals that sexual isolation promotes lake–stream divergence in stickleback, although the strength of this reproductive barrier is relatively modest.

Materials and methods

Study design

Our experimental strategy was to release a male from each ecotype into outdoor mesocosms and to allow each individual to establish a nest territory. (In

threespine stickleback, nest construction and brood care is performed by the male alone.) Then, we released a single lake or stream female ready to spawn into each mesocosm and recorded with which male the female mated. Our prediction was that if the lake and stream ecotypes are at least partially sexually isolated, within-ecotype mating should be more frequent than between-ecotype mating. This experimental approach simultaneously offered the opportunity for a female to choose between two males (two-choice situation), for each male to either accept or reject the female as mate partner (no-choice situation) and for the two males to display antagonistic (intrasexual) interactions. No effort was made to disentangle these female and male behavioural components; the goal of our study was an overall test of sexual isolation between lake and stream ecotypes, considering the actual spawning as the mating phenotype most relevant to reproductive isolation.

Our study design differs from the one used predominantly in investigations of sexual isolation between stickleback ecotypes or species, that is a female no-choice setting allowing for interactions between a gravid female and only a single male maintaining a nest (e.g. Hatfield & Schluter, 1996; Albert, 2005; Olafsdottir *et al.*, 2006; Furin *et al.*, 2012; Head *et al.*, 2013). However, choice and no-choice designs can produce different results, the former generally being more sensitive; hence, the choice of the design should reflect mate encounter opportunities in the focal organismal system in nature (Wagner, 1998; Coyne *et al.*, 2005; Dougherty & Shuker, 2015). In our study system, population densities are very high (Moser *et al.*, 2016) and male territories are small, thus plausibly allowing gravid females to inspect multiple males within minutes. We therefore considered a design allowing a female to choose among territorial males most biologically meaningful.

As study populations, we used stickleback from Lake Constance, a very large and genetically well-mixed population (Moser *et al.*, 2012; Roesti *et al.*, 2015; sampling occurred at the ROM site described in Berner *et al.*, 2010), and from a tributary to that lake (the NID stream population in Berner *et al.*, 2010). These populations exhibit highly divergent life styles (pelagic vs. benthic) and associated differentiation in trophic and predator-mediated morphology and in life history (Berner *et al.*, 2010; Moser *et al.*, 2012, 2015). This phenotypic differentiation is at least partly the outcome of divergent natural selection: first, a marker-based genome scan identified numerous signatures of selection in localized genome regions between these populations (Roesti *et al.*, 2015). Second, a field transplant experiment performed in the focal stream habitat revealed that the resident stream population had much higher fitness than lake fish (and lake–stream hybrids) and hence is locally adapted (Moser *et al.*, 2016). Nevertheless, lake and stream ecotypes in the Lake Constance basin hybridize, as shown by molecular signatures of

introgression from the lake into tributary populations (Roesti *et al.*, 2015). The opportunity for hybridization arises because although Lake Constance stickleback breed along the shore of the large lake (D. Berner and D. Moser, personal observation), they also use the lower reaches of tributaries for reproduction. In these regions, the breeding ranges of lake and stream fish overlap (Roesti *et al.*, 2015; Marques *et al.*, 2016), thus motivating an investigation of sexual barriers between the ecotypes.

Mating trials

Our experiment required two stickleback males to simultaneously maintain a nest. Direct qualitative observation of the lake population at a natural breeding site indicated that males maintain nesting territories at a distance of around 2 m in the wild (Daniel Berner, personal observation; see also Kynard, 1978; Goldschmidt & Bakker, 1990). We thus constructed 24 total outdoor mesocosms 230 cm long, 30 cm wide and 30 cm high (approximate water volume: 180 L) in the shaded courtyard of the Zoological Institute of the University of Basel. The mesocosms consisted of a wooden frame coated with 1-mm-thick, black PVC pond liner and were fitted with an overflow to avoid flooding by rain. To increase stability and to buffer water temperature, the mesocosms were arranged in triplets (Fig. S1 shows images of the experimental facility).

