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1   **An experimental test of predation's effect upon behaviour and trait correlations**

2   **in threespine stickleback**

3

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10
11   **Running title:** Stickleback behaviour correlates with armour
12

Abstract:

13

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*

Introduction

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. Jiggens 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 ~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).

10

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, ρ= 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; ρ= -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, ρ= -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 then 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 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.

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	Treatment				PC1				Treatment x PC1			
Novel Tank Test	df	F	P	95% CI	df	F	P	95% CI	df	F	P	95% CI
Mean Vertical Position	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
Latency to upper tank	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
Distance traveled	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												
Mean horizontal position	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
Shoaling preference	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
Distance traveled	1,4	5.69	0.08	-3266-252	1,95	0.00	0.94	-784-511	1,95	0.52	0.47	-571-1226

	Treatment				PC2				Treatment x PC2			
Novel Tank Test	df	F	P	95% CI	df	F	P	95% CI	df	F	P	95% CI
Mean Vertical Position	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
Latency to upper tank	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
Distance traveled	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												
Mean horizontal position	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
Shoaling preference	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
Distance traveled	1,4	5.88	0.07	-3269-189	1,95	0.09	0.77	-176-1378	1,95	4.52	**0.04**	-2380- -82

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