Habitat Choice and Female Preference in a Polymorphic Stickleback Population

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ABSTRACT

Background: A small pond, c. 90 years old, near Bern, Switzerland contains a population of threespine stickleback (Gasterosteus aculeatus) with two distinct male phenotypes. Males of one type are large, and red, and nest in the shallow littoral zone. The males of the other are small and orange, and nest offshore at slightly greater depth. The females in this population are phenotypically highly variable but cannot easily be assigned to either male type.

Question: Is the existence of two sympatric male morphs maintained by substrate-associated male nest site choice and facilitated by female mate preferences?

Organisms: Male stickleback caught individually at their breeding sites. Females caught with minnow traps.

Methods: In experimental tanks, we simulated the slope and substrate of the two nesting habitats. We then placed individual males in a tank and observed in which habitat the male would build his nest. In a simultaneous two-stimulus choice design, we gave females the choice between a large, red male and a small, orange one. We measured female morphology and used linear mixed effect models to determine whether female preference correlated with female morphology.

Results: Both red and orange males preferred nesting in the habitat that simulated the slightly deeper offshore condition. This is the habitat occupied by the small, orange males in the pond itself. The proportion of females that chose a small orange male was similar to that which chose a large red male. Several aspects of female phenotype correlated with the male type that a female preferred.

Keywords: Gasterosteus aculeatus, behavioral mate choice, color polymorphism, sympatric divergence
INTRODUCTION

The evolution of reproductive isolation between populations, eventually leading to fully isolated distinct species, may be driven by many factors including natural and sexual selection and may most often require geographic isolation (Coyne & Orr, 2004). For speciation to happen with gene flow in the absence of geographic isolation however, strong disruptive selection and assortative mating are needed (Bolnick & Fitzpatrick, 2007). Disruptive sexual selection is thought to be more powerful than disruptive natural selection in promoting speciation, because it automatically leads to non-random mating and thus to the coupling of a diverging force and reproductive isolation (Kirkpatrick & Ravigne, 2002). Disruptive sexual and natural selection acting in concert may be even more powerful, as ecological differentiation is probably a pre-requisite to the coexistence of two incipient species in sympatry (Maan & Seehausen, 2011). As the whole speciation process usually cannot be observed within a human lifetime, the study of its driving forces often focuses on ecotypes or sexual morphs (e.g. male color polymorphism, Gray & McKinnon, 2007) at various stages of divergence that may, or may not, ultimately become largely reproductively isolated species (Nosil et al., 2009).

The threespine stickleback (Gasterosteus aculeatus species complex) is an important model system in evolutionary biology that helps us to understand the evolution of reproductive isolation between divergently adapted populations or within polymorphic populations (McKinnon & Rundle, 2002; Kitano et al., 2009). Since the last glacial retreat ~10-15 kyrs ago, ancestral marine stickleback have repeatedly colonized distinct freshwater habitats and subsequently adapted therein, leading to divergence in many traits (McKinnon & Rundle, 2002; Hendry et al., 2013) and to variable degrees of reproductive isolation among the evolved ecotypes (Boughman, 2001; McKinnon & Rundle, 2002; Boughman et al., 2005;
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Hendry et al., 2013). The evolution of reproductive isolation between distinct stickleback
ecotypes can be driven by divergent natural selection (Rundle et al., 2000; Arnegard et al.,
2014) and/or through ecologically-dependent sexual selection (Boughman, 2001; Cooper et
al., 2011).

Although many distinct stickleback ecotypes have been described that occupy a wide
range of habitats including streams, lakes and the marine environment, very few stickleback
ecotypes are known to coexist in sympatry, suggesting that disruptive selection may often not
be sufficient to initiate speciation in stickleback (Hendry et al., 2009; Bolnick, 2011) or that
assortative mating often does not evolve (Raeymaekers et al., 2010; Räsänen et al., 2012;
Seehausen & Wagner, 2014). Cases of fully sympatric ecotypes are described from eight
lakes in British Columbia, Canada (Gow et al., 2008), from lakes in Alaska (Cresko & Baker,
1996), Far Eastern Russia (Ziuganov, 1995) and Iceland (Kristjánsson et al., 2002a;
Ólafsdóttir et al., 2006). Two of the Canadian species pairs have been studied in great detail
and in these cases it appears that strong disruptive natural selection and environmentally-
dependent sexual selection together led to reproductive isolation between coexisting benthic
and limnetic stickleback species (McPhail, 1984; Hatfield & Schluter, 1996; Hatfield &
Schluter, 1999; Boughman, 2001; Conte & Schluter, 2013; Arnegard et al., 2014).