To offer nesting sites, the outer 25 cm on each side of each mesocosm were covered with a 1 cm deep layer of sand and fine gravel. The rest of the mesocosm was unsuitable for nest construction (nests were always built on this sand substrate). In addition, we placed a brick (18 × 10 × 14 cm) in the centre of each mesocosm as shelter for the female, with the holes parallel to the mesocosm's long axis. This brick did not constrain the movement of the fish within the mesocosms. For instance, males were commonly observed collecting nesting material, courting females and chasing the other male in the mesocosm sector opposite to their own nesting territory. For further shelter, a cover was fixed at both ends and in the centre of each mesocosm (Fig. S1). As nest construction material, we added to each nesting site approximately 500 green and black polyester sewing threads of 6 cm length (Barber *et al.*, 2001; Moser *et al.*, 2015), 0.8 g of filaments of the cyanobacterium *Nostoc flagelliforme* ('fat choy') and four thalli of *Brachythecium* sp. moss.

Our experiment was performed with reproductively mature fish caught in the wild at the ROM (Lake Constance) and NID (inlet stream) site (Berner *et al.*, 2010) during the breeding season of 2016. To ensure that the experimental individuals were representative of the entire breeding cohort, we sampled field specimens in the beginning (13 April), in the middle (20 May) and towards the end of the breeding season (14 June) (we

found no indication of asynchrony in breeding time between the populations; see also Hanson *et al.*, 2016). To ensure a sufficient number of experimental fish from both sexes, we collected in each sampling round approximately 30 males and 50–80 females per ecotype. To initiate the trials, each mesocosm was stocked with one lake and one stream male. The choice of these individuals was random with respect to phenotype. The males were not marked because the ecotypes differed clearly in body size and nuptial coloration. However, to preclude potential ambiguities, we recorded the initial body mass of all experimental males. Males not used immediately and all females were transferred to the laboratory and distributed among 20 50–120 L aquaria under long-day (summer) conditions (details provided in Moser *et al.*, 2015), keeping the sexes separate. Both the males in the mesocosms and the laboratory fish were fed defrosted chironomid larvae (bloodworms) – the prey consumed predominantly by both study populations on breeding grounds (Moser *et al.*, 2012) – and frozen *Daphnia* to satiation at least twice daily. The mesocosms additionally offered live prey and were probably self-sustaining, as indicated by frequent observations of live chironomid and other diptera larvae during experimental work.

To stimulate nest building, the males in each mesocosm were exposed to a single gravid lake or stream female twice a day (in the morning and afternoon), randomizing the order of the female ecotype every day. Females for stimulation were chosen haphazardly from the laboratory aquaria, confined in transparent 10 × 10 × 10 cm plastic containers with a mesh cover (thus allowing for olfactory interactions), placed on top of the brick in the centre of the mesocosms for 1 h and then released back into the laboratory aquaria. Once daily, both nesting sites in all mesocosms were inspected for new nests. A nest was considered ready when it exhibited a clearly distinguishable entrance (a representative example is shown in Fig. S2). In cases in which all nesting material at a nesting site seemed depleted, we immediately offered additional material to avoid constraints on nest construction.

When both males in a mesocosm had built a nest (always on the opposed sides), a single female ready for reproduction was chosen from the laboratory aquaria and transferred to a transparent container. The container was placed on top of the brick for 20 min to offer the female the opportunity to perceive both males. After this acclimatization, we removed the mesh cover, thus releasing the female into the mesocosm. The choice of experimental females was random with respect to their phenotype, and we used lake and stream ecotypes alternately. The following day, we recaptured the female and inspected her abdomen to check whether she had spawned. If this was not the case, we again inspected the female 24 h later (spawning always occurred within 48 h). After the female had

