The LINNEAN SOCIETY of London BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2016, **119**, 117–125. With 1 figure.

An experimental test of the effect of predation upon behaviour and trait correlations in the threespine stickleback

SARA E. MILLER*, KIERAN M. SAMUK and DIANA J. RENNISON

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

Received 30 December 2015; revised 30 January 2016; accepted for publication 31 January 2016

Benthic and limnetic threespine stickleback *(Gasterosteus aculeatus)* are a classic example of ecological speciation. Behavioural and armour divergence between these species has been predicted to be the result of divergent selection driven in part by differential predation from cutthroat trout *(Oncorhynchus clarki)*. To experimentally test this prediction, we reared split families of benthic–limnetic hybrids in the presence or absence of trout predation. Our results show that the presence of trout had little effect upon stickleback behaviour. We then compared performance in behavioural assays among stickleback that varied in armour to test if armour morphology correlates with behaviour. Our measurements revealed trait correlations between several behaviours and components of armour morphology. Trout predation did not result in an increased correlation between traits, therefore differential trout predation between benthics and limnetics is unlikely to be the cause of these correlations. The presence of trait correlations in advanced generation hybrids suggests that pleiotropy or linkage between genes underlying behaviour and armour morphology may be greater than previously appreciated. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **119**, 117–125.

KEYWORDS: adaptation – armour – ecological speciation – *Gasterosteus aculeatus* – pleiotropy – selection experiment – shoaling.

INTRODUCTION

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

Benthic and limnetic threespine stickleback (*Gasterosteus aculeatus sp.*) are a classic example of ecological speciation. The two species have evolved in sympatry in five lakes in coastal British Columbia (Schluter & McPhail, 1992). The species differ in many morphological and behavioural traits. Relative to benthics, limnetics have longer spines and more lateral plates (Vamosi, 2002). Nesting males of the two species exhibit habitat isolation (Southcott *et al.*, 2013). Limnetics have an increased shoaling preference (Vamosi & Schluter, 2002; Wark *et al.*, 2011), and are generally higher in the water column (Larson, 1976). In comparison, benthics are more often solitary (Vamosi & Schluter, 2002; Odling-Smee, Boughman & Braithwaite, 2008; Wark *et al.*, 2011), and prefer to be lower in the water column (Larson, 1976). Limnetics primarily eat zooplankton in the open water while benthics consume macroinvertebrates in the littoral zone (Schluter & McPhail, 1992). In the open water, limnetics encounter cutthroat trout *(Oncorhynchus clarