
Habitat choice and female preference in a polymorphic stickleback population

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ABSTRACT

Background: A small pond near Bern, Switzerland that is about 90 years old 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. Males of the other type 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. Female stickleback caught with minnow traps.

Methods: In experimental tanks, we simulated the slope and substrate of the two nesting habitats. Males were placed individually in a tank and we observed in which habitat they chose to build their 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. Females showed a broad and bimodal preference distribution, with one group of females choosing the small, orange male and the other females showing a weak tendency to prefer the large, red male. Several aspects of female phenotype correlated with the male type that a female preferred.

Keywords: behavioural mate choice, colour polymorphism, *Gasterosteus aculeatus*, sympatric divergence, threespine stickleback.

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

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selection and may most often require geographic isolation (Coyne and Orr, 2004). For speciation to happen with gene flow in the absence of geographic isolation, however, strong disruptive selection and assortative mating are necessary (Bolnick and 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 and Ravigne, 2002). Disruptive sexual and natural selection acting in concert may be even more powerful, as ecological differentiation is probably a prerequisite to the co-existence of two incipient species in sympatry (Maan and 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 colour polymorphism (Gray and McKinnon, 2007)] at various stages of divergence, which 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, as it helps us to understand the evolution of reproductive isolation between divergently adapted populations or within polymorphic populations (McKinnon and Rundle, 2002; Kitano *et al.*, 2009). Since the last glacial retreat (*c.* 10,000–15,000 years ago), ancestral marine stickleback have repeatedly colonized distinct freshwater habitats and subsequently adapted therein, leading to divergence in many traits (McKinnon and Rundle, 2002; Hendry *et al.*, 2013) and to variable degrees of reproductive isolation among the evolved ecotypes (Boughman, 2001; McKinnon and Rundle, 2002; Boughman *et al.*, 2005; 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 co-exist in sympatry, suggesting that disruptive selection may often not be sufficient to initiate speciation in sticklebacks (Hendry *et al.*, 2009; Bolnick, 2011) or that assortative mating often does not evolve (Raeymaekers *et al.*, 2010; Räsänen *et al.*, 2012; Seehausen and Wagner, 2014). Cases of fully sympatric ecotypes are described from eight lakes in British Columbia, Canada (Gow *et al.*, 2008), and from lakes in Alaska (Cresko and Baker, 1996), Far Eastern Russia (Ziuganov, 1995), and Iceland (Kristjánsson *et al.*, 2002; 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 co-existing benthic and limnetic stickleback species (McPhail, 1984; Hatfield and Schluter, 1996, 1999; Boughman, 2001; Conte and Schluter, 2013; Arnegard *et al.*, 2014).

Here we study a case of sympatric polymorphism within a large pond, the Jordeweier (Zeller *et al.*, 2012; Marques *et al.*, 2016), which lies in the invasive range of stickleback in Switzerland (Lucek *et al.*, 2010; Roy *et al.*, 2015). This pond, measuring approximately 50 × 60 m with a maximum depth of just 3 m, harbours two distinct male stickleback phenotypes (Marques *et al.*, 2016). ‘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 differ markedly in their nuptial coloration and nest habitat, females cannot readily be assigned to either phenotype,