spawned, she was removed from the mesocosm and each nest was assigned to the corresponding male ecotype. The latter was done by observing brood care or nest maintenance behaviour by each male. These observations occurred either directly, or by analysing videos taken by underwater cameras (GoPro Hero3+) placed in front of the nests. Once the nests were assigned to their male, we retrieved both nests from the mesocosm and recorded in which nest the female had spawned. The two males were then also removed from the mesocosm, and together with the female euthanized with an overdose of Koi Med Sleep (Fishmed, Rain, Switzerland), weighed to the nearest 0.01 g and preserved in absolute ethanol. All experimental individuals were thus used for a single trial only. In total, we performed 58 trials, with a balanced representation of experimental lake and stream females ($N = 29$). For a subset of trials ($N = 28$), the three experimental fish were additionally photographed together on a white background under standardized indoor light conditions, immediately after catching them from the mesocosm. Finally, the mesocosm was supplied with fresh substrate and nesting material and restocked with a new lake and stream male. Throughout the experiment, we regularly performed water changes to avoid turbidity in the mesocosms.

All females involved in our trials oviposited into a nest. To confirm successful fertilization within the mesocosms, the clutches obtained at the end of 32 haphazardly chosen trials were incubated in the laboratory as in Moser *et al.* (2015). All these clutches proved well fertilized and displayed normal embryonic development. Mortality in the mesocosms was zero.

Additional phenotypic measurements

Besides testing for assortative mating between lake and stream stickleback, our experiment offered the opportunity to investigate whether the ecotypes differed in traits potentially important to reproductive behaviour. The first of these traits was body size. Divergence in body size has often been inferred to underlie sexual isolation in other threespine stickleback ecotype or species comparisons (Nagel & Schluter, 1998; Ishikawa & Mori, 2000; McKinnon *et al.*, 2004, 2012; Boughman *et al.*, 2005; Conte & Schluter, 2013). Body size differences between our ecotypes were quantified based on the male and female body mass measurements taken at the end of each trial. Although body mass is used as body size proxy throughout the paper, we additionally quantified body size as standard length measured from the photographs by using tpsDig2.32 (Rohlf, 2015), to facilitate comparisons with other studies.

Second, we used the photographs to examine whether lake and stream male stickleback differed in nuptial coloration. In threespine stickleback, males typically express at least some orange or red colour in the

cheek, throat and chest region. The extent and intensity of this coloration appears to reflect a male's vigour (Milinski & Bakker, 1990; Candolin, 1999a; Pike *et al.*, 2007), represents a social signal of a male's territorial dominance (Moodie, 1972; Kynard, 1978; Bakker & Sevenster, 1983; Rowland, 1984; Candolin, 1999b; Lackey & Boughman, 2013; Candolin & Tukiainen, 2015) and serves as a cue in female mate choice within stickleback populations (McLennan & McPhail, 1990; Milinski & Bakker, 1990; Candolin, 1999b; Braithwaite & Barber, 2000; Pike *et al.*, 2007). Moreover, divergence in male nuptial coloration has been suggested to influence sexual isolation between co-occurring stickleback groups (Boughman *et al.*, 2005; Feller *et al.*, 2016). Divergence in nuptial colour has been observed qualitatively in our previous field work on lake and stream stickleback from the Lake Constance region (see also Marques *et al.*, 2016), with more intense red coloration in lake fish, but this has never been quantified formally. To do so, we defined an area based on skeletal landmarks in the stickleback's cheek and throat region well suited to capture male breeding coloration (Fig. 1a). For this area, we recorded the mean red, green and blue value (linear RGB) as provided by the histogram function in GIMP2 (using median values produced similar results). These values were then used to derive the hue (dominant wavelength) with the *rgb2hsv* function from the R package *grDevices* and to calculate the relative luminance (perceived brightness) as $0.2126R + 0.7152G + 0.0722B$ (International Telecommunication Union, recommendation BT.709; <http://www.itu.int/rec/R-REC-BT.709>). Although tailored to the human eye, these metrics should be informative for

