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Habitat Choice and Female Preference in a Polymorphic Stickleback Population

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ABSTRACT

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

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

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

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

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

42 *Keywords:* *Gasterosteus aculeatus*, behavioral mate choice, color polymorphism, sympatric
43 divergence

44

45 INTRODUCTION

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

61 The threespine stickleback (*Gasterosteus aculeatus* species complex) is an important
62 model system in evolutionary biology that helps us to understand the evolution of
63 reproductive isolation between divergently adapted populations or within polymorphic
64 populations (McKinnon & Rundle, 2002; Kitano *et al.*, 2009). Since the last glacial retreat
65 ~10-15 kyrs ago, ancestral marine stickleback have repeatedly colonized distinct freshwater
66 habitats and subsequently adapted therein, leading to divergence in many traits (McKinnon &
67 Rundle, 2002; Hendry *et al.*, 2013) and to variable degrees of reproductive isolation among
68 the evolved ecotypes (Boughman, 2001; McKinnon & Rundle, 2002; Boughman *et al.*, 2005;

69 Hendry *et al.*, 2013). The evolution of reproductive isolation between distinct stickleback
70 ecotypes can be driven by divergent natural selection (Rundle *et al.*, 2000; Arnegard *et al.*,
71 2014) and/or through ecologically-dependent sexual selection (Boughman, 2001; Cooper *et*
72 *al.*, 2011).

73 Although many distinct stickleback ecotypes have been described that occupy a wide
74 range of habitats including streams, lakes and the marine environment, very few stickleback
75 ecotypes are known to coexist in sympatry, suggesting that disruptive selection may often not
76 be sufficient to initiate speciation in stickleback (Hendry *et al.*, 2009; Bolnick, 2011) or that
77 assortative mating often does not evolve (Raeymaekers *et al.*, 2010; Räsänen *et al.*, 2012;
78 Seehausen & Wagner, 2014). Cases of fully sympatric ecotypes are described from eight
79 lakes in British Columbia, Canada (Gow *et al.*, 2008), from lakes in Alaska (Cresko & Baker,
80 1996), Far Eastern Russia (Ziuganov, 1995) and Iceland (Kristjánsson *et al.*, 2002a;
81 Ólafsdóttir *et al.*, 2006). Two of the Canadian species pairs have been studied in great detail
82 and in these cases it appears that strong disruptive natural selection and environmentally-
83 dependent sexual selection together led to reproductive isolation between coexisting benthic
84 and limnetic stickleback species (McPhail, 1984; Hatfield & Schluter, 1996; Hatfield &
85 Schluter, 1999; Boughman, 2001; Conte & Schluter, 2013; Arnegard *et al.*, 2014).

86 Here we study a case of sympatric polymorphism within a large pond, the Jordeweier
87 (Zeller *et al.*, 2012; Marques *et al.*, in review), which lies in the invasive range of stickleback
88 in Switzerland (Lucek *et al.*, 2010; Roy *et al.*, 2015). This pond, measuring approximately
89 50x60m with a maximum depth of just three meters, harbors two distinct male stickleback
90 phenotypes (Marques *et al.*, in review): “Nearshore” males breed on steep clay-like shore
91 substrate under overhanging trees, where they build concealed nests with small entries, show
92 a deep red throat coloration and are large and more deep-bodied (Figs. 1b, 1d). “Offshore”
93 males breed on the flat, muddy bottom of the open pond, build large, open “crater”-like nests
94 and show an orange throat coloration as well as a pale, almost white body pigmentation

95 during the breeding season (Figs. 1a, 1c). In contrast to the territorial males that strongly
96 differ in their nuptial coloration and nest habitat, females cannot readily be assigned to either
97 phenotype although large variation in female size and shape exists. Given the small
98 geographic scale and the lack of evidence for dietary differentiation even among males
99 (Marques *et al.*, in review), females are likely to explore all habitats and thus encounter both
100 male types. Consequently, the diverging phenotypes occur in full sympatry as defined by
101 Gavrillets (2003), when ‘mating is random in respect to the place of birth of the mating
102 partner’ (p. 2198; Gavrillets, 2003). Importantly, these phenotypes must have evolved only
103 recently, because stickleback were introduced in the central parts of Switzerland in the 1920s
104 (Lucek *et al.*, 2010). The Jordeweiher population is composed of mitochondrial haplotypes of
105 two distinct genetic lineages originating from different parts of Europe that have formed a
106 large hybrid zone in the Swiss midlands including the Jordeweiher pond (population 'EYM' in
107 Roy *et al.*, 2015).

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

121 habitat-matching scenario, we expected both male types to build their nests in the
122 experimental habitat that matches their wild type habitat best, an optimum presumably
123 influenced by substrate color relevant to camouflage and sexual signaling against different
124 backgrounds (Reimchen, 1989).

125 Second, we studied the distribution of female preferences for one or the other male type
126 using a simultaneous two-stimulus choice design (Fig. 2). Many previous studies have
127 demonstrated the importance of male nuptial coloration, body size and body shape for female
128 choice (e.g. Reimchen, 1989; Milinski & Bakker, 1990; Nagel & Schluter, 1998; Boughman,
129 2001; Boughman *et al.*, 2005; Conte & Schluter, 2013; Head *et al.*, 2013). Several studies on
130 threespine stickleback found directional selection for males with bright red throats (Milinski
131 & Bakker, 1990; Bakker & Mundwiler, 1994; Baube *et al.*, 1995; Cubillos & Guderley, 2000;
132 Flamarique *et al.*, 2013), but others found divergent female mate preferences for alternative
133 male morphs in sympatric benthic and limnetic stickleback species pairs (Boughman, 2001;
134 Boughman *et al.*, 2005). We tested whether the distribution of female mating preferences in
135 the Jordeweiher pond was compatible with either directional selection for redder males or
136 with divergent selection for alternative male phenotypes. Furthermore, body size and body
137 shape matching between males and females have been shown to be additional important
138 components of reproductive isolation between sympatric limnetic and benthic stickleback
139 species (Nagel & Schluter, 1998; Kraak *et al.*, 1999; Conte & Schluter, 2013; Head *et al.*,
140 2013). We thus also tested whether females prefer similarly-sized males. Finally, we assessed
141 whether females with different preferences differed in linear morphological traits or in body
142 shape.

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

147 **METHODS**

148 **Fish collection**

149 We conducted male nest site habitat choice experiments in two years, 2011 and 2015,
150 and the female preference experiment in one year, 2015. In 2011, we caught 16 nearshore and
151 10 offshore males between May 16th and May 23rd in the Jordeweier pond (Wohlen, Bern,
152 Switzerland, 46°57'24" N, 7°23'21" E) using unbaited minnow traps placed in the respective
153 habitats. In 2015, ten males of each type were caught at their breeding sites with hand nets
154 during scuba diving between May 15th and May 22nd. Only adult males in full breeding
155 coloration from both years and only males that showed territorial or nesting behavior
156 (digging, transfer of material, fanning or guarding) in the pond in 2015 were used for the male
157 habitat choice experiment. We refer to males as 'offshore' or 'nearshore' males in the
158 remainder of our study, depending on the habitat where they were captured.

159 For the female preference experiment, we caught 65 gravid females using unbaited
160 minnow traps between May 15th and June 26th 2015 and kept them in groups of maximum 18
161 fish in 72 liter tanks. An additional 20 gravid females were caught for the stimulation of nest
162 building in the habitat choice experiments. Following the experimental trials, all males from
163 2011 and females caught in 2015 were released back into the Jordeweier, while the 20 males
164 caught in 2015 were anesthetized and euthanized in a clove oil solution in accordance with
165 granted permits. Fish collection, experimentation and euthanasia followed the Swiss fisheries
166 and veterinary legislation in concordance with the federal food safety and veterinary office
167 (FSVO), the cantonal veterinary office in Bern (Veterinärdienst Kanton Bern, permit numbers
168 BE57/11 and BE66/13), the fishery authorities of the canton Bern and the fishery rights
169 owner, Augsburger AG, Hinterkappelen, Switzerland.

170 **Morphological measurements**

171 We quantified male nuptial coloration of males caught in 2015 from standardized
172 cuvette photographs. For this, stickleback were transferred into a plexiglas cuvette, transferred
173 into a box lined with black velvet to exclude unwanted ambient light sources and
174 photographed with an SLR camera (Canon 7D, Canon, Japan), mounted with a 85 mm lens
175 and illuminated by two external flashes from both sides of the camera lens. To control for
176 potential plasticity in male coloration, we photographed each male at several time points
177 during the experiments. First, we took underwater photos (Canon PowerShot D10, Canon,
178 Japan) of each male taken in its breeding habitat in the pond. We used these underwater
179 photos to qualitatively assess potential color changes between capture and taking cuvette
180 pictures and did not detect any qualitative change in throat color in this short period.
181 Immediately after capture, no later than two minutes after the male had been captured under
182 water, we took the first standardized cuvette photograph. A second cuvette photograph was
183 taken after completing the habitat choice experiment when males had spent 22 ± 9 SD days in
184 the aquaria. In addition, males used in the female mate preference trials (see below) were
185 photographed once more, after completion of the mate preference trials, 14 (pair 60/62) and
186 24 days (other male pairs) after the last cuvette photograph. We also photographed females
187 immediately after the trials, using the same standardized setup.

188 We measured throat coloration (hue) of each male on standardized cuvette pictures by
189 measuring median red, green and blue (RGB) values from pixels in a 1mm^2 circle below the
190 eye in a well-lit area consisting mostly of erythrophores and lacking melanophores (i.e. dark
191 spots) using ImageJ v1.49 (Schneider *et al.*, 2012). Based on median RGB values, we
192 calculated the median hue angle for throat coloration of each male, using the formula h_{Preucil}
193 $\text{circle} = 60^\circ * (G-B) / (R-B)$ applicable to RGB values with $R \geq G \geq B$ (Preucil, 1953).
194 Differences in coloration between wild caught nearshore and offshore individuals were
195 compared using a Kruskal-Wallis test, and differences between time points (i.e. after
196 capturing and after the habitat choice experiment) were compared using a paired *t*-test.

197 We placed 33 landmarks on the best photograph of each fish using tpsDig v2.17 (Rohlf,
198 2015) to obtain measurements for 16 linear traits (Fig. 3b, Table 1). For all females analyzed
199 in the female mate preference experiment, we also used a subset of the landmarks (Fig. 3a) to
200 analyze body shape (landmarks 1, 10-15), head shape (landmarks 1-5), body and head shape
201 combined (landmarks 1-5, 10-15) and body, head and eye shape combined (landmarks 1-15)
202 in MorphoJ 1.06d (Klingenberg, 2011). We tested whether allometric slopes for each linear
203 trait differed between male color morphs in an ANCOVA with standard length as independent
204 variable, the trait as dependent variable and morph as covariate. As we did not find significant
205 differences in slopes between male color morphs, we assumed that slopes would be uniform
206 among the females and performed a size correction with all females combined for linear traits
207 in R 3.2.1 (R Development Core Team, 2013) and for shape in MorphoJ by taking the
208 residuals of a linear regression of the respective linear trait or shapes against standard length
209 (Reist, 1980).

210 **Male habitat choice experiment**

211 The male habitat choice experiment was divided into two parts, testing first for substrate
212 only and then including a substrate-slope combination that simulates the two nesting
213 environments in the pond. The experiment focusing solely on substrate was conducted in
214 2011. Here, each male was placed in an individual 72 liter tank containing two flower pots at
215 opposite ends of the tank. One pot was filled with 8cm of sand, covered by 5cm of mud from
216 the Jordeweiher pond, simulating the offshore substrate. The second pot was filled with sand
217 and covered by tree leaves from the pond, simulating the nearshore substrate. The second
218 experiment simulating both differences in substrate and slope was conducted in 2015, where
219 each male was transferred into a 72 liter tank divided into two equally-sized compartments:
220 One compartment contained a flat, ~6cm thick layer of substrate taken from the middle of the
221 Jordeweiher pond, simulating the offshore habitat (Fig. 2a). The other compartment,

222 simulating the nearshore habitat, contained substrate taken from the shore of Jordeweiher that
223 was set along a 36° angle from the horizontal, representing the average angle at which
224 nearshore males build nests in the Jordeweiher (D. A. Marques, unpublished data).