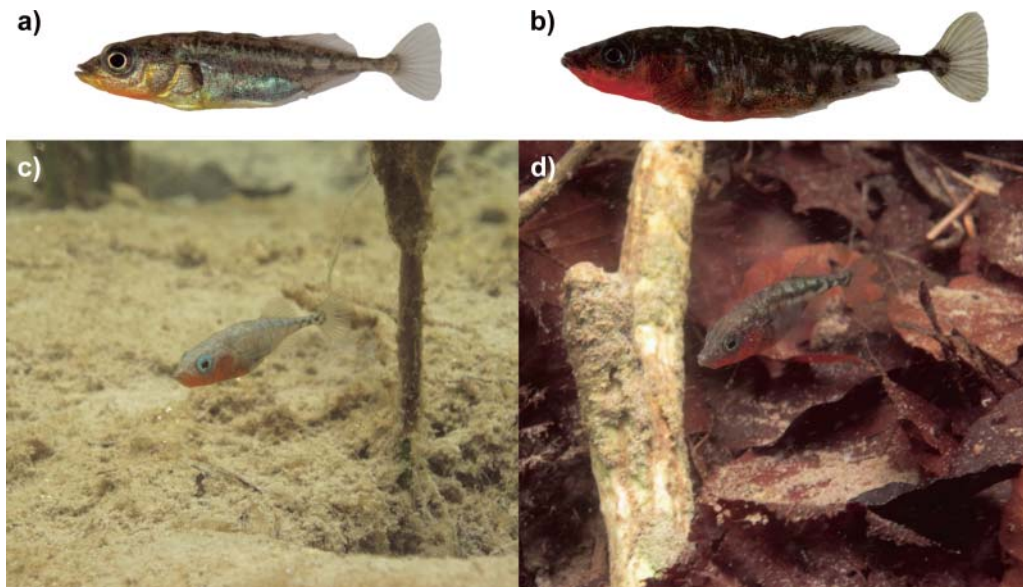


Fig. 1. Breeding threespine stickleback males from the Jordeweier pond near Bern, Switzerland. ‘Offshore’ (a, c) and ‘nearshore’ (b, d) males are shown in the respective habitats (c, d) and on colour-standardized photographs (a, b). Note the difference in throat and body colour as well as the different 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.

although large variation in female size and shape is observed. Given the small geographic scale and the lack of evidence for dietary differentiation even among males (Marques *et al.*, 2016), females are likely to explore all habitats and thus encounter both male types. Consequently, the diverging phenotypes occur in full sympatry, defined by Gavrillets as when ‘mating is random in respect to the place of birth of the mating partner’ (2003, p. 2918). 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 Jordeweier 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 Jordeweier pond [population ‘EYM’ in Roy *et al.* (2015)].

The aim of the present study was to explore two behavioural 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 investigated 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 colour types breed in different habitats: (1) competitive exclusion, whereby the dominant type excludes the subdominant type from the habitat preferred by both; or (2) habitat matching, where 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 and 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 matched their wild-type habitat best, an optimum presumably influenced by substrate colour relevant to camouflage and sexual signalling 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 (see Fig. 3 below). Many previous studies have demonstrated the importance of male nuptial coloration, body size, and body shape for female choice (e.g. Reimchen, 1989; Milinski and Bakker, 1990; Nagel and Schluter, 1998; Boughman, 2001; Boughman *et al.*, 2005; Conte and Schluter, 2013; Head *et al.*, 2013). Several studies on threespine stickleback found directional selection for males with bright red throats (Milinski and Bakker, 1990; Bakker and Mundwiler, 1994; Baube *et al.*, 1995; Cubillos and 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 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 and Schluter, 1998; Kraak *et al.*, 1999; Conte and 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 wished to assess the potential for behavioural 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 16 and 23 May 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 15 and 22 May. Only adult males in full breeding coloration from both years and only males that showed territorial or nesting behaviour (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 in which they were captured.

For the female preference experiment, we caught 65 gravid females between 15 May and 26 June 2015 using unbaited minnow traps and kept them in groups of a maximum 18 fish in 72-litre 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 anaesthetized and euthanized in a clove oil

solution in accordance with granted permits. Fish collection, experimentation, and euthanasia followed Swiss fisheries and veterinary legislation in accordance 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 of Bern, and the fishery rights owner, Augsburger AG, Hinterkappelen, Switzerland.

Morphological measurements

We quantified nuptial coloration of males caught in 2015 from standardized cuvette photographs. For this, stickleback were transferred into a plexiglas cuvette, placed in a box lined with black velvet to exclude unwanted ambient light, and photographed with an SLR camera (Canon 7D, Canon, Japan), mounted with an 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 images (Canon PowerShot D10, Canon, Japan) of each male in its breeding habitat in the pond. We used these underwater photos to qualitatively assess potential colour changes between capture and taking cuvette pictures and did not detect any qualitative change in throat colour in this short period. Then, no more than 2 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 days (mean \pm SD) 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 1 mm^2 circle below the eye in a well-lit area consisting mostly of erythrophores and lacking melanophores (i.e. dark spots) using ImageJ v.1.49 (Schneider *et al.*, 2012). Based on median RGB values, we calculated the median hue angle for throat coloration of each male, using the formula $h_{\text{Preucil circle}} = 60^\circ * (G - B) / (R - B)$ 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 capture 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 v.2.17 (Rohlf, 2015) to obtain measurements for 15 linear traits (Fig. 2, Table 1). For all females analysed in the female mate preference experiment, we also used a subset of the landmarks (Fig. 2a) to analyse 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 v.1.06d (Klingenberg, 2011). We tested whether allometric slopes for each linear trait differed between male colour morphs in an ANCOVA with standard length as the independent variable, each trait as the dependent variable, and morph as a covariate. As we did not find significant differences in slopes between male colour 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 v.3.2.1 (R Development Core Team, 2013)

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

Trait	Abbreviation	Linear mixed-effect model statistics			Pearson's correlation coefficient
		β_{trait}	$t_{2,31}$	<i>P</i> -value	
Standard length	SL	0.008	1.02	0.317	0.14
Head length	HL	0.448	4.16	<0.001	0.39
Snout length	SnL	0.131	0.55	0.586	0.06
Eye diameter	ED	0.839	3.95	<0.001	0.53
Eye area	EA	0.137	4.00	<0.001	0.53
Upper jaw length	UJL	0.266	1.61	0.117	0.25
First spine length	FSL	-0.013	-0.12	0.902	-0.02
Second spine length	SSL	0.026	0.23	0.819	0.00
Pelvic spine length	PSL	-0.008	-0.09	0.925	-0.05
Body depth 1	BD1	0.068	0.72	0.478	0.10
Body depth 2	BD2	-0.026	-0.34	0.733	-0.05
Basal length of dorsal fin	BLD	0.028	0.35	0.731	0.00
Basal length of anal fin	BLA	-0.003	-0.03	0.975	-0.03
Caudal peduncle depth	CPD	0.500	1.07	0.293	0.21
Caudal peduncle length	CPL	-0.127	-1.38	0.176	-0.17
Total length pectoral fin	TLP	-0.035	-0.31	0.757	-0.11
All traits PC1	PC1 (35.9%)	-0.123	-3.76	<0.001	-0.50
All traits PC2	PC2 (19.4%)	0.019	0.35	0.728	0.01
All traits PC3	PC3 (13.2%)	0.064	1.01	0.320	0.11
All traits PC4	PC4 (11.6%)	-0.071	-1.04	0.307	-0.18
All traits PC5	PC5 (6.5%)	-0.099	-1.02	0.316	-0.19

Note: The linear mixed-effect model fixed-effect regression coefficients (β_{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 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 simulated the two nesting environments in the pond. The experiment focusing solely on substrate was conducted in 2011. Each male was placed individually in a 72-litre tank containing two flowerpots at opposite ends of the tank. One pot was filled with 8 cm of sand, covered by 5 cm of mud from the Jordeweier 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, when each male was transferred into a 72-litre tank divided into two compartments of equal size. One compartment contained a flat, ~6 cm thick layer of substrate taken from the middle of the Jordeweier pond, simulating the offshore habitat (Fig. 3a); the other compartment, simulating the nearshore habitat, contained substrate taken from the shore

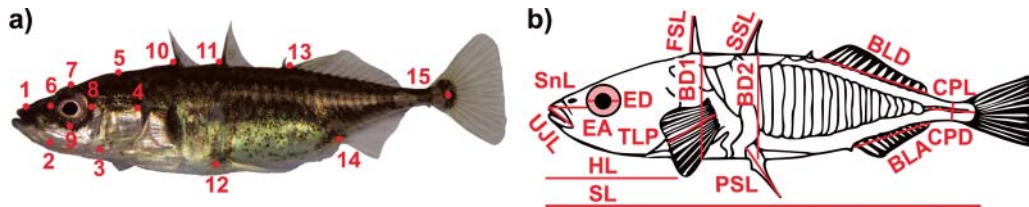


Fig. 2. (a) Standardized cuvette photograph of a female with the 15 landmarks used for geometric morphometrics in this study indicated in red. (b) Linear measurements used in this study: standard length (SL), head length (HL), snout length (SnL), eye diameter (ED), eye area (EA), upper jaw length (UJL), 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).

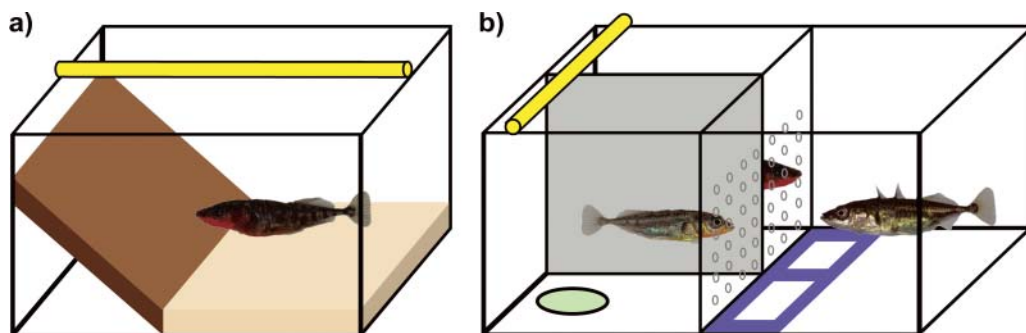


Fig. 3. Experimental setup for (a) the male habitat choice experiment and (b) the female mate preference experiment. In (a), single males could choose to build their nests in a steep part with nearshore substrate (brown) or a flat part with offshore mud substrate (light brown). In (b), females could 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. Yellow tubes represent light sources in the two settings.

of the Jordeweier and was set along a 36° angle from the horizontal, representing the average angle at which nearshore males build nests in the Jordeweier (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-hour day. For the habitat choice experiment, we installed a fluorescent tube lamp with a colour temperature of 2700 K (Philips, Amsterdam, The Netherlands) over each tank, 10 cm above the water surface in the middle of the tank parallel to its long side (Fig. 3a). The fish were fed daily with 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 5 minutes (following Frommen and 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 4 weeks had elapsed.

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 behaviour 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. In five 96-litre tanks, each divided into two compartments measuring $29 \times 40 \times 30$ cm, perforated clear plastic was used to allow exchange of both olfactory and visual cues between the two compartments (Fig. 3b). One compartment was further subdivided into two parts, each measuring $29 \times 19 \times 30$ cm using non-transparent dark grey plastic. To control for external stimuli, the tank was further visually isolated from its surroundings by opaque black plastic sheets. We used the same lighting 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 nest (Fig. 3b). 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, giving 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 allowed them to acclimatize for one day. We checked whether males continued to care for their nests after transfer and found that all males started to repair the transferred nest or build a new one either immediately or after 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. We began each female preference trial by placing a female in the large compartment, allowing 30 minutes for acclimatization. After 30 minutes, the opaque plastic partition was lifted and the interactions were filmed for 30–50 minutes. We analysed 29 minutes of each trial, after (1) both males had seen the female, (2) both males showed courtship behaviour [zigzag dance and an attempt to bite the female (Östlund-Nilsson, 2007)], and (3) the female had entered the second of the two 'contact zones', a 7×19 cm area in front of each male compartment (Fig. 3b).

In the female mate preference experiment, none of the 65 females used 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 pair saw 10–14 different females (pair 60/62: 10 females; pair 66/71: 14 females; pair 67/76: 14 females; pair 61/78: 13 females; pair 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 being extruded if the female was gravid (Nagel and 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 (pair 60/62: 6 females; pair 66/71: 9 females; pair 67/76: 5 females; pair 61/78: 7 females; pair

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 v.1.0 (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 courtship behaviour. A preference score for nearshore males (NPS) was calculated as the proportion of the total time in the contact zones in which 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 data set (for details, see Langfelder *et al.*, 2008). The parameters used for this method were a minimal cluster size of 10 individuals, 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 male closer to their own size. Discriminant function analyses on the four body shape categories were conducted to assess whether females preferring one or the other of the male phenotypes (NPS <0.5 or >0.5 respectively) could be distinguished by her own body shape. Significance levels were estimated using a permutation test with 1000 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), although all nearshore males except one 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. Twelve 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 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: $P = 0.511$), or for males that were more similar in body size to the choosing female (binomial test: $P = 0.511$).

Females that preferred red nearshore males had larger eyes and a longer head than females that preferred orange offshore males, as 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, which explained 35.9% of the total variance, showed a significant association with NPS (Table 1, Fig. 5b). Again, PC1 was 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 orange offshore males (NPS < 0.5) from those that preferred red nearshore males (NPS > 0.5) based on body shape, head shape or eye shape (Hotelling's T^2 : body = 10.6, $P = 0.60$; head = 6.4, $P = 0.50$; body + head = 33.5, $P = 0.54$; 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 that preferred orange offshore males (NPS < 0.4) and those with intermediate to nearshore red-biased preferences (NPS > 0.4 ; Hotelling's T^2 : body = 10.8, $P = 0.63$; head = 3.0, $P = 0.86$; body + head = 28.5, $P = 0.67$; body + head + eyes = 126.0, $P = 0.30$).

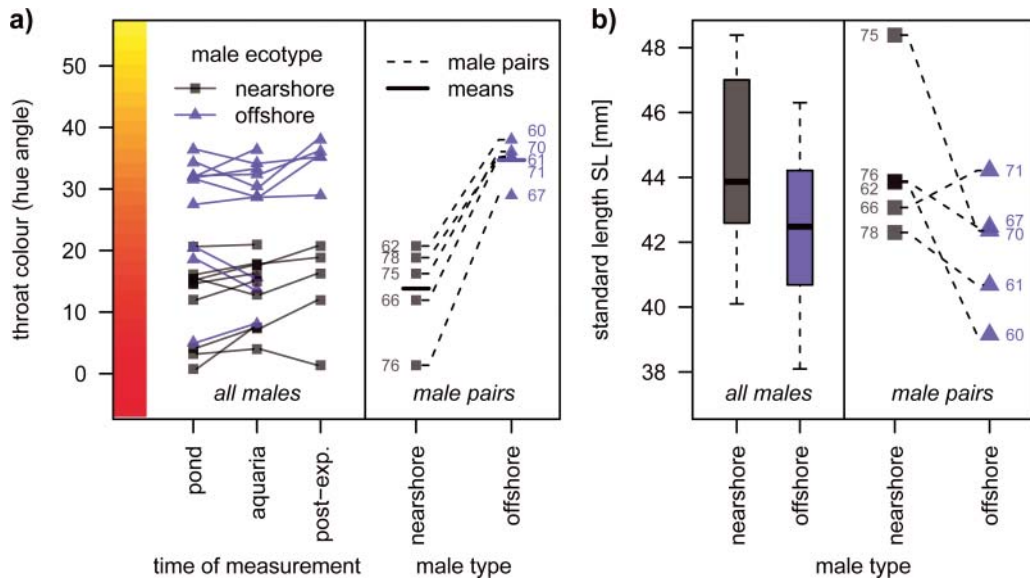


Fig. 4. (a) Throat coloration of the 20 males caught in 2015 did not change between measurements taken directly after capture ('pond'), after the male nest habitat choice experiment ('aquaria', after 22 ± 9 SD days under standardized light conditions), and after the female mate preference experiment ('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 differ significantly 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.

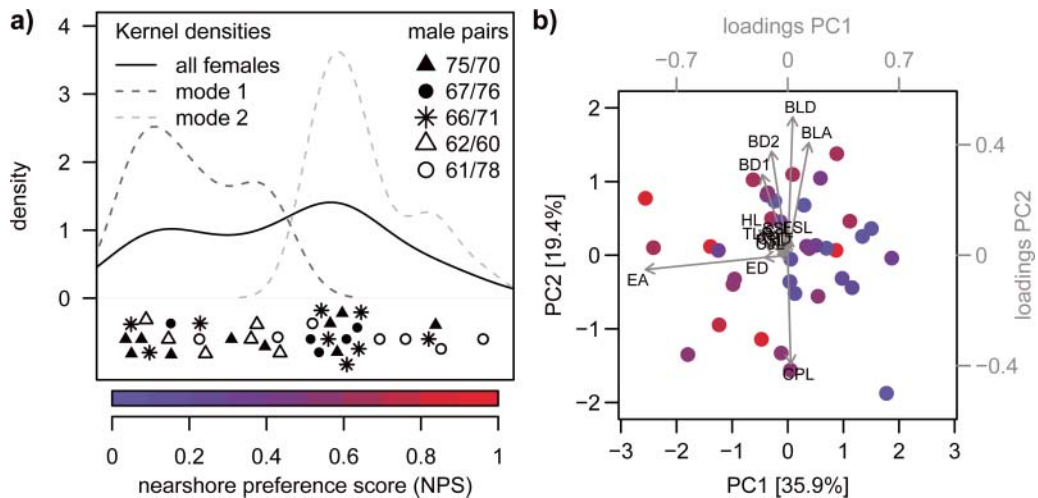


Fig. 5. (a) Kernel density function of the female preference score (quantified as the preference for the nearshore male, NPS) across the 37 wild-caught females that we tested. Kernel densities are shown for all individuals combined (black line) or separately for each identified multivariate mode (grey dashed lines). Each data point is one female, grouped by the five male pairs used in the trials (see key for 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 colours indicate the female's preference score for nearshore or offshore males, as shown in the colour bar at the x-axis in (a).

DISCUSSION

In a population of threespine stickleback that is less than 90 years old, where two phenotypically distinct male morphs breed in sympatry, we explored two behavioural 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, both of which 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). Under the competitive exclusion scenario, we expected all males to breed in the nearshore-like experimental habitat, assuming that larger/redder nearshore males are dominant and thus occupy the preferred habitat in the pond. Under the habitat-matching scenario, we expected males of the two morphs to breed in the habitat that mimicked their nest habitat in the pond. 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 contrasts with the results of studies on other stickleback populations that demonstrated the dominance of larger and brighter red males over smaller and duller red males (Bakker and 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 not tested in our experiment may be equally or more important to male habitat choice, such as divergent light conditions, habitat complexity or water depth (Candolin and Voigt, 2003; Bolnick *et al.*, 2015). By using experimental tanks with a depth of 0.3 m, water depth in our experiment deviated from natural conditions in the pond where males breed at depths of 0.5–3 m (Marques *et al.*, 2016). Bolnick *et al.* (2015) showed recently that water depth can be a very strong predictor for male mating success, and thus the males in our experiment may have chosen the offshore-like habitat because it was the habitat closest to the natural depth of water 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 of being lost. Another factor not tested in our experiment, which is also strongly influenced by water depth and has important consequences for social signalling (Seehausen *et al.*, 2008), is light. While we used standardized light conditions in our experiment, the nearshore habitat in Jordeweiher pond is characterized by dynamic, heterogeneous light conditions caused by overhanging trees, roots, and branches. The light in our experimental setup thus may have resembled more the offshore part of the pond in that respect, which receives direct sunlight throughout the day, while with respect to water depth, the light spectrum may have been closer to that of the shallower, nearshore part of the pond. Habitat complexity also varies in the pond, as roots, branches, and leaf litter are largely restricted to the nearshore habitat, but we did not account for this 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 favourable conditions for

reproduction (Candolin and Voigt, 2003). Predation and habitat-dependent sexual signalling may have shaped male habitat preferences in the Jordeweier 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 it is also closer to the water surface, where predatory birds are a threat. The offshore habitat, on the other hand, is deeper and thus protected from 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 sighting each of trout *Salmo trutta* and northern pike *Esox lucius*) may be negligible in this pond compared with 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 the two habitats. Habitat-dependent sexual signalling may be mainly influenced by light conditions and substrate colour 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 Jordeweier 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 favouring the offshore males to those clearly favouring 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 surprising, however, and different from studies of other populations just a few kilometres downstream of the Jordeweier (Milinski and 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 did not quantify 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 favoured orange offshore males. Indeed, the light conditions in the experimental setup (i.e. 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 or for a preference 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 and Schluter, 2013; Head *et al.*, 2013), is 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 behaviour between sympatric species of stickleback (Schluter, 1993), where they may reflect assortative mating related to feeding (Snowberg and Bolnick, 2008; Bolnick and Paull, 2009). This association between preferences for either of two male colour morphs and the female's morphology in the Jordeweier population could thus indicate the presence of some level of assortative mating in this system. Indeed, in another study on the Jordeweier stickleback, we found that orange males have on average shorter heads and marginally smaller eyes [corrected for body size (Marques *et al.*, 2016)], suggesting a correlation between male colour and morphology with female morphology and preferences.

Are the Jordeweier sticklebacks undergoing sensory drive speciation? Sensory drive

speciation is characterized by: (1) divergence in the male sexual signalling trait, (2) divergence in female preferences, and (3) the environment-dependence of (1) and (2) (Boughman, 2002; Seehausen *et al.*, 2008). In the Jordeweier, we have indications for environment-dependence, as male throat colour was consistently associated with nearshore and offshore habitat (Marques *et al.*, 2016). In another study, we found differentiation at several places in the genome correlated with these colour morphs (Marques *et al.*, 2016), suggesting a possible genetic basis for this colour 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 colour 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 requires further investigation. Furthermore, 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 them requires further analysis.

CONCLUSIONS

The nest-site habitat choice of two sympatric male colour 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 conditions, 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 colour morph. We may have underestimated potential assortative mating in our experimental setup, as we did not take account of 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 in the evolution of reproductive isolation in sympatry.

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