

1 **An experimental test of predation's effect upon behaviour and trait correlations**
2 **in threespine stickleback**

3

4 Sara E. Miller^{1*}, Kieran M. Samuk¹, Diana J. Rennison¹

5

6 ¹Department of Zoology, University of British Columbia, Vancouver, BC, Canada

7 *Corresponding author

8 Telephone: (604) 822-3383, Fax: (604) 822-2416, Email: miller@zoology.ubc.ca

9

10

11 **Running title:** Stickleback behaviour correlates with armour

12

13 **Abstract:**

14 Benthic and limnetic threespine stickleback (*Gasterosteus aculeatus*) are a classic
15 example of ecological speciation. Behavioural divergence between these species has been
16 predicted to be the result of divergent selection driven in part by differential predation from
17 cutthroat trout (*Oncorhynchus clarki*). To experimentally test this prediction, we reared
18 split families of benthic-limnetic hybrids in the presence or absence of trout predation. Our
19 results show that the presence of trout had little effect upon stickleback behaviour. We
20 then compared performance in behavioural assays among stickleback that varied in armour.
21 Our measurements also revealed trait correlations between several behaviours and
22 components of armour morphology. The strength of the correlations between traits did not
23 differ between predation treatments therefore differential predation between benthics and
24 limnetics is unlikely to be the cause of these correlations. The presence of trait correlations
25 in advanced generation hybrids suggests that pleiotropy or linkage between genes
26 underlying behaviour and armour morphology may be greater than previously appreciated.

27

28 *Keywords: adaptation – armour – ecological speciation – Gasterosteus aculeatus –*

29 *pleiotropy - shoaling – selection experiment*

30 **Introduction**

31 Ecological speciation occurs when reproductive isolation evolves as a consequence of
32 divergent natural selection between contrasting environments (Schluter, 2009; Nosil, 2012).
33 While there are many examples of ecological speciation in nature, our understanding of the
34 underlying mechanisms remains incomplete (Rundle & Nosil, 2005; Nosil, 2012). Divergent
35 selection can occur in response to differences in resource availability and as a result of
36 biotic interactions such as predation, competition, or intraguild predation (Schluter, 2000;
37 2009; Miller, Metcalf, & Schluter, 2015). Experimental studies have shown that differential
38 predation can lead to the evolution of divergent morphological traits (e.g. Jiggins et al.
39 2001; Vamosi & Schluter, 2002; Rundle *et al.*, 2003; Nosil & Crespi, 2006; Langerhans,
40 Gifford, & Joseph, 2007; Diabaté *et al.*, 2008; Marchinko, 2009; Svanbäck & Eklöv, 2011).
41 However, less attention has been given to the role of divergent selection in the evolution of
42 behavioural diversity.

43 Benthic and limnetic threespine stickleback (*Gasterosteus aculeatus sp.*) are a classic
44 example of ecological speciation; the two species have evolved in sympatry in five lakes in
45 coastal British Columbia (Schluter & McPhail, 1992). The species differ in many
46 morphological and behavioural traits. Limnetics primarily eat zooplankton in the open
47 water while benthics consume macroinvertebrates in the littoral zone (Schluter & McPhail,
48 1992). In the open water, limnetics encounter cutthroat trout (*Oncorhynchus clarki*) more
49 frequently (Reimchen, 1994). Consequently, many of the phenotypic differences between
50 the species are thought to be the result of differential predation on limnetics by trout
51 (Vamosi & Schluter, 2002). Relative to benthics, limnetics have longer spines and more
52 lateral plates (Vamosi, 2002). Limnetics also have an increased shoaling preference

53 (Vamosi & Schluter, 2002; Wark *et al.*, 2011), and are generally found higher in the water
54 column (Larson, 1976). In comparison, benthics are more often solitary (Vamosi &
55 Schluter, 2002; Odling-Smee, Boughman, & Braithwaite, 2008; Wark *et al.*, 2011), and
56 prefer to be lower in the water column (Larson, 1976).

57 To determine if a trait is the target of divergent selection, indirect evidence from
58 observational or comparative studies is insufficient (Schluter, 2009). The presence of
59 aquatic predators can co-vary with environmental factors (*e.g.* abiotic conditions, food
60 resources) (Jackson, Peres-Neto, & Olden, 2001). Therefore it is necessary to use
61 controlled experiments manipulating the presence/absence of predators to confirm that trait
62 shifts are caused by divergent selection from predation. Comparing trait shifts between
63 species is further problematic because species have fixed differences in many traits. As a
64 result, it is difficult to separate the trait(s) that are the target of divergent selection from
65 those traits that are genetically linked but not under direct selection. Predation may also
66 lead to selection for correlations between advantageous combinations of behaviour and
67 defence morphology (Sinervo & Svensson, 2002; Murren, 2012). Creating advanced
68 generation crosses between species with divergent phenotypes can create trait
69 combinations not normally seen in the wild. When such crosses are combined with
70 predator manipulation, it is possible to test if predation is responsible for changes in traits
71 and trait correlations.

72 We experimentally tested the hypothesis that differences in behaviour between
73 benthic and limnetic stickleback are the result of divergent selection from cutthroat trout
74 predation. Benthic-limnetic hybrid families were introduced into large, naturalistic
75 experimental ponds in the presence/absence of trout predation. Experimental stickleback

76 reproduced annually in the ponds and underwent two generations of differential selection
77 prior to measurement in behavioural assays. We measured two putative anti-predator
78 behaviours, which have been previously shown to differ between the two species -
79 preferred position in the water column and shoaling preference (Larson, 1976; Vamosi,
80 2002; Kozak & Boughman, 2008; Wark *et al.*, 2011). Behaviours that differ consistently
81 between control and predation ponds can be interpreted to arise in response to trout
82 predation. We then tested for correlations between behaviour and defensive armour, and
83 compared the strength of these correlations between treatments. If trout predation selects
84 for combinations of behaviour and defensive armour, trait correlation will be greater in the
85 predation treatment.

86

87 **Methods**

88 *Experimental Design*

89 In May 2011, four F1 crosses were made between wild-caught benthic females and
90 limnetic males from Paxton Lake, Texada Island. These F1 crosses were reared in 300L
91 tanks in the laboratory without predators for one year until adulthood. In May 2012, adult
92 stickleback were collected from First Lake, an advanced generation hybrid population. First
93 Lake is a small shallow lake on Texada Island that was founded in 1981 with Paxton Lake
94 benthic x limnetic F1 stickleback (McPhail, 1993). We consider this population to be a
95 single family of \sim F29 benthic-limnetic hybrids at the time of sampling. The First Lake
96 population was included in the study because the greater number of recombination events
97 this population has undergone affords us the opportunity to investigate the effect of linkage
98 on adaptation.

99 In May 2012, the five hybrid families (Four F1s and one First Lake) were introduced
100 in a split plot design to pairs of semi-natural ponds (n=21-31 individuals/pond; 10 ponds
101 total) at the University of British Columbia's experimental pond facilities. Each paired pond
102 contained a single family. Stickleback bred in all experimental ponds creating F2s or ~F30s
103 (First Lake ponds) in the summer of 2012. In the summer of 2013, the F2/F30 stickleback
104 bred to form a F3/F31 generation. All behavioural assays were conducted on adult
105 stickleback from the 2013 (F3/F31) cohort.

106 The experimental ponds are 25m x 15m with a shallow littoral area and a 6m deep
107 open water region. These ponds contain a natural assemblage of food resources and
108 contain invertebrate and avian predators. For each set of paired ponds, one pond was
109 randomly assigned to a predation treatment and the other pond to a control treatment.
110 Adult Cutthroat trout were collected from Placid Lake in the Malcolm Knapp Research
111 Forest. Two trout were added to each predation pond in September 2012. The trout died
112 in the summer of 2013 and were replaced with three new trout in September 2013.

113

114 *Behavioural Assays*

115 Behavioural assays were conducted from November 8-14, 2013, in tanks adjacent to
116 the experimental ponds. Twelve randomly chosen stickleback were collected from each
117 pond with unbaited minnow traps (n=120 total). Paired ponds were tested sequentially,
118 alternating between treatments. Sticklebacks were transferred in a bucket from the pond
119 to the behavioural assay area for a 15-minute acclimation period prior to the start of the
120 behavioural trials. At that time, each stickleback was placed into an individual mesh basket
121 inside a larger aquarium so that we could follow the behaviour of individuals across assays.

122 Behavioural tests were conducted in the following order: stickleback were tested in the
123 novel tank test, returned to the holding basket for 15 minutes, and then tested in the
124 shoaling assay.

125 The novel tank diving test measures stickleback movement and position in a new
126 tank. Vertical position in the water column of a tank has been used as a proxy for habitat
127 usage in guppies and stickleback (Larson, 1976; Torres-Dowdall *et al.*, 2012; Miller *et al.*,
128 2015). It has also been found that anxiety in zebrafish (e.g. following exposure to alarm
129 pheromones) leads to a reduction in exploration and a lower position in a tank (Egan *et al.*,
130 2009; Cachat *et al.*, 2010; Stewart *et al.*, 2012). During the trial, a focal fish was gently
131 introduced to the top centre of an empty unfamiliar 35.5 cm x 22 cm x 20 cm tank and
132 allowed to move freely for 630 seconds. All assays were recorded with wireless D-Link
133 DCS-930L webcams (DLink Corporation, Taiwan). We excluded the first 30 seconds of each
134 assay as the introduction of a stickleback often resulted in erratic movement (Miller *et al.*,
135 2015). Videos were subsampled to 0.5 frames per second using VirtualDub software
136 (www.virtualdub.org). The MtrackJ plugin (Meijering, Dzyubachyk, & Smal, 2012) in
137 ImageJ (Schneider, Rasband, & Eliceiri, 2012) was used to measure the x and y coordinates
138 of the focal fish every 2 seconds. We calculated the mean vertical position of the focal fish,
139 the latency to enter the upper half of the tank, and the distance that the focal fish travelled
140 during the assay.

141 The second assay assesses shoaling preference by measuring the time that the focal
142 stickleback spends near a stimulus shoal (Vamosi, 2002; Kozak & Boughman, 2008; Wark
143 *et al.*, 2011). Assay tanks were 75 cm x 30 cm x 46 cm with two 10 cm end compartments
144 on either side of the tank that were separated from a large centre arena with window

145 screen (Figure S1). Ten stimulus stickleback (shoal) were added to one end compartment
146 and two stimulus stickleback (distractor) were added to the other end compartment (Wark
147 et al., 2011). The stimulus sticklebacks were limnetic stickleback from Priest Lake reared at
148 the experimental pond facility. This population was unrelated and unfamiliar to the
149 experimental stickleback and was chosen because individuals have a high shoaling
150 tendency (Wark et al., 2011) and were similar in size to the experimental stickleback. At
151 the start of the shoaling assay, the focal stickleback was gently introduced into the centre
152 arena and was allowed to move for 630 seconds. We measured the x and y coordinates of
153 the focal fish every 2 seconds following the method used in the novel tank test. We used
154 two metrics to assess shoaling behaviour: the mean horizontal position in the tank
155 (shoaling position), and the time that the focal fish spends within one body length of the
156 experimental shoal (shoaling preference).

157 As a result of camera error, two trials were not analysed. Following Wark *et al.*
158 (2011), we excluded trials in which the focal fish did not move during the trial (novel tank
159 n=10; shoaling n=12). In total, 110 novel tank trials and 108 shoaling trials were
160 measured.

161

162 *Armour*

163 Immediately following the shoaling assay, stickleback were euthanized in MS-222
164 and fixed in 10% formalin. Specimens were later stained with alizarin red to highlight bony
165 structures following established protocols (Peichel et al., 2001). On the left side of each
166 stained specimen we measured the length of length of the first and second dorsal spines,
167 pelvic spine, pelvic girdle, the number of lateral plates and standard length. Specimens

168 lacking an armour component were assigned a value of zero. Lateral plate number and
169 standard length were not significantly correlated. All other armour traits were positively
170 correlated with standard length and were size corrected to the average length (43.82 mm)
171 using the equation $Y_i = X_i - \beta(L_i - \bar{L})$. Where Y_i is the size-adjusted trait, X_i is the original
172 trait, β is the regression coefficient of the original trait values on standard length, L_i is the
173 standard length of the individual and \bar{L} is the average length (Vamosi, 2002). For second
174 dorsal spine, pond had a significant effect on β and thus this trait was size corrected
175 independently for each pond (pond did not have a significant effect for other traits). A
176 principal component analysis (PCA) of the correlation matrix of size-corrected armour traits
177 was used to visualize the overall defensive armour of each stickleback. The first principal
178 component (PC1) accounted for 40.9% of the variation in stickleback armour and primarily
179 describes the pelvic spine and pelvic girdle (Table S1). The second principal component
180 (PC2) accounted for 25.8% of the variation and describes the length of the first and second
181 dorsal spine.

182

183 *Statistical Analysis*

184 A linear mixed effects model was used to test if performance in behavioural assays
185 differed between treatments and if armour traits affected these behaviours. Principal
186 component score, treatment, and population (Paxton Lake or First Lake) were fixed factors.
187 Pond and family were random factors. Population was not a significant covariate and was
188 dropped from the final model.

189 All traits were not normally distributed. Therefore, Spearman's rank correlations
190 were used to evaluate the correlations between armour and behavioural measurements.

191 Confidence intervals for trait correlations were calculated by bootstrapping (1000 replicates)
192 with RVAideMemoire (Hervé, 2014). For traits with significant correlations, we compared
193 the magnitude of the correlations between treatments using the Wilcoxon signed-rank test
194 on Spearman rank correlations calculated separately for each pond. All statistical analysis
195 were conducted in R (version 3.1) (R Core Team, 2014)

196

197 **Results**

198 The presence of trout did not have a measurable effect upon stickleback behaviour
199 (Table 1; Figure S2). Predation and control ponds did not differ in vertical position in the
200 water column, the latency to enter the upper half of the tank, or distance travelled during
201 the novel tank assay. Fish from all ponds spent more time shoaling than the random
202 expectation, regardless of treatment (one sample t-test: effect size = ____, $t_{10}=9.29$,
203 $P<0.0001$). In the shoaling assay, we observed a trend of increased time spent with the
204 shoal (shoaling preference) in the control ponds for four of the five families (Treatment:
205 effect size = ____, $F_{1,4}=3.24$, $P=0.15$), and focal fish from control ponds travelled more
206 during the assay (Figure 1; Treatment: effect size = ____, $F_{1,4}=5.69$, $P=0.08$), although
207 these results were not significant.

208 We observed variation in armour traits among experimental families (Table S2). PC1
209 differentiated stickleback with robust pelvic armour (limnetic-like) and stickleback with
210 reduced pelvic armour (benthic-like), while PC2 separated individuals with longer dorsal
211 spines (limnetic-like) from those with reduced dorsal spines (benthic-like). Predation and
212 control ponds did not differ in PC1 (Treatment: $F_{1,4}=0.43$, $P=0.55$), PC2 (Treatment:
213 $F_{1,4}=2.5$, $P=0.18$), or standard length (Treatment: $F_{1,4}=0.19$, $P=0.69$).

214 There was a positive correlation between PC1 score and mean vertical position
215 during the novel tank test (Figure 1A; Spearman's rank correlation coefficient, $\rho = 0.261$,
216 $P = 0.006$, 95% CI: 0.068-0.442). Individuals with increased pelvic armour preferred a
217 higher vertical position in the water column (PC1: $F_{4,97} = 4.10$, $P = 0.045$). There was a
218 negative correlation between PC2 and distance travelled during the novel tank test (Figure
219 1C; $\rho = -0.260$, $P = 0.006$, 95% CI: -0.428 - 0.071). PC2 and distance travelled during the
220 shoaling assay were not correlated (Table S3), but there was a significant Treatment x PC2
221 interaction ($F_{1,95} = 4.52$, $P = 0.04$). One individual had an extreme value for PC2. However,
222 the correlation between these traits remained significant when this point was removed
223 (without point, $\rho = -0.245$, $P = 0.01$). Behaviour was not correlated with standard length
224 (Table S3). All other armour and behaviour correlations were non-significant (Table 1,
225 Table S3).

226 Trout predation did not change the strength of the correlations between PC1 and
227 water column position (Figure 1B; Wilcoxon signed-rank test, $z = 9$, $n = 5$, $P = 0.812$), or PC2
228 and distance travelled during the water column assay (Figure 1D; $z = 5$, $n = 5$, $P = 0.625$).
229

230 **Discussion**

231 Divergent selection from trout predation has been hypothesized to be an important
232 driver of behavioural differences between benthic and limnetic stickleback (e.g. Larson,
233 1976; Vamosi, 2002; Vamosi & Schluter, 2004; Wark *et al.*, 2011). We reared families of
234 benthic-limnetic hybrids in naturalistic experimental ponds in the presence or absence of
235 trout predation. Contrary to predictions, there was no significant difference in behaviour

236 between predation and control ponds. Instead, armour morphology was a stronger
237 predictor of behaviour than trout predation.

238

239

240 *Stickleback Behaviour*

241 Preferred position in the water column did not differ between predation and control
242 ponds. Stickleback in predation ponds had a decreased shoaling preference, but this result
243 was non-significant. If differences in benthic and limnetic behaviour are not the
244 consequence of divergent selection from trout predation, then behavioural differences may
245 be the result of selection from other factors that differ between the benthic and limnetic
246 habitats. For example, benthics forage for invertebrates in the littoral zone, while limnetics
247 eat zooplankton near the surface of the water (Larson, 1976; Odling-Smee et al., 2008).
248 Therefore differences in water column preference may be due to divergence in diet and/or
249 foraging behaviour between the two species. Similarly, limnetics are frequently observed in
250 large aggregations (Larson, 1976) and have a stronger shoaling preference than benthics
251 (Vamosi, 2002; Kozak & Boughman, 2008; Wark et al., 2011). The differences in shoaling
252 behaviour in the lakes may be due to differences in the structural complexity and amount of
253 open space between the two environments (Odling-Smee et al., 2008) rather than a
254 consequence of increased trout predation. A shift in resource or habitat use could also
255 have driven changes in shoaling preference. Compared to control ponds, predation ponds
256 had a decrease in population density and a shift in diet towards benthic resources (S.
257 Rudman, per. comm.). Selection for benthic-like trophic characteristics may have led to a
258 decrease in shoaling preference. Trout predation may have also led to non-consumptive

259 changes in behaviour by reducing competition and increasing intimidation in the open water
260 environment (Preisser, Bolnick, & Benard, 2005).

261 The experimental ponds provide an improvement over behavioural studies
262 conducted in mesocosms or in the laboratory because we were able to manipulate
263 experimental subjects in a natural environment. Our findings suggest that differential
264 predation alone is unlikely to explain the large differences in shoaling behaviour and water
265 column preference observed in the wild. However, the paired design limited the statistical
266 power of this experiment to detect small differences in behaviour between treatments.
267 Additionally, behaviours were assayed at a single end point, therefore if paired ponds did
268 not start at the same trait value this would decrease our ability to detect a treatment effect.

269

270 *Correlation between morphology and behaviour*

271 The likelihood that an individual escapes a predation event may be determined by
272 an interaction between behavioural and morphological traits (e.g. Brodie, 1992; Dewitt,
273 1999; Buskirk & McCollum, 2000; Relyea, 2001). We found a correlation between
274 behavioural traits and bony armour. Armour PC1 (increased pelvic armour) was associated
275 with a higher position in the water column and armour PC2 (longer dorsal spines) was
276 associated with increased movement during the water column assay. Functionally these
277 associations match the greater pelvic armour and preference for a higher water column
278 position found in limnetics (Larson, 1976). A previous study by Grand (2000) found that
279 within benthic stickleback that those individuals with reduced pelvic armour were less bold
280 than individuals with increased pelvic armour. Behavioural traits have high variance and any
281 measurement error can decrease the correlation between traits (Whitlock & Schluter,

282 2014), as a result, correlations between these traits in the wild are likely greater than
283 reported in this study.

284 The correlations we found between armour morphology and behaviour could result
285 from genetic linkage or pleiotropy (Schlosser & Wagner, 2004). Several inferences can be
286 made regarding the possible genetic basis of the correlations. Recombination events in
287 advanced generation hybrids should uncouple most traits that were genetically linked in
288 limnetics and benthics, aside from regions of especially low recombination. Yet three
289 generations of recombination were insufficient to break up the association between armour
290 and behaviour in the F3 families and >30 generations of recombination in First Lake ponds
291 did not decrease the correlation. The maintenance of these correlations in spite of
292 genome-wide recombination indicates that tight genetic linkage or pleiotropy underlies
293 these associations.

294 Prior studies in stickleback support a role for tight linkage or pleiotropy between
295 behaviour and morphology. Lateral plate number and body orientation during schooling
296 have been genetically mapped to the same chromosomal segment (Greenwood et al.
297 2013). A single gene (*Ectodysplasin*) in this low recombination region has been previously
298 shown to have pleiotropic effects upon lateral plate development, neuromast position,
299 schooling behaviour, and salinity preference (Barrett et al. 2009; Wark & Peichel, 2009;
300 Wark et al. 2012; Mills et al. 2014). A recent study has also uncovered a correlation
301 between anti-predator behaviour and pigmentation in juvenile stickleback (Kim & Velando,
302 2015), suggesting that these correlations may be more widespread than previously
303 appreciated.

304 When certain trait combinations are preferentially favoured, natural selection may
305 directly or indirectly lead to an increase in the correlation between these traits (Sinervo &
306 Svensson, 2002; Murren, 2012). While we describe a correlation between multiple armour
307 and behavioural traits, the strength of these correlations did not differ between treatments.
308 Therefore we were unable to support the hypothesis that trout predation is the causal
309 mechanism for the associations. However, the lack of change in correlation between
310 treatments could be a consequence of the limited power of our experiment, or insufficient
311 variation in correlation for selection to act upon. Trout may have also played an important
312 role during the historical divergence between benthic and limnetic stickleback. Therefore,
313 while trout predation may not be the proximate cause for the correlation between defence
314 morphology and behaviour, it may be the ultimate cause for this association. Future work
315 examining the genetic basis of these traits will be required to elucidate the role of
316 pleiotropy and tight linkage in behaviour and armour morphology in the threespine
317 stickleback.

318
319

320 *Acknowledgements:*

321 We would like to thank Kira Delmore for loan of the cameras and Andy LeBlanc for technical
322 assistance. Seth Rudman and Dolph Schluter (D.S.) assisted with the set-up of the
323 predation experiment and helpful comments to the manuscript. The experimental ponds
324 were created with a grant from the Canadian Fund for Innovation (D.S.) and this project
325 was funded by an NSERC discovery grant (D.S), and graduate fellowship (D.J.R). Data will
326 be archived in the Dryad digital repository.

327
328

329

330 **References:**

331

332 **Barrett RDH, Vines TH, Bystriansky JS, Schulte PM. 2009.** Should I stay or should I
333 go? The Ectodysplasin locus is associated with behavioural differences in threespine
334 stickleback. *Biology Letters* **5**: 788–791.

335 **Brodie ED III. 1992.** Correlational selection for color pattern and antipredator behavior in
336 the garter snake *Thamnophis ordinoides*. *Evolution*: 1284–1298.

337 **Buskirk V. 2000.** Functional mechanisms of an inducible defence in tadpoles: morphology
338 and behaviour influence mortality risk from predation. *Journal of Evolutionary Biology* **13**:
339 336–347.

340 **Cachat JM, Stewart A, Grossman L, Gaikwad S, Kadri F, Chung KM, Wu N, Wong**
341 **K, Roy S, Suciú C, et al. 2010.** Measuring behavioral and endocrine responses to novelty
342 stress in adult zebrafish. *Nature protocols* **5**: 1786–1799.

343 **Dewitt T, Sih A, Hucko J. 1999.** Trait compensation and cospecialization in a freshwater
344 snail: size, shape and antipredator behaviour. *Animal Behaviour* **58**: 397–407.

345 **Diabaté A, Dabiré RK, Heidenberger K, Crawford J, Lamp WO, Culler LE,**
346 **Lehmann T. 2008.** Evidence for divergent selection between the molecular forms of
347 *Anopheles gambiae*: role of predation. *BMC evolutionary biology* **8**: 5.

348 **Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat**
349 **SI, Bartels BK, Tien AK, Tien DH, et al. 2009.** Understanding behavioral and
350 physiological phenotypes of stress and anxiety in zebrafish. *Behavioural brain research*
351 **205**: 38–44.

352 **Grand TC. 2000.** Risk-Taking by Threespine Stickleback (*Gasterosteus aculeatus*) Pelvic
353 Phenotypes: Does Morphology Predict Behaviour? *Behaviour* **137**: 889–906.

354 **Greenwood AK, Wark AR, Yoshida K, Peichel CL. 2013.** Genetic and Neural
355 Modularity Underlie the Evolution of Schooling Behavior in Threespine Sticklebacks. *Current*
356 *Biology* **23**: 1884–1888.

357 **Hervé M. 2014.** RVAideMemoire: Diverse Basic Statistical and Graphical Functions,
358 Version 0.9-52. Available at <https://cran.r-project.org/package=RVAideMemoire>
359

360 **Jackson DA, Peres-Neto PR, Olden JD. 2001.** What controls who is where in
361 freshwater fish communities - the roles of biotic, abiotic, and spatial factors. *Canadian*
362 *Journal of Fisheries and Aquatic Sciences* **58**:157-170.

363

364 **Jiggins CD, Naisbit RE, Coe RL, Mallet J. 2001.** Reproductive isolation caused by
365 colour pattern mimicry. *Nature* **411**: 302–305.

366 **Kim SY, Velando A. 2015.** Phenotypic integration between antipredator behavior and

367 camouflage pattern in juvenile sticklebacks. *Evolution* **69**: 830–838.

368 **Kozak GM, Boughman JW. 2008.** Experience influences shoal member preference in a
369 species pair of sticklebacks. *Behavioral Ecology* **19**: 667–676.

370 **Kozak GM, Boughman JW. 2012.** Plastic responses to parents and predators lead to
371 divergent shoaling behaviour in sticklebacks. *Journal of Evolutionary Biology* **25**: 759–769.

372 **Langerhans RB, Gifford ME, Joseph EO. 2007.** Ecological speciation in *Gambusia*
373 fishes. *Evolution* **61**: 2056–2074.

374 **Larson GL. 1976.** Social behavior and feeding ability of two phenotypes of *Gasterosteus*
375 *aculeatus* in relation to their spatial and trophic segregation in a temperate lake. *Canadian*
376 *Journal of Zoology* **54**: 107–121.

377 **Marchinko KB. 2009.** Predation's role in repeated phenotypic and genetic divergence of
378 armor in threespine stickleback. *Evolution* **63**: 127–138.

379 **McPhail JD. 1993.** Ecology and evolution of sympatric sticklebacks (*Gasterosteus*): origin
380 of the species pairs. *Canadian Journal of Zoology* **71**: 515–523.

381 **Meijering E, Dzyubachyk O, Smal I. 2012.** Methods for cell and particle tracking.
382 *Methods in enzymology* **504**: 183–200.

383 **Mills MG, Greenwood AK, Peichel CL. 2014.** Pleiotropic effects of a single gene on
384 skeletal development and sensory system patterning in sticklebacks. *EvoDevo* **5**: 1–10.

385 **Miller SE, Metcalf DM, Schluter D.** Intraguild predation leads to genetically based
386 character shifts in the threespine stickleback. *Evolution* **69**: 3194–3203.

387 **Murren CJ. 2012.** The Integrated Phenotype. *Integrative and Comparative Biology* **52**:
388 64–76.

389 **Nosil P. 2012.** *Ecological Speciation*. Oxford, UK: Oxford University Press.

390 **Nosil P, Crespi BJ. 2006.** Experimental evidence that predation promotes divergence in
391 adaptive radiation. *Proceedings of the National Academy of Sciences* **103**: 9090–9095.

392 **Odling-Smee LC, Boughman JW, Braithwaite VA. 2008.** Sympatric species of
393 threespine stickleback differ in their performance in a spatial learning task. *Behavioral*
394 *Ecology and Sociobiology* **62**: 1935–1945.

395 **Peichel CL, Nereng KS, Ohgi KA, Cole BL, Colosimo PF, Buerkle CA, Schluter D,**
396 **Kingsley DM. 2001.** The genetic architecture of divergence between threespine
397 stickleback species. *Nature* **414**: 901–905.

398 **Preisser EL, Bolnick DI, Benard MF. 2005.** Scared to death? The effects of intimidation
399 and consumption in predator-prey interactions. *Ecology* **86**: 501–509.

- 400 **R Core Team. 2014.** *R: A language and environment for statistical computing*. Vienna: R
401 Foundation for Statistical Computing. Available at: <http://www/R-project.org>.
- 402 **Reimchen T. 1994.** Predators and morphological evolution in threespine stickleback. In:
403 M.A. Bell and S.A. Foster, eds. *The evolutionary biology of the threespine stickleback*.
404 Oxford, U.K.: Oxford Univ. Press, 240-276.
- 405 **Relyea RA. 2001.** Morphological and Behavioral Plasticity of Larval Anurans in Response
406 to Different Predators. *Ecology* **82**: 523–540.
- 407 **Rundle HD, Nosil P. 2005.** Ecological speciation. *Ecology Letters* **8**:336-352.
- 408 **Rundle HD, Vamosi S, Schluter D. 2003.** Experimental test of predation's effect on
409 divergent selection during character displacement in sticklebacks. *Proceedings of the*
410 *National Academy of Sciences* **100**: 14943–14948.
- 411 **Schluter D. 2000.** *The ecology of adaptive radiation*. Oxford, UK: Oxford University Press.
- 412 **Schluter D. 2009.** Evidence for ecological speciation and its alternative. *Science* **323**:
413 737–741.
- 414 **Schluter D, McPhail JD. 1992.** Ecological character displacement and speciation in
415 sticklebacks. *The American naturalist* **140**: 85–108.
- 416 **Schlosser G, Wagner GP. 2004.** *Modularity in Development and Evolution*. Chicago, IL:
417 University of Chicago Press.
- 418 **Schneider CA, Rasband WS, Eliceiri KW. 2012.** NIH Image to ImageJ: 25 years of
419 image analysis. *Nature methods* **9**: 671–675.
- 420 **Sinervo B, Svensson E. 2002.** Correlational selection and the evolution of genomic
421 architecture. *Heredity* **89**: 329–338.
- 422 **Stewart A, Gaikwad S, Kyzar E, Green J, Roth A, Kalueff AV. 2012.** Modeling anxiety
423 using adult zebrafish: A conceptual review. *Neuropharmacology* **62**: 135–143.
- 424 **Svanbäck R, Eklöv P. 2011.** Catch me if you can-predation affects divergence in a
425 polyphenic species. *Evolution* **65**: 3515–3526.
- 426 **Torres-Dowdall J, Handelsman CA, Reznick DN, Ghalambor CK. 2012.** Local
427 adaptation and the evolution of phenotypic plasticity in Trinidadian guppies (*Poecilia*
428 *reticulata*). *Evolution* **66**: 3432–3443.
- 429 **Vamosi S. 2002.** Predation sharpens the adaptive peaks: survival trade-offs in sympatric
430 sticklebacks. *Annales Zoologici Fennici* **39**: 237–248.
- 431 **Vamosi S, Schluter D. 2002.** Impacts of trout predation on fitness of sympatric
432 sticklebacks and their hybrids. *Proceedings of the Royal Society B: Biological Sciences* **269**:

- 433 923–930.
- 434 **Vamosi S, Schluter D. 2004.** Character shifts in the defensive armor of sympatric
435 sticklebacks. *Evolution* **58**: 376–385.
- 436 **Wark AR, Peichel CL. 2009.** Lateral line diversity among ecologically divergent
437 threespine stickleback populations. *Journal of Experimental Biology* **213**: 108–117.
- 438 **Wark AR, Wark BJ, Lageson TJ, Peichel CL. 2011.** Novel methods for discriminating
439 behavioral differences between stickleback individuals and populations in a laboratory
440 shoaling assay. *Behavioral Ecology and Sociobiology* **65**: 1147–1157.
- 441 **Wark AR, Mills MG, Dang LH, Chan YF, Jones FC, Brady SD, Absher DM,**
442 **Grimwood J, Schmutz J, Myers RM, et al. 2012.** Genetic architecture of variation in
443 the lateral line sensory system of threespine sticklebacks. *G3* **2**: 1047–1056.
- 444 **Whitlock MC, Schluter D. 2009.** *The Analysis of Biological Data*. Greenwood Village, CO:
445 Roberts & Company.
- 446
- 447

Novel Tank Test	Treatment				PC1				Treatment x PC1			
	df	F	P	95% CI	df	F	P	95% CI	df	F	P	95% CI
<i>Mean Vertical Position</i>	1,4	1.42	0.30	-29.4-4.7	1,97	4.10	0.05	-0.8-17.3	1,97	0.14	0.71	-15.0-10.2
<i>Latency to upper tank</i>	1,4	1.12	0.35	-37.3-77.5	1,97	0.17	0.68	-6.5-35.6	1,97	2.69	0.10	-55.1-5.2
<i>Distance traveled</i>	1,4	0.98	0.38	-4026-1808	1,97	0.78	0.38	-886-1027	1,97	0.61	0.44	-820-1888

Shoaling Assay

<i>Mean horizontal position</i>	1,4	0.71	0.45	-1.5-0.77	1,95	0.04	0.83	-0.5-0.4	1,95	0.03	0.86	-0.7-0.6
<i>Shoaling preference</i>	1,4	3.24	0.15	-84.0-11.9	1,95	0.24	0.62	-23.7-16	1,95	0.00	0.98	-28.9-29.7
<i>Distance traveled</i>	1,4	5.69	0.08	-3266-252	1,95	0.00	0.94	-784-511	1,95	0.52	0.47	-571-1226

Novel Tank Test	Treatment				PC2				Treatment x PC2			
	df	F	P	95% CI	df	F	P	95% CI	df	F	P	95% CI
<i>Mean Vertical Position</i>	1,4	1.33	0.31	-34.2-15.7	1,97	0.49	0.49	-16.4-6.0	1,97	0.38	0.54	-11.0-20.8
<i>Latency to upper tank</i>	1,4	0.85	0.41	-44.8-86.6	1,97	0.02	0.88	-39.6-12.5	1,97	3.6	0.06	-1.9-75.6
<i>Distance traveled</i>	1,4	0.90	0.4	-1838-1585	1,97	1.69	0.20	-1696-609	1,97	0.02	0.88	-1838-1585

Shoaling Assay

<i>Mean horizontal position</i>	1,4	0.75	0.44	-1.2-0.6	1,95	1.78	0.19	-0.6-0.5	1,95	2.3	0.13	-1.4-0.2
<i>Shoaling preference</i>	1,4	3.27	0.15	-82.7-12.8	1,95	0.81	0.37	-38.0-11.1	1,95	0.39	0.54	-26.1-49.9
<i>Distance traveled</i>	1,4	5.88	0.07	-3269-189	1,95	0.09	0.77	-176-1378	1,95	4.52	0.04	-2380- -82

448