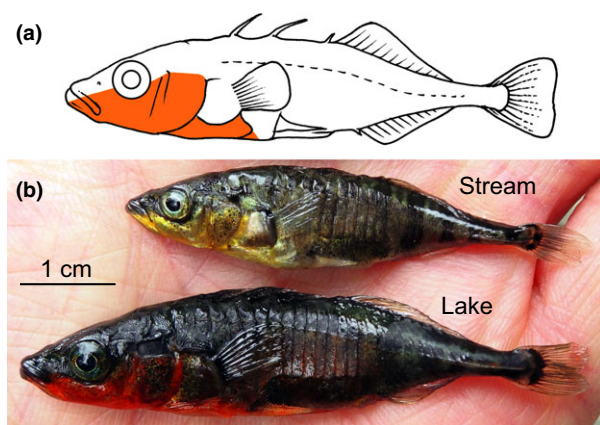


Fig. 1 (a) The shading indicates the body region considered for the measurement of hue and luminance from photographs of stickleback males involved in the mate choice trials. (b) Representative example of an experimental lake–stream male pairing, highlighting the difference in body size and breeding coloration.

our study, given the similarity between the stickleback and human visual systems (Lythgoe, 1979; Rowe *et al.*, 2006). The RGB measurements were performed without any prior colour correction to the photographs. The justification is that each image displayed *both* males involved in a given mating trial. Variation in image quality thus potentially added noise to our investigation of differences in breeding coloration, but introduced no bias related to ecotype.

The third phenotype investigated was nest size. In threespine stickleback, nest size has been shown to vary among males within and among populations, some of this variation having a genetic basis (Kitano *et al.*, 2008; Rushbrook *et al.*, 2008; Raeymaekers *et al.*, 2009). The size (and structural aspects) of the nest has further been suggested to allow females to assess male quality and to influence mating decisions (Barber *et al.*, 2001; Ostlund-Nilsson & Holmlund, 2003; Head *et al.*, 2016; see also Goldschmidt & Bakker, 1990; Nagel & Schluter, 1998; Candolin *et al.*, 2007), thus potentially contributing to sexual isolation between populations divergent in nest characteristics (Olafsdottir *et al.*, 2006; Kitano *et al.*, 2008; Raeymaekers *et al.*, 2009). In our study, both nests were removed from a mesocosm when terminating a mating trial. For a subset of the trials ($N = 32$), this was done in a standardized way by lifting the nests from underneath, allowing substrate not attached to the nest to drop off (stickleback males use a secreted glue for nest building). These nests were then transferred to Petri dishes, dried for 72 h at 50 °C and weighed to the nearest 10 mg. Quantifying nest size as nest surface measured crudely from nest photographs taken with a reference scale produced very similar results (details not presented), confirming that dry mass was a meaningful index of nest size.

Data analysis

To test for assortative mating between lake and stream stickleback, we used a generalized linear mixed-effect model (*glmer* function implemented in the *lme4* R package) with an experimental male's mating success as binary response (logit link function). Female ecotype and male ecotype (scored relative to the focal female's ecotype: 'same' or 'different') were defined as categorical predictors, and female identity was specified as grouping variable (the full model syntax is given in Appendix S1). Our specific interest was in two model terms: first, the male ecotype main effect tested the hypothesis that the outcome of a mating trial was random with respect to the ecotypes of the males (i.e. an overall test of assortative mating). Second, the male ecotype by female ecotype interaction term evaluated whether the strength of assortative mating differed between lake and stream females.

Differences between the lake and stream fish in body size, breeding coloration (hue and luminance) and nest

size were visualized by kernel density plots. Corresponding *P*-values were generated through permutation tests with 9999 iterations, using the absolute difference between the ecotype medians as test statistic (Manly, 2007). For body size, this was done separately in each sex.