Here we study a case of sympatric polymorphism within a large pond, the Jordeweiher
(Zeller et al., 2012; Marques et al., in review), which lies in the invasive range of stickleback
in Switzerland (Lucek et al., 2010; Roy et al., 2015). This pond, measuring approximately
50x60m with a maximum depth of just three meters, harbors two distinct male stickleback
phenotypes (Marques et al., in review): “Nearshore” males breed on steep clay-like shore
substrate under overhanging trees, where they build concealed nests with small entries, show
a deep red throat coloration and are large and more deep-bodied (Figs. 1b, 1d). “Offshore”
males breed on the flat, muddy bottom of the open pond, build large, open “crater”-like nests
and show an orange throat coloration as well as a pale, almost white body pigmentation
during the breeding season (Figs. 1a, 1c). In contrast to the territorial males that strongly
differ in their nuptial coloration and nest habitat, females cannot readily be assigned to either
phenotype although large variation in female size and shape exists. Given the small
geographic scale and the lack of evidence for dietary differentiation even among males
(Marques et al., in review), females are likely to explore all habitats and thus encounter both
male types. Consequently, the diverging phenotypes occur in full sympatry as defined by
Gavrilets (2003), when ‘mating is random in respect to the place of birth of the mating
partner’ (p. 2198; Gavrilets, 2003). Importantly, these phenotypes must have evolved only
recently, because stickleback were introduced in the central parts of Switzerland in the 1920s
(Lucek et al., 2010). The Jordeweiher population is composed of mitochondrial haplotypes of
two distinct genetic lineages originating from different parts of Europe that have formed a
large hybrid zone in the Swiss midlands including the Jordeweiher pond (population 'EYM' in
Roy et al., 2015).

This study aims to explore two behavioral aspects that could lead to the evolution of
reproductive isolation associated with divergence in male phenotypes: male nest site habitat
choice and female mate preferences. First, we investigate the role of two characteristics of the
nest site habitats, substrate and slope, in mediating the male’s choice of a nesting site in the
absence of competition. Two scenarios could explain why the two color types breed in
different habitats: Competitive exclusion, in which the dominant type would exclude the
subdominant type from the habitat preferred by both, or habitat matching, when the two male
types choose alternative nest habitats that best match their phenotype(Edelaar et al., 2008;
Bolnick et al., 2009). Larger and redder males have been shown to be dominant in other
stickleback populations (Bakker & Sevenster, 1983; Östlund-Nilsson, 2007) and shallower
habitat to be preferred in some populations (Bolnick et al., 2015). If competitive exclusion
was driving the observed distribution of males in the pond, all males should build nests in the
simulated nearshore habitat in an experimental setup without competition. In contrast, under a
habitat-matching scenario, we expected both male types to build their nests in the experimental habitat that matches their wild type habitat best, an optimum presumably influenced by substrate color relevant to camouflage and sexual signaling against different backgrounds (Reimchen, 1989).

Second, we studied the distribution of female preferences for one or the other male type using a simultaneous two-stimulus choice design (Fig. 2). Many previous studies have demonstrated the importance of male nuptial coloration, body size and body shape for female choice (e.g. Reimchen, 1989; Milinski & Bakker, 1990; Nagel & Schluter, 1998; Boughman, 2001; Boughman et al., 2005; Conte & Schluter, 2013; Head et al., 2013). Several studies on threespine stickleback found directional selection for males with bright red throats (Milinski & Bakker, 1990; Bakker & Mundwiler, 1994; Baube et al., 1995; Cubillos & Guderley, 2000; Flamarique et al., 2013), but others found divergent female mate preferences for alternative male morphs in sympatric benthic and limnetic stickleback species pairs (Boughman, 2001; Boughman et al., 2005). We tested whether the distribution of female mating preferences in the Jordeweiher pond was compatible with either directional selection for redder males or with divergent selection for alternative male phenotypes. Furthermore, body size and body shape matching between males and females have been shown to be additional important components of reproductive isolation between sympatric limnetic and benthic stickleback species (Nagel & Schluter, 1998; Kraak et al., 1999; Conte & Schluter, 2013; Head et al., 2013). We thus also tested whether females prefer similarly-sized males. Finally, we assessed whether females with different preferences differed in linear morphological traits or in body shape.

By elucidating the role of male habitat choice and female mate preference among the strikingly phenotypically divergent Jordeweiher stickleback morphs (Fig. 1), we aim to estimate the potential for behavioral reproductive isolation to evolve in association with sympatric differentiation among male phenotypes.
METHODS

Fish collection

We conducted male nest site habitat choice experiments in two years, 2011 and 2015, and the female preference experiment in one year, 2015. In 2011, we caught 16 nearshore and 10 offshore males between May 16th and May 23rd in the Jordeweiher pond (Wohlen, Bern, Switzerland, 46°57′24″ N, 7°23′21″ E) using unbaited minnow traps placed in the respective habitats. In 2015, ten males of each type were caught at their breeding sites with hand nets during scuba diving between May 15th and May 22nd. Only adult males in full breeding coloration from both years and only males that showed territorial or nesting behavior (digging, transfer of material, fanning or guarding) in the pond in 2015 were used for the male habitat choice experiment. We refer to males as ‘offshore’ or ‘nearshore’ males in the remainder of our study, depending on the habitat where they were captured.