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

238 A nesting trial was deemed to be successful and the chosen substrate subsequently
239 recorded, once a nest was clearly visible or when a male showed repeated nesting behavior
240 including digging, transfer of material, gluing or fanning in the same place (Östlund-Nilsson,
241 2007). We tested if male habitat choice was expected by chance ($p = 0.5$ due to equal area
242 size of both habitats) or if one habitat was preferred over the other by performing binomial
243 tests in R.

244 **Female mate choice experiment**

245 We measured female mate preference following the protocol of Frommen and Bakker
246 (2006), using a simultaneous two-stimulus setup, which allows the detection of directional

247 biases in mating preferences on a population level. A set of five 96 liter tanks were each
248 divided into two compartments measuring 29x40x30cm using perforated clear plastic
249 allowing exchange of both olfactory and visual cues between both compartments (Fig. 2b).
250 One compartment was further subdivided into two parts, each measuring 29x19x30cm using
251 non-transparent dark grey plastic. The tank was moreover visually isolated from the
252 surroundings by opaque black plastic sheets to control for external stimuli. We simulated the
253 same light condition as for the nesting experiment, with the difference that the light source
254 was placed perpendicular to the tank's long edge at its rear edge over the male's nests (Fig.
255 2b). A video camera above each tank recorded each experimental trial. Following successful
256 nest building in the habitat choice experiment, we transferred ten males (five nearshore and
257 five offshore, producing five male pair combinations) together with their nests to the smaller
258 compartments of the test tank (one male of each morph per test tank) and gave them an
259 acclimatization time of one day. We checked whether males continued to care for their nests
260 after transfer and found that all males either started repairing the transferred nest or building a
261 new nest immediately or after the first exposure to a female. The large compartment was
262 visually isolated from the male compartments using removable non-transparent plastic. We
263 chose each pair of males to represent typical nearshore and offshore phenotypes based on
264 their location in the pond, body size and throat coloration. Each female preference trial started
265 by placing a female in the large compartment, allowing 30 min of acclimatization time. After
266 acclimatization, the opaque plastic partition was lifted and the interactions were filmed for 30-
267 50 min. We analyzed 29 min of each trial, after i) both males had seen the female, ii) both
268 males showed courtship behavior (zig-zag dance and attempts at biting the female; Östlund-
269 Nilsson, 2007), and iii) the female had entered the second of both 'contact zones', a 7x19cm
270 area in front of each male compartment (Fig. 2b).

271 In the female mate preference experiment, none of the 65 females used in the
272 experiment had previously been exposed to any of the males during captivity and each female