Finally, we performed two tests to explore whether assortative mating was influenced by body size differences among the experimental fish. We first hypothesized that if lake and stream ecotypes mate preferentially with partners of matching size (Conte & Schluter, 2013), the difference in body size between an experimental female and the male of the opposite ecotype should be smaller in trials in which the female actually mated with this male than in trials resulting in within-ecotype mating. We thus calculated the absolute body size difference between the female and the male of the opposite ecotype for each trial and carried out a generalized linear model using R's *glm* function with the female–male size difference and female ecotype as predictors, and mating outcome (within-ecotype or between-ecotype) as binary response. In a second model, we replaced the female–male body size difference by the size difference between the two males in a trial as predictor to test whether females were more likely to mate with a partner from the opposite ecotype when the body size difference between the two males was relatively small. Analogous analyses exploring a potential influence of male breeding coloration or nest size on mating were not performed because for these traits the data set was prohibitively small. All analyses and graphing were performed in R (R Development Core Team, 2016). The complete raw data set is available from the Dryad Digital Repository (<http://dx.doi.org/10.5061/dryad.78p62>).

Results

We obtained clear support for assortative mating in our stickleback system: lake and stream fish mated preferentially, although not consistently, with partners from their own ecotype (Fig. 2; male ecotype main effect $P = 0.005$; further details presented in Appendix S1). This positive assortative mating was symmetric (male–female ecotype interaction $P = 0.69$), with a bias towards within-ecotype mating of close to 70% in both populations (lake: 69%, binomial 95% confidence interval = 49–85%; stream: 66%, 95% CI = 46–82%).

The lake and stream ecotypes differed in all additional traits investigated (Fig. 3; two-sided $P = 0.0001$ for all permutation tests). Specifically, in both sexes, median body mass in lake fish was more than twice as large as in stream fish, and the overlap between the trait distributions was minimal. Measured as standard length, lake males were still 37% larger than stream males, with completely nonoverlapping size ranges (lake median = 57.9 mm, stream median = 42.4 mm.).

Likewise, lake and stream males differed strongly in breeding coloration: lake males displayed deep red throats, whereas the same area was orange and brighter in stream males (representative males are shown in Fig. 1b). The larger lake males also constructed larger nests than stream males, median nest size differing two-fold between the ecotypes.

Finally, we found no indication that body size differences among the experimental fish substantially determined the outcome of the mating trials: when mating occurred between partners from the opposite ecotype, the size difference between a female and the male of the opposed ecotype was only trivially smaller than in trials resulting in within-ecotype mating (Fig. 4 top; main effect of size difference $P = 0.36$; full results presented in Appendix S2). Likewise, we found no evidence of a material influence of the size difference between the two experimental males on the probability of between-ecotype mating (Fig. 4 bottom; $P = 0.38$; Appendix S2).

Discussion

Our study demonstrates the presence of positive and symmetric assortative mating in parapatric lake and stream stickleback. With an average probability of within-ecotype mating of 0.68 across the populations, however, assortment is clearly incomplete. This mating probability corresponds to a strength of premating reproductive isolation in the order of 0.36 (according to equation 4A in Sobel & Chen, 2014, based on the relative proportion of between-ecotype matings) in areas where lake and stream stickleback breeding ranges overlap and hybridization can occur (values of 0 and 1 would indicate random mating and a complete sexual barrier to gene flow between the populations, respectively). Sexual isolation alone is thus insufficient to allow strong genetic divergence in our parapatric lake–stream stickleback, but promotes divergence alongside other, stronger reproductive barriers identified in these populations (Moser *et al.*, 2016). This resembles other organismal systems in the early stages of speciation in which strong overall reproductive isolation arises from a combination of multiple incomplete barriers to gene

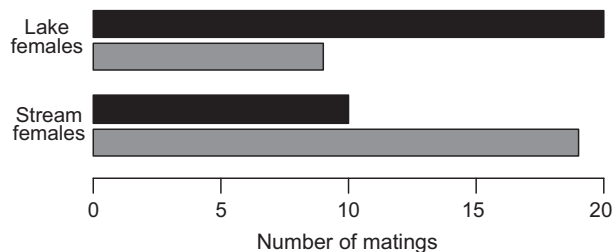


Fig. 2 Number of times lake and stream stickleback females mated with lake males (black bars) and stream males (grey bars). Total sample size is 29 in each female group.

flow (Ramsey *et al.*, 2003; Nosil, 2007; Lowry *et al.*, 2008a,b; Eroukhanoff *et al.*, 2011).