For the female preference experiment, we caught 65 gravid females using unbaited minnow traps between May 15th and June 26th 2015 and kept them in groups of maximum 18 fish in 72 liter tanks. An additional 20 gravid females were caught for the stimulation of nest building in the habitat choice experiments. Following the experimental trials, all males from 2011 and females caught in 2015 were released back into the Jordeweiher, while the 20 males caught in 2015 were anesthetized and euthanized in a clove oil solution in accordance with granted permits. Fish collection, experimentation and euthanasia followed the Swiss fisheries and veterinary legislation in concordance with the federal food safety and veterinary office (FSVO), the cantonal veterinary office in Bern (Veterinärdienst Kanton Bern, permit numbers BE57/11 and BE66/13), the fishery authorities of the canton Bern and the fishery rights owner, Augsburger AG, Hinterkappelen, Switzerland.

Morphological measurements
We quantified male nuptial coloration of males caught in 2015 from standardized cuvette photographs. For this, stickleback were transferred into a plexiglas cuvette, transferred into a box lined with black velvet to exclude unwanted ambient light sources and photographed with an SLR camera (Canon 7D, Canon, Japan), mounted with a 85 mm lens and illuminated by two external flashes from both sides of the camera lens. To control for potential plasticity in male coloration, we photographed each male at several time points during the experiments. First, we took underwater photos (Canon PowerShot D10, Canon, Japan) of each male taken in its breeding habitat in the pond. We used these underwater photos to qualitatively assess potential color changes between capture and taking cuvette pictures and did not detect any qualitative change in throat color in this short period. Immediately after capture, no later than two minutes after the male had been captured under water, we took the first standardized cuvette photograph. A second cuvette photograph was taken after completing the habitat choice experiment when males had spent 22 ± 9 SD days in the aquaria. In addition, males used in the female mate preference trials (see below) were photographed once more, after completion of the mate preference trials, 14 (pair 60/62) and 24 days (other male pairs) after the last cuvette photograph. We also photographed females immediately after the trials, using the same standardized setup.

We measured throat coloration (hue) of each male on standardized cuvette pictures by measuring median red, green and blue (RGB) values from pixels in a 1mm² circle below the eye in a well-lit area consisting mostly of erythrophores and lacking melanophores (i.e. dark spots) using ImageJ v1.49 (Schneider et al., 2012). Based on median RGB values, we calculated the median hue angle for throat coloration of each male, using the formula $h_{\text{Preucil}} = 60^\circ \times (G-B) / (R-B)$ applicable to RGB values with $R \geq G \geq B$ (Preucil, 1953). Differences in coloration between wild caught nearshore and offshore individuals were compared using a Kruskal-Wallis test, and differences between time points (i.e. after capturing and after the habitat choice experiment) were compared using a paired $t$-test.
We placed 33 landmarks on the best photograph of each fish using tpsDig v2.17 (Rohlf, 2015) to obtain measurements for 16 linear traits (Fig. 3b, Table 1). For all females analyzed in the female mate preference experiment, we also used a subset of the landmarks (Fig. 3a) to analyze body shape (landmarks 1, 10-15), head shape (landmarks 1-5), body and head shape combined (landmarks 1-5, 10-15) and body, head and eye shape combined (landmarks 1-15) in MorphoJ 1.06d (Klingenberg, 2011). We tested whether allometric slopes for each linear trait differed between male color morphs in an ANCOVA with standard length as independent variable, the trait as dependent variable and morph as covariate. As we did not find significant differences in slopes between male color morphs, we assumed that slopes would be uniform among the females and performed a size correction with all females combined for linear traits in R 3.2.1 (R Development Core Team, 2013) and for shape in MorphoJ by taking the residuals of a linear regression of the respective linear trait or shapes against standard length (Reist, 1980).

**Male habitat choice experiment**

The male habitat choice experiment was divided into two parts, testing first for substrate only and then including a substrate-slope combination that simulates the two nesting environments in the pond. The experiment focusing solely on substrate was conducted in 2011. Here, each male was placed in an individual 72 liter tank containing two flower pots at opposite ends of the tank. One pot was filled with 8cm of sand, covered by 5cm of mud from the Jordeweiher pond, simulating the offshore substrate. The second pot was filled with sand and covered by tree leaves from the pond, simulating the nearshore substrate. The second experiment simulating both differences in substrate and slope was conducted in 2015, where each male was transferred into a 72 liter tank divided into two equally-sized compartments: One compartment contained a flat, ~6cm thick layer of substrate taken from the middle of the Jordeweiher pond, simulating the offshore habitat (Fig. 2a). The other compartment,
simulating the nearshore habitat, contained substrate taken from the shore of Jordeweiher that
was set along a 36° angle from the horizontal, representing the average angle at which
nearshore males build nests in the Jordeweiher (D. A. Marques, unpublished data).

All tanks were visually isolated from each other with black plastic sheets to avoid
interactions among males. Airflow and light conditions were standardized for all tanks,
simulating an 18 hours day. For the habitat choice experiment, we installed a fluorescent tube
lamp with a color temperature of 2,700 K (Philips, Amsterdam, The Netherlands) over each
tank, 10cm above the water surface, in the middle of a tank parallel to its long side (Fig. 2a).
The fish were fed daily with frozen chironomid larvae. The males were given one day of
acclimatization in the tanks. On the second day, we stimulated each male to build a nest by
visually presenting a randomly selected gravid female for five minutes (following Frommen
& Bakker, 2006). On the third day, a female was submerged into the male tank in a perforated
plastic jar to allow stimulation by olfactory cues. Males that had not built a nest after the first
two stimulations were subsequently stimulated every second day, alternating between visual
only and visual plus olfactory stimulations, until they had built a nest or until a maximum of
four weeks after the start of the experiment.

A nesting trial was deemed to be successful and the chosen substrate subsequently
recorded, once a nest was clearly visible or when a male showed repeated nesting behavior
including digging, transfer of material, gluing or fanning in the same place (Östlund-Nilsson,
2007). We tested if male habitat choice was expected by chance (p = 0.5 due to equal area
size of both habitats) or if one habitat was preferred over the other by performing binomial
tests in R.

**Female mate choice experiment**

We measured female mate preference following the protocol of Frommen and Bakker
(2006), using a simultaneous two-stimulus setup, which allows the detection of directional
biases in mating preferences on a population level. A set of five 96 liter tanks were each divided into two compartments measuring 29x40x30cm using perforated clear plastic allowing exchange of both olfactory and visual cues between both compartments (Fig. 2b).

One compartment was further subdivided into two parts, each measuring 29x19x30cm using non-transparent dark grey plastic. The tank was moreover visually isolated from the surroundings by opaque black plastic sheets to control for external stimuli. We simulated the same light condition as for the nesting experiment, with the difference that the light source was placed perpendicular to the tank’s long edge at its rear edge over the male’s nests (Fig. 2b). A video camera above each tank recorded each experimental trial. Following successful nest building in the habitat choice experiment, we transferred ten males (five nearshore and five offshore, producing five male pair combinations) together with their nests to the smaller compartments of the test tank (one male of each morph per test tank) and gave them an acclimatization time of one day. We checked whether males continued to care for their nests after transfer and found that all males either started repairing the transferred nest or building a new nest immediately or after the first exposure to a female. The large compartment was visually isolated from the male compartments using removable non-transparent plastic. We chose each pair of males to represent typical nearshore and offshore phenotypes based on their location in the pond, body size and throat coloration. Each female preference trial started by placing a female in the large compartment, allowing 30 min of acclimatization time. After acclimatization, the opaque plastic partition was lifted and the interactions were filmed for 30-50 min. We analyzed 29 min of each trial, after i) both males had seen the female, ii) both males showed courtship behavior (zig-zag dance and attempts at biting the female; Östlund-Nilsson, 2007), and iii) the female had entered the second of both ‘contact zones’, a 7x19cm area in front of each male compartment (Fig. 2b).

In the female mate preference experiment, none of the 65 females used in the experiment had previously been exposed to any of the males during captivity and each female
was tested only once with a single male pair. The five male pairs were used multiple times and each saw 10-14 different females (pair 60/62: 10 females; 66/71: 14 females; 67/76: 14 females; 61/78: 13 females; 70/75: 14 females). We assessed the gravidity of each female after a trial by gently applying pressure to the abdomen, which resulted in eggs extruding if the female was gravid (Nagel & Schluter, 1998). We only considered a trial successful if a female was gravid and spent at least 30% of the trial time in the contact zones in front of the two males. Applying these conditions, we considered 37 trials for analysis (60/62: 6 females; 66/71: 9 females; 67/76: 5 females; 61/78: 7 females; 70/75: 10 females). For each trial, we measured the time a female spent in front of either male, starting after the female had inspected both males, as well as the time she spent outside the contact zones using JWatcherTM v1.0 (Blumstein et al., 2006). We also qualitatively confirmed that both males in a pair courted the females during the experimental trial. We observed that the first reactions of the males towards the female occurred within 2-3 minutes (5 minutes in one trial) after exposure, and this was followed by relentless courting behavior. A preference score for nearshore males (NPS) was calculated as the proportion of the total time in the contact zones a female spent in front of the nearshore male (preference score for offshore male = 1 - NPS). We tested the distribution of female mate preferences for multimodality using the dynamic tree cut clustering method (Langfelder et al., 2008). This method identifies clusters based on an Euclidean distance tree among data points without prior assumptions of the number of inferred clusters and thus provides an unbiased estimate for the number of distinct clusters or modes present in a given dataset (see Langfelder et al., 2008 for details). The parameters used for this method were 10 individuals as minimal cluster size, a maximal scatter of 0.75 and a maximal distance of 0.90 for assignment. To test if female mate preferences can be predicted by any of the measured linear or shape traits, we used linear mixed effect models, testing for relationships between NPS and each linear trait separately as well as between NPS and the first five principal component
(PC) axes for all size corrected linear traits combined and for the four body shape combinations (head, body, head+body, head+eye+body shape), respectively. We entered size-corrected trait values as predictors into the model and used male pairs as a random effect. To account for multiple testing of the same hypothesis, p-values for the linear mixed effect models were corrected using a false discovery rate correction. We further performed binomial tests to infer whether females would prefer the larger male or the males closer to their own size. Discriminant function analyses on the four body shape categories were conducted to test if the females that preferred either male phenotype (NPS <0.5 or >0.5 respectively) could be distinguished by their own body shape. Significance levels were estimated using a permutation test with 1,000 replicates as implemented in MorphoJ (Klingenberg, 2011).

RESULTS

Male phenotypes

Throat coloration, estimated by the median hue angle, differed significantly between nearshore and offshore males (Kruskal-Wallis test, $\chi^2 = 9.61, P = 0.002$) and remained different between the two groups after males were transferred from the field to standardized substrate and light conditions in aquaria ($\chi^2 = 6.0, P = 0.014$, Fig. 4a). While on average, male throat coloration did not change during the male nest habitat choice experiment (i.e. between ‘aquaria’ and ‘post-experiment’ in Fig. 4a, paired t-test, $t_{1,18} = 0.6, P = 0.542$), all males shifted towards slightly higher hue values over the course of the female mate preference experiment (paired t-test, $t_{1,9} = 3.0, P = 0.015$, Fig. 4a). However, in all male pairs used in the female preference trials, the nearshore males had redder throats than the offshore males (Kruskal-Wallis test, $\chi^2 = 6.82, P = 0.009$, Fig. 4a). Average standard length was not significantly different between the male types (t-test, $t_{1,18} = 1.6, P = 0.127$, Fig. 4b), but all but one nearshore male were larger than their paired offshore male in the female mate preference trials (Fig. 4b).
Male nest habitat choice

We first tested for male habitat choice based on a single habitat characteristic: substrate. 12 nearshore and five offshore males built their nest on the muddy, offshore-like substrate, while the remaining four nearshore and five offshore males failed to build a nest. No male of either type built its nest on the nearshore type substrate. Consequently, the offshore type substrate was clearly preferred overall (binomial test, $P < 0.001$) and no difference was observed between the male types.

We then tested for male habitat choice based on two habitat characteristics: substrate and slope. Six offshore and six nearshore males built their nests in the flat offshore-like compartment of the experimental tank and one offshore and one nearshore male each built their nests in the steep, nearshore-like compartment, while three males of each type failed to build a nest. Consequently, offshore-like habitat was again clearly preferred overall (binomial test, $P = 0.013$) and no difference was observed between the male types.

Female mate preferences

We observed considerable variation in female mate preference with many females showing strong preferences for the male of either one or the other type (Fig. 5a), suggesting a broad distribution of female mate preferences across the entire population. The dynamic tree cut method supported two modes in the distribution of preference scores, one mode comprised of females clearly preferring the orange offshore male (mode 1: mean NPS = 0.21 ± 0.14 SD, Fig. 5a) and another mode for females without strong preferences, but with a tendency towards choosing the red nearshore male (mode 2: mean NPS = 0.66 ± 0.13 SD, Fig. 5a). We did not detect any preference for larger males (binomial test, $P = 0.511$), nor for the males that are more similar in body size to the choosing female (binomial test, $P = 0.511$).

Females preferring red nearshore males showed larger eyes and a longer head than females preferring orange offshore males, suggested by significant positive associations.
between eye diameter, eye area, head length and NPS in females (Table 1). When all size-corrected linear traits were combined into a principal component analysis (Fig. 5b), PC1, explaining 35.9% of the total variance, showed a significant association with NPS (Table 1, Fig. 5b). Again, PC1 is dominated by eye area, eye diameter and head length (Fig. 5b), confirming the association of female preference with these traits.

In contrast to linear traits, we did not find any associations between female shape traits and NPS (shape PCs, all \( P > 0.1 \), results not shown). Similarly, a discriminant analysis of shape traits failed to separate females that preferred offshore orange males (NPS < 0.5) from females that preferred nearshore red males (NPS > 0.5) based on body shape, head shape or eye shape (Hotelling’s \( T^2_{body} = 10.6, P = 0.60; T^2_{head} = 6.4, P = 0.50, T^2_{body+head} = 33.5, P = 0.54, T^2_{body+head+eyes} = 84.2, P = 0.59 \)). The same was true when we considered only females with stronger preferences (i.e. NPS > 0.6 and < 0.4 respectively; results not shown). We also tested whether females in the two modes of the preference distribution could be distinguished on body shape with a discriminant analysis, but could not detect significant differences in body shape between females preferring offshore orange males (NPS < 0.4) and females with intermediate to nearshore red-biased preferences (NPS > 0.4, \( T^2_{body} = 10.8, P = 0.63; T^2_{head} = 3.0, P = 0.86, T^2_{body+head} = 28.5, P = 0.67, T^2_{body+head+eyes} = 126.0, P = 0.30 \)).

**DISCUSSION**

Studying a population of threespine stickleback that is less than 90 years old, where two phenotypically distinct male morphs breed in sympatry, we have explored two behavioral traits with relevance for possible reproductive isolation between the morphs (habitat choice of nesting males and female mate preferences). We found that male nest habitat choice could not be experimentally replicated based on two factors, substrate and slope, that both differ among nesting habitats in nature and could be simulated in our aquaria. In the absence of intra- and interspecific interactions, all males preferred to build their nest in the offshore-like habitat.
regardless of male phenotype in our experiment. This outcome is contrary to our predictions
both under a competitive exclusion scenario and under a habitat matching scenario (Edelaar et
al., 2008): In the competitive exclusion scenario, we expected all males would breed in the
nearshore-like experimental habitat, assuming that larger / redder nearshore males may be
dominant and thus occupy the preferred habitat in the pond. In the habitat matching scenario,
we expected males of the two morphs to breed in the habitat that mimicked their nest habitat
in the pond. Taken as such, our results could suggest that the redder and larger nearshore
males might not actually be dominant over the orange, smaller males in nature in the
Jordeweiher population and that the nearshore habitat of red males may be the less preferred
habitat. This would be in contrast to expectations from studies on other stickleback
populations that demonstrated the dominance of larger and brighter red males over smaller
and duller red males (Bakker & Sevenster, 1983; Östlund-Nilsson, 2007) and a study that
showed male preference for shallower habitats (Bolnick et al., 2015).

Alternatively, our results could suggest that other habitat characteristics, besides
substrate and slope, which were not tested in our experiment may be equally or more
important to male habitat choice, e.g. divergent light conditions, habitat complexity or water
depth (Candolin & Voigt, 2003; Bolnick et al., 2015). With available experimental tanks of
0.3m depth, water depth in our experiment deviated from natural conditions in the pond
where males breed in 0.5-3m depth (Marques et al., in review). Bolnick et al. (2015) showed
recently that water depth can be a very strong predictor for male mating success and thus,
males in our experiment may have chosen the offshore-like habitat because it was the habitat
closer to the natural situation in water depth for both morphs. Given that the Jordeweiher
pond experiences occasional water level fluctuation due to hydropower usage, depth may also
play a role in assessing the risk of losing a nest: nests placed in very shallow water are at risk
to be lost. Another factor not tested in our experiment, which is also strongly influenced by
water depth and has important consequences for social signaling (Seehausen et al., 2008), is
light environment: While we used standardized light conditions in the experiment, the nearshore habitat in the pond is characterized by a dynamic, heterogeneous light environment caused by overhanging trees, roots and branches. The light in our experimental setup thus may have resembled more the offshore part in the pond in that respect, which receives direct sunlight all day, while in respect to water depth, the light spectrum may have been closer to the shallower nearshore part. Habitat complexity, another factor not incorporated in our experiment, also varies in the pond, as roots, branches and leaf litter is largely restricted to nearshore habitat, but were not present in our nearshore habitat model. Enclosure transplant experiments are needed to test whether male nest habitat choice can be recreated by embracing all the factors that differ between the two alternative habitats.

Often, the distribution of individuals within a habitat is the outcome of conflicting demands, such as predator avoidance, food availability and favorable conditions for reproduction (Candolin & Voigt, 2003). Predation and habitat-dependent sexual signaling may have shaped male habitat preferences in the Jordeweiher stickleback population. The substrate of the nearshore region is darker and shows more structural complexity and may thus allow better concealment from visual predators, but at the same time is closer to the water surface, where predatory birds would have access. The offshore habitat on the other hand is deeper and thus protected from the predatory birds but more open and thus vulnerable to fish predation. However, predation by birds (Common Kingfisher *Alcedo atthis*, Grey Heron *Ardea cinerea*) and fish (single sightings of trout *Salmo trutta* and Northern Pike *Esox Lucius*) may be negligible in this pond as opposed to nearby streams (Zeller *et al.*, 2012). Furthermore, the predation pressure by large dragonfly larvae (*Anax* and *Aeshna* sp.), the dominant predators in the pond, may not differ between habitats. Habitat-dependent sexual signaling may be mainly influenced by light environment and substrate color in the two habitats, selecting males to maximize visibility to females (Reimchen, 1989), likely in a trade-off with camouflage against different backgrounds protecting the males from predators.
Females from the Jordeweiher pond showed a broad distribution of mate preferences when given one orange offshore and one red nearshore male to choose from, ranging from individuals clearly favoring the offshore males to those clearly favoring the nearshore males, while others lacked a preference for either type. Two distinct modes occur, one with females preferring orange offshore males and the other with less choosy females that tend to prefer redder nearshore males (Fig. 5a). Maybe most surprisingly however, and different from other studies that used populations just a few kilometers downstream of the Jordeweiher (Milinski & Bakker, 1990), we found no general preference for the redder of two males and hence, there seems to be no directional sexual selection on red coloration in this pond population. Rather, our results are compatible with the presence of a mate preference polymorphism among females. In our experiment, we have not quantified the possible environment-dependence of such mate preferences, but the lack of a clear preference mode for red nearshore males suggests that the experimental conditions may have favored orange offshore males. Indeed, the light environment in the experimental setup with direct light and a flat floor, may have more closely resembled the offshore environment, potentially undermining the expression of a stronger preference for red nearshore males.

We did not find evidence for size matching mate preferences in females nor for preferences towards the larger of two males. Hence, one mechanism that has been shown to facilitate reproductive isolation between sympatric stickleback ecotypes, namely size-assortative mating (McKinnon et al., 2004; Boughman et al., 2005; Conte & Schluter, 2013; Head et al., 2013) seems unlikely to operate in this population. Interestingly, we found that females with different mate preferences also differed in morphology: females with smaller eyes and shorter heads seemed to prefer orange offshore males. In other populations these traits have been shown to be associated with differences in feeding behavior between sympatric species of stickleback (Schluter, 1993), where they may reflect some assortative mating related to feeding (Snowberg & Bolnick, 2008; Bolnick & Paull, 2009).
association between preferences for either of two male color morphs and the female’s morphology in the Jordeweiher population could thus indicate the presence of some level of assortative mating in this system. Indeed, in another study on the Jordeweiher stickleback, we found that orange males have on average shorter heads and marginally smaller eyes (corrected for body size; Marques et al., in review), suggesting a correlation of male color and morphology with female morphology and preferences.

Are the Jordeweiher stickleback undergoing sensory drive speciation? Sensory drive speciation is characterized by i) divergence in male sexual signaling trait ii) divergence in female preferences and iii) the environment-dependence of i and ii (Boughman, 2002; Seehausen et al., 2008). In the Jordeweiher, we have indications for environment-dependence as male throat color was consistently associated with nearshore and offshore habitat. In another study, we found differentiation at several places in the genome correlated with these color morphs (Marques et al., in review), suggesting a possible genetic basis for this color polymorphism. Here, we showed a broad and bimodal distribution of female mating preferences and an association between female phenotype and female mating preference for two male morphs (Fig. 5, Table 1). If both male color traits and female preferences are heritable and are under environment-dependent selection, a sensory drive mechanism could promote the evolution of reproductive isolation in our studied population, consistent with incipient sympatric speciation. Whether female preferences are heritable and whether they are environment-dependent or have evolved under environment-dependent selection needs further investigation. Also, the role of the hybrid swarm origin of this population (Lucek et al., 2010; Roy et al., 2015) in generating variation in male traits, habitat choice and female preferences or potential correlations between these requires further analyses.

Conclusions
Nest site habitat choice of two sympatric male color morphs of stickleback with distinct nesting habits in nature could not be reproduced in our aquarium experiment, based on two factors, substrate and slope. This suggests that nest site choice in nature may be determined by additional factors, such as water depth, light environment, habitat complexity and intra- and interspecific interactions. At the same time, our experiments revealed a broad and bimodal female mate preference distribution in this population and identified significant associations between female morphology (eye size, head length) and mating preference for males of either color morph. We may underestimated potential assortative mating with this experimental setup, by not taking into account nest and habitat differences among males, or aspects of phenotype that depend on these differences (e.g. phenotypically plastic light/dark body coloration, relative contrast of nuptial coloration against the background). However, our results indicate the presence of some assortative mating in this population, consistent with an incipient stage of sympatric speciation. Future experiments using field enclosures might help to better quantify the presence of male- and habitat-assortative mating and their potential for evolving reproductive isolation in sympatry.

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Ziuganov, V.V. 1995. Reproductive Isolation Among Lateral Plate Phenotypes (Low, Partial, Complete) of the Threespine Stickleback, Gasterosteus Aculeatus, From the White Sea Basin and the Kamchatka Peninsula, Russia. *Behaviour*, **132**: 1173-1181.
### Table 1. Morphological linear traits measured in females and tests of associations between nearshore preference score (NPS) and each linear trait and their aggregate PC scores. The linear mixed-effect model fixed effect regression coefficients ($\beta_{\text{trait}}$) and associated test statistics are given. P-values significant after correcting for multiple testing using a false discovery rate correction are shown in bold and highlighted with a single asterisk.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Abbr.</th>
<th>Linear mixed-effect model statistics</th>
<th>Pearson's correlation coefficient</th>
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<td></td>
<td>$\beta_{\text{trait}}$</td>
<td>$t_{2,31}$</td>
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<td>All traits PC1</td>
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<td>All traits PC5</td>
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FIGURES

Figure 1. Breeding threespine stickleback males from the Jordeweiher pond near Bern, Switzerland. ‘Offshore’ (a, c) and ‘nearshore’ (b, d) males are shown in the respective habitats (c, d) and on color-standardized photographs (a, b). Note the difference in throat and body color as well as the different habitats these male types breed in. In (c), the offshore male guards its large deep and open crater nest on the pond bottom, while the nearshore male’s nest in (d) is hidden under branches and leaves.

Figure 2. Experimental setup for the (a) male habitat choice experiment and the (b) female mate preference experiment. In (a), single males could choose to build their nests in a steep part with nearshore substrate (brown) or a flat part with offshore mud substrate (light brown). In (b), females could simultaneously choose between two males – one offshore and one nearshore in separate compartments, visually isolated from each other. Holes in the plexiglas divider to the female’s compartment allowed for olfactory exchange. Each male had a nest in its compartment (green circle). The time spent in a contact zone (blue areas) in front of each male’s compartment was used to assess female mate preferences. Light sources in the two settings are indicated by yellow tubes.

Figure 3. (a) Standardized cuvette photograph of a female with the 15 landmarks used for geometric morphometrics in this study are indicated in red. (b) Linear measurements used in this study: standard length (SL), head length (HL), snout length (SnL), eye diameter (ED), eye area (EA), upper jaw length (UJL), first spine length (FSL), second spine length (SSL), pelvic spine length (PSL), body depth at first spine (BD1) and second spine (BD2), basal length of dorsal fin (BLD) and anal fin (BLA), caudal peduncle depth (CPD) and length (CPL) and total length of the pectoral fin (TLP).
Figure 4. (a) Throat coloration of the 20 males caught in 2015 did not change between measurements taken directly after capture (‘pond’), after the male nest habitat choice experiment, (‘aquaria’, after 22 ± 9 SD days under standardized light conditions) and after the female mate preference experiment (‘post-exp.’, 24 days and 14 days later for four male pairs and pair 60/62, respectively). The five male pairs used in the preference trials differed consistently in throat coloration. (b) Average body size did not significantly differ between nearshore and offshore males caught in 2015. Among the male pairs used in the female preference experiment the nearshore male was larger than the offshore male in all but one male pair.

Figure 5: (a) Kernel density function of the female preference score (quantified as the preference for the nearshore male, NPS) across the 37 wild caught females that we tested. Kernel densities are shown for all individuals combined (black line) or separately for each identified multivariate mode (grey dashed lines). Each data point is one female, grouped by the five male pairs used in the trials (symbols). (b) PCA and trait loadings of all size-corrected linear traits among the 37 females used in the mate preference experiment. Arrows indicate the loadings of each linear trait and point colors indicate the female’s preference score for nearshore or offshore males, as shown in the color bar at the x-axis in (a).
(a) Kernel densities
- all females
- mode 1
- mode 2

(b) Loadings PC1 and PC2
- PC1 [35.9%]
- PC2 [19.4%]

Male preference score (NPS)
- nearshore preference score

Density
- all females
- mode 1
- mode 2

PC1 loadings
- HL
- SnL
- ED
- EA
- UJL
- FSL
- SSL
- PSL
- BD1
- BD2
- BLD
- BLA
- CPD
- CPL
- TLP

PC2 loadings