273 was tested only once with a single male pair. The five male pairs were used multiple times
274 and each saw 10-14 different females (pair 60/62: 10 females; 66/71: 14 females; 67/76: 14
275 females; 61/78: 13 females; 70/75: 14 females). We assessed the gravidity of each female
276 after a trial by gently applying pressure to the abdomen, which resulted in eggs extruding if
277 the female was gravid (Nagel & Schluter, 1998). We only considered a trial successful if a
278 female was gravid and spent at least 30% of the trial time in the contact zones in front of the
279 two males. Applying these conditions, we considered 37 trials for analysis (60/62: 6 females;
280 66/71: 9 females; 67/76: 5 females; 61/78: 7 females; 70/75: 10 females). For each trial, we
281 measured the time a female spent in front of either male, starting after the female had
282 inspected both males, as well as the time she spent outside the contact zones using
283 JWatcher™ v1.0 (Blumstein *et al.*, 2006). We also qualitatively confirmed that both males
284 in a pair courted the females during the experimental trial. We observed that the first reactions
285 of the males towards the female occurred within 2-3 minutes (5 minutes in one trial) after
286 exposure, and this was followed by relentless courting behavior. A preference score for
287 nearshore males (NPS) was calculated as the proportion of the total time in the contact zones
288 a female spent in front of the nearshore male (preference score for offshore male = 1 - NPS).
289 We tested the distribution of female mate preferences for multimodality using the dynamic
290 tree cut clustering method (Langfelder *et al.*, 2008). This method identifies clusters based on
291 an Euclidean distance tree among data points without prior assumptions of the number of
292 inferred clusters and thus provides an unbiased estimate for the number of distinct clusters or
293 modes present in a given dataset (see Langfelder *et al.*, 2008 for details). The parameters used
294 for this method were 10 individuals as minimal cluster size, a maximal scatter of 0.75 and a
295 maximal distance of 0.90 for assignment.

296 To test if female mate preferences can be predicted by any of the measured linear or
297 shape traits, we used linear mixed effect models, testing for relationships between NPS and
298 each linear trait separately as well as between NPS and the first five principal component

299 (PC) axes for all size corrected linear traits combined and for the four body shape
300 combinations (head, body, head+body, head+eye+body shape), respectively. We entered size-
301 corrected trait values as predictors into the model and used male pairs as a random effect. To
302 account for multiple testing of the same hypothesis, p-values for the linear mixed effect
303 models were corrected using a false discovery rate correction. We further performed binomial
304 tests to infer whether females would prefer the larger male or the males closer to their own
305 size. Discriminant function analyses on the four body shape categories were conducted to test
306 if the females that preferred either male phenotype (NPS <0.5 or >0.5 respectively) could be
307 distinguished by their own body shape. Significance levels were estimated using a
308 permutation test with 1,000 replicates as implemented in MorphoJ (Klingenberg, 2011).

309 **RESULTS**

310 **Male phenotypes**

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

324 **Male nest habitat choice**

325 We first tested for male habitat choice based on a single habitat characteristic: substrate.
326 12 nearshore and five offshore males built their nest on the muddy, offshore-like substrate,
327 while the remaining four nearshore and five offshore males failed to build a nest. No male of
328 either type built its nest on the nearshore type substrate. Consequently, the offshore type
329 substrate was clearly preferred overall (binomial test, $P < 0.001$) and no difference was
330 observed between the male types.

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

337 **Female mate preferences**

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

347 Females preferring red nearshore males showed larger eyes and a longer head than
348 females preferring orange offshore males, suggested by significant positive associations

349 between eye diameter, eye area, head length and NPS in females (Table 1). When all size-
350 corrected linear traits were combined into a principal component analysis (Fig. 5b), PC1,
351 explaining 35.9 % of the total variance, showed a significant association with NPS (Table 1,
352 Fig. 5b). Again, PC1 is dominated by eye area, eye diameter and head length (Fig. 5b),
353 confirming the association of female preference with these traits.

354 In contrast to linear traits, we did not find any associations between female shape traits
355 and NPS (shape PCs, all $P > 0.1$, results not shown). Similarly, a discriminant analysis of
356 shape traits failed to separate females that preferred offshore orange males ($NPS < 0.5$) from
357 females that preferred nearshore red males ($NPS > 0.5$) based on body shape, head shape or
358 eye shape (Hotelling's $T^2_{\text{body}} = 10.6$, $P = 0.60$; $T^2_{\text{head}} = 6.4$, $P = 0.50$, $T^2_{\text{body+head}} = 33.5$, $P =$
359 0.54 , $T^2_{\text{body+head+eyes}} = 84.2$, $P = 0.59$). The same was true when we considered only females
360 with stronger preferences (i.e. $NPS > 0.6$ and < 0.4 respectively; results not shown). We also
361 tested whether females in the two modes of the preference distribution could be distinguished
362 on body shape with a discriminant analysis, but could not detect significant differences in
363 body shape between females preferring offshore orange males ($NPS < 0.4$) and females with
364 intermediate to nearshore red-biased preferences ($NPS > 0.4$, $T^2_{\text{body}} = 10.8$, $P = 0.63$; $T^2_{\text{head}} =$
365 3.0 , $P = 0.86$, $T^2_{\text{body+head}} = 28.5$, $P = 0.67$, $T^2_{\text{body+head+eyes}} = 126.0$, $P = 0.30$).

366 **DISCUSSION**

367 Studying a population of threespine stickleback that is less than 90 years old, where two
368 phenotypically distinct male morphs breed in sympatry, we have explored two behavioral
369 traits with relevance for possible reproductive isolation between the morphs (habitat choice of
370 nesting males and female mate preferences). We found that male nest habitat choice could not
371 be experimentally replicated based on two factors, substrate and slope, that both differ among
372 nesting habitats in nature and could be simulated in our aquaria. In the absence of intra- and
373 interspecific interactions, all males preferred to build their nest in the offshore-like habitat

374 regardless of male phenotype in our experiment. This outcome is contrary to our predictions
375 both under a competitive exclusion scenario and under a habitat matching scenario (Edelaar *et*
376 *al.*, 2008): In the competitive exclusion scenario, we expected all males would breed in the
377 nearshore-like experimental habitat, assuming that larger / redder nearshore males may be
378 dominant and thus occupy the preferred habitat in the pond. In the habitat matching scenario,
379 we expected males of the two morphs to breed in the habitat that mimicked their nest habitat
380 in the pond. Taken as such, our results could suggest that the redder and larger nearshore
381 males might not actually be dominant over the orange, smaller males in nature in the
382 Jordeweier population and that the nearshore habitat of red males may be the less preferred
383 habitat. This would be in contrast to expectations from studies on other stickleback
384 populations that demonstrated the dominance of larger and brighter red males over smaller
385 and duller red males (Bakker & Sevenster, 1983; Östlund-Nilsson, 2007) and a study that
386 showed male preference for shallower habitats (Bolnick *et al.*, 2015).

387 Alternatively, our results could suggest that other habitat characteristics, besides
388 substrate and slope, which were not tested in our experiment may be equally or more
389 important to male habitat choice, e.g. divergent light conditions, habitat complexity or water
390 depth (Candolin & Voigt, 2003; Bolnick *et al.*, 2015). With available experimental tanks of
391 0.3m depth, water depth in our experiment deviated from natural conditions in the pond
392 where males breed in 0.5-3m depth (Marques *et al.*, in review). Bolnick *et al.* (2015) showed
393 recently that water depth can be a very strong predictor for male mating success and thus,
394 males in our experiment may have chosen the offshore-like habitat because it was the habitat
395 closer to the natural situation in water depth for both morphs. Given that the Jordeweier
396 pond experiences occasional water level fluctuation due to hydropower usage, depth may also
397 play a role in assessing the risk of losing a nest: nests placed in very shallow water are at risk
398 to be lost. Another factor not tested in our experiment, which is also strongly influenced by
399 water depth and has important consequences for social signaling (Seehausen *et al.*, 2008), is

400 light environment: While we used standardized light conditions in the experiment, the
401 nearshore habitat in the pond is characterized by a dynamic, heterogeneous light environment
402 caused by overhanging trees, roots and branches. The light in our experimental setup thus
403 may have resembled more the offshore part in the pond in that respect, which receives direct
404 sunlight all day, while in respect to water depth, the light spectrum may have been closer to
405 the shallower nearshore part. Habitat complexity, another factor not incorporated in our
406 experiment, also varies in the pond, as roots, branches and leaf litter is largely restricted to
407 nearshore habitat, but were not present in our nearshore habitat model. Enclosure transplant
408 experiments are needed to test whether male nest habitat choice can be recreated by
409 embracing all the factors that differ between the two alternative habitats.

410 Often, the distribution of individuals within a habitat is the outcome of conflicting
411 demands, such as predator avoidance, food availability and favorable conditions for
412 reproduction (Candolin & Voigt, 2003). Predation and habitat-dependent sexual signaling
413 may have shaped male habitat preferences in the Jordeweier stickleback population. The
414 substrate of the nearshore region is darker and shows more structural complexity and may
415 thus allow better concealment from visual predators, but at the same time is closer to the
416 water surface, where predatory birds would have access. The offshore habitat on the other
417 hand is deeper and thus protected from the predatory birds but more open and thus vulnerable
418 to fish predation. However, predation by birds (Common Kingfisher *Alcedo atthis*, Grey
419 Heron *Ardea cinerea*) and fish (single sightings of trout *Salmo trutta* and Northern Pike *Esox*
420 *Lucius*) may be negligible in this pond as opposed to nearby streams (Zeller *et al.*, 2012).
421 Furthermore, the predation pressure by large dragonfly larvae (*Anax* and *Aeshna* sp.), the
422 dominant predators in the pond, may not differ between habitats. Habitat-dependent sexual
423 signaling may be mainly influenced by light environment and substrate color in the two
424 habitats, selecting males to maximize visibility to females (Reimchen, 1989), likely in a trade-
425 off with camouflage against different backgrounds protecting the males from predators.

426 Females from the Jordeweiher pond showed a broad distribution of mate preferences
427 when given one orange offshore and one red nearshore male to choose from, ranging from
428 individuals clearly favoring the offshore males to those clearly favoring the nearshore males,
429 while others lacked a preference for either type. Two distinct modes occur, one with females
430 preferring orange offshore males and the other with less choosy females that tend to prefer
431 redder nearshore males (Fig. 5a). Maybe most surprisingly however, and different from other
432 studies that used populations just a few kilometers downstream of the Jordeweiher (Milinski
433 & Bakker, 1990), we found no general preference for the redder of two males and hence,
434 there seems to be no directional sexual selection on red coloration in this pond population.
435 Rather, our results are compatible with the presence of a mate preference polymorphism
436 among females. In our experiment, we have not quantified the possible environment-
437 dependence of such mate preferences, but the lack of a clear preference mode for red
438 nearshore males suggests that the experimental conditions may have favored orange offshore
439 males. Indeed, the light environment in the experimental setup with direct light and a flat
440 floor, may have more closely resembled the offshore environment, potentially undermining
441 the expression of a stronger preference for red nearshore males.

442 We did not find evidence for size matching mate preferences in females nor for
443 preferences towards the larger of two males. Hence, one mechanism that has been shown to
444 facilitate reproductive isolation between sympatric stickleback ecotypes, namely size-
445 assortative mating (McKinnon *et al.*, 2004; Boughman *et al.*, 2005; Conte & Schluter, 2013;
446 Head *et al.*, 2013) seems unlikely to operate in this population. Interestingly, we found that
447 females with different mate preferences also differed in morphology: females with smaller
448 eyes and shorter heads seemed to prefer orange offshore males. In other populations these
449 traits have been shown to be associated with differences in feeding behavior between
450 sympatric species of stickleback (Schluter, 1993), where they may reflect some assortative
451 mating related to feeding (Snowberg & Bolnick, 2008; Bolnick & Paull, 2009). This

452 association between preferences for either of two male color morphs and the female's
453 morphology in the Jordeweiher population could thus indicate the presence of some level of
454 assortative mating in this system. Indeed, in another study on the Jordeweiher stickleback, we
455 found that orange males have on average shorter heads and marginally smaller eyes (corrected
456 for body size; Marques *et al.*, in review), suggesting a correlation of male color and
457 morphology with female morphology and preferences.

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

475 **Conclusions**

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

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641

642 **TABLES**

643 **Table 1.** Morphological linear traits measured in females and tests of associations between
 644 nearshore preference score (NPS) and each linear trait and their aggregate PC scores. The linear
 645 mixed-effect model fixed effect regression coefficients (β_{trait}) and associated test statistics are
 646 given. P-values significant after correcting for multiple testing using a false discovery rate
 647 correction are shown in bold and highlighted with a single asterisk.

648

Trait	Abbr.	Linear mixed-effect model statistics			Pearson's correlation coefficient
		β_{trait}	$t_{2,31}$	p-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

649

650 **FIGURES**

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

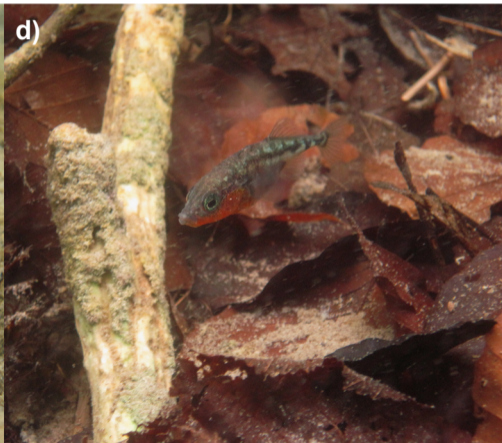
657 **Figure 2.** Experimental setup for the (a) male habitat choice experiment and the (b) female
658 mate preference experiment. In (a), single males could choose to build their nests in a steep part
659 with nearshore substrate (brown) or a flat part with offshore mud substrate (light brown). In
660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

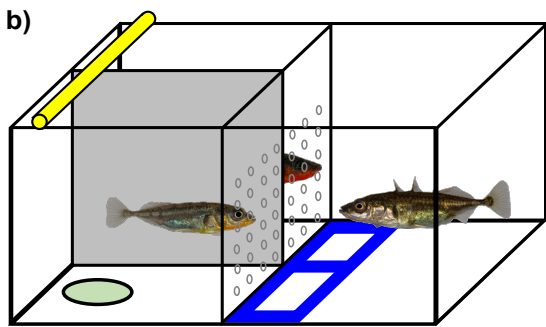
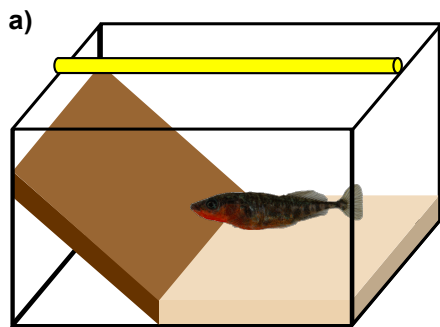
666 **Figure 3.** (a) Standardized cuvette photograph of a female with the 15 landmarks used for
667 geometric morphometrics in this study are indicated in red. (b) Linear measurements used in
668 this study: standard length (SL), head length (HL), snout length (SnL), eye diameter (ED), eye
669 area (EA), upper jaw length (UJL), first spine length (FSL), second spine length (SSL), pelvic
670 spine length (PSL), body depth at first spine (BD1) and second spine (BD2), basal length of
671 dorsal fin (BLD) and anal fin (BLA), caudal peduncle depth (CPD) and length (CPL) and total
672 length of the pectoral fin (TLP).

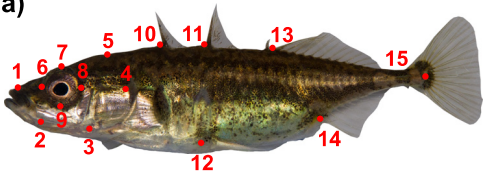
673 **Figure 4.** (a) Throat coloration of the 20 males caught in 2015 did not change between
674 measurements taken directly after capture ('pond'), after the male nest habitat choice
675 experiment, ('aquaria', after 22 ± 9 SD days under standardized light conditions) and after the
676 female mate preference experiment ('post-exp.', 24 days and 14 days later for four male pairs
677 and pair 60/62, respectively). The five male pairs used in the preference trials differed
678 consistently in throat coloration. (b) Average body size did not significantly differ between
679 nearshore and offshore males caught in 2015. Among the male pairs used in the female
680 preference experiment the nearshore male was larger than the offshore male in all but one male
681 pair.

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

690





a)**b)**