Despite the identification of substantial sexual isolation (this study) and selection against migrants and hybrids between the ecotypes (Moser *et al.*, 2016), assessing the total (cumulative) strength of reproductive

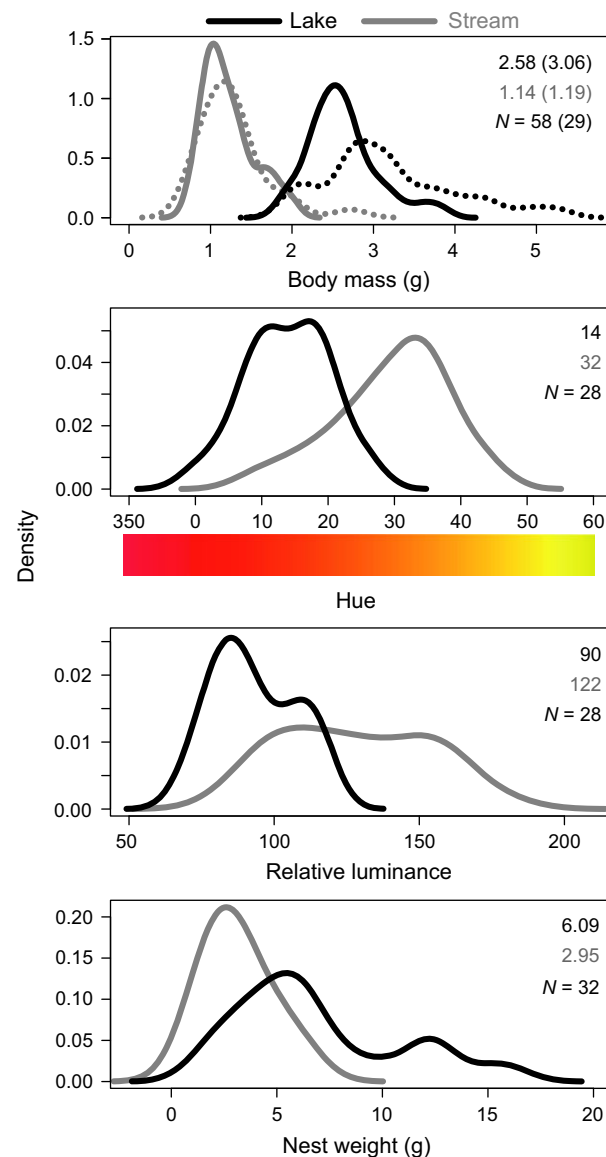


Fig. 3 Difference between lake and stream stickleback males in body size, in two components of breeding coloration (hue, the dominant wavelength; relative luminance, the perceived brightness) and in the size of the nest. For body size, female data are also presented (dotted lines). Numbers within the plots indicate the median value of each ecotype and sample size within each ecotype (female values in parentheses). Note that hue values refer to an angular scale (0–360°). For ease of interpretation, the relevant sector is visualized below the axis.

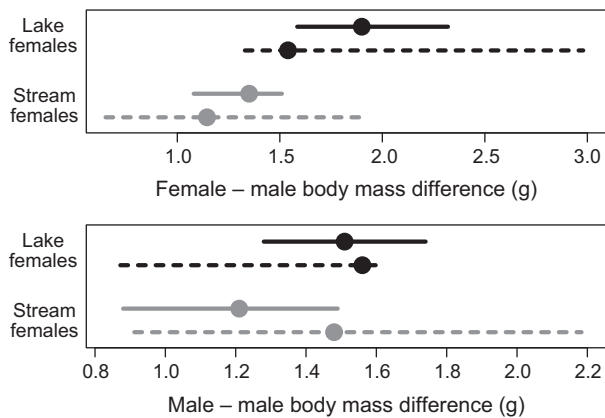


Fig. 4 Difference in body size between stickleback females and males of the opposite ecotype (top) and difference in body size between the two experimental males (bottom) in trials resulting in within-ecotype (solid lines) and between-ecotype (dashed lines) mating. The data are visualized by medians and bootstrap 95% confidence intervals (10 000 resamples), calculated separately for trials involving lake and stream females. Sample sizes are 20 (within-ecotype) and 9 (between-ecotype) for lake females, and 19 and 10 for stream females.

isolation between the lake and the stream populations, and the relative contribution of each individual barrier to total isolation (Coyne & Orr, 1989; Sobel & Chen, 2014), remains difficult. The reason is that ecogeographic isolation (Ramsey *et al.*, 2003; Sobel *et al.*, 2010) arising directly from the parapatric arrangement of the lake and stream habitats, and possibly nonrandom dispersal caused by some form of habitat preference (Rice, 1987; Bolnick *et al.*, 2009; Edelaar & Bolnick, 2012; Webster *et al.*, 2012; Berner & Thibert-Plante, 2015), might already greatly reduce gene flow between the ecotypes, thus discounting the importance of the isolating barriers identified thus far. Therefore, a more complete understanding of reproductive isolation in our lake and stream stickleback system now requires quantitative information on the extent of overlap in the reproductive range and on the magnitude and life stage of dispersal between the habitats.

Potential mechanisms underlying assortative mating

An important question emerging from our study concerns the mechanistic basis of assortative mating. A phenotype of particular importance to this question is body size, because body size may both be used as a cue in female and male mate choice and influence territorial interactions among males. Indeed, body size is the trait most consistently inferred to underlie sexually based reproductive isolation in other stickleback systems (Nagel & Schluter, 1998; Ishikawa & Mori, 2000; McKinnon *et al.*, 2004, 2012; Boughman *et al.*, 2005; Conte & Schluter, 2013). In our experiment, lake fish

were dramatically larger than their stream counterparts, a pattern consistent with field data from lake and stream stickleback in the Lake Constance basin collected in previous years (Lucek *et al.*, 2012; Moser *et al.*, 2012, 2015). The body size differences among populations in this system largely reflect a by-product of phenotypic plasticity in life history, presumably driven by the exploitation of differential food resources (Moser *et al.*, 2012, 2015). Specifically, foraging on benthic prey typically permits an annual life cycle with small reproductive body size in the stream populations, whereas pelagic foraging appears less rewarding, generally allowing the lake fish to breed only after 2 years – albeit at larger size. We thus hypothesized (Moser *et al.*, 2015) that reproductive isolation between the ecotypes is promoted directly by plastic divergence in a phenotype pivotal to sexual behaviour (Thibert-Plante & Hendry, 2011; Fitzpatrick, 2012). Intriguingly, our present results do not support this idea: we detect sexual isolation but find little indication that this isolation is related to the striking population difference in body size.

Beside body size, our lake and stream stickleback populations differ greatly in male nuptial coloration and nest size. Both traits might be involved in assortative mating between the ecotypes (Boughman *et al.*, 2005; Kitano *et al.*, 2008; Raeymaekers *et al.*, 2009; Feller *et al.*, 2016), although we made no attempt to explore this possibility with our data because sample sizes and hence statistical power were substantially lower than for body size (see Fig. 3). A question thus potentially important to understanding the basis of sexual isolation concerns the extent to which these differences reflect adaptive genetic divergence vs. phenotypic plasticity. For male nuptial coloration, population differentiation has been shown to have a genetic component in other stickleback systems (Lewandowski & Boughman, 2008; Malek *et al.*, 2012; Yong *et al.*, 2016). Moreover, in habitats with low visibility (due to stained water or structural complexity), the benefits of the red male ornament in sexual and social interactions seem reduced, thus promoting the evolution of reduced (or alternative) nuptial coloration (Reimchen, 1989; Boughman, 2001; Candolin *et al.*, 2007; Lackey & Boughman, 2013). It is possible that structural differences between lake and stream habitat in the Lake Constance basin have driven adaptive divergence in male breeding coloration (recall that the Lake Constance population mostly breeds on the lake shore – only a small proportion reproduces in the lower reaches of tributaries). Alternatively, the difference in male coloration may simply reflect differential endocrine states resulting directly from plastic lake–stream divergence in age and size at maturity and is perhaps also influenced by different availabilities between the habitats in carotenoids (Grether *et al.*, 1999), compounds underlying red coloration

that stickleback cannot synthesize themselves (Goodwin, 1984).

Our study is consistent with previous work in demonstrating that larger stickleback ecotypes also build larger nests (Kitano *et al.*, 2008), a difference shown to have a genetic basis in a lake and stream system where this has been tested (Raeymaekers *et al.*, 2009; see also Rushbrook *et al.*, 2008). Since female clutch size scales with body size among lake and stream populations from our study system (Moser *et al.*, 2012) and male stickleback often tend clutches from several females simultaneously (Wootton, 1976; Kynard, 1978), it is tempting to speculate that body size divergence requires correlated evolution of nest size to satisfy space requirements. To evaluate these ideas, it would be valuable to investigate the genetic basis of divergence in male nuptial coloration and nest characteristics through field transplant or common garden experiments, and to examine the costs and benefits of variation in these traits in the two habitats.

We find that lake and stream stickleback differ in several phenotypes commonly considered relevant to sexual isolation – and likely in additional ones such as courting behaviour or territorial aggression not considered in our study. Also, lake and stream fish from our study region have diverged by natural selection, so that lake–stream F1 hybrids show reduced fitness relative to pure resident individuals in the wild (Moser *et al.*, 2016). This hybrid inferiority clearly reflects genetic divergence, as it was observed in individuals derived from controlled laboratory lines. The coincidence of both strong lake–stream differences in sexually important traits and genetically based hybrid inferiority raises the question why sexual isolation is not more complete. Why has selection against the production of hybrid offspring with a poor prospect of reproduction not favoured the spread of alleles driving stronger discrimination against mating partners from the opposite ecotype – that is reinforcement (Dobzhansky, 1940; Servedio & Kirkpatrick, 1997; Coyne & Orr, 2004)? A possible explanation is that the geographic arrangement of the lake and stream habitat is not conducive to reinforcement: the opportunity for hybridization between lake and stream ecotypes is limited to a small fraction of each population's breeding range, making the direct selection of alleles promoting stronger sexual isolation within each population ineffective (Liou & Price, 1994; Servedio & Noor, 2003; Coyne & Orr, 2004). We thus expect that sexual isolation in this stickleback system will remain a fortuitous by-product of genetic and/or plastic ecological divergence.

Conclusions

Based on mating trials in mesocosms, we have demonstrated the presence of sexually based reproductive isolation in lake and stream stickleback.

Certainly facilitating divergence between the ecotypes from the two habitats, this sexual barrier remains incomplete despite strong differences between the ecotypes in multiple traits generally believed to be important to reproductive behaviour. This highlights that the strength of sexual isolation between diverging taxa is difficult to predict and should be measured directly. Future studies should now identify the mechanism(s) underlying assortative mating and quantify the relative contribution of male vs. female reproductive behaviour to sexual isolation. Details on dispersal behaviour and breeding ranges would further allow appraising the importance of sexual and other reproductive barriers to total isolation between lake and stream stickleback. Finally, the comparison of sexual isolation among population pairs in different spatial contexts using similar methodology may reveal the influence of geography on the opportunity for sexual barriers to evolve.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article: **Figure S1** Photographs of the experimental mesocosm facility.

Figure S2 Photograph of a representative stickleback nest.

Appendix S1 Summary of the test of assortative mating.

Appendix S2 Summary of the two tests of an influence of body size on assortative mating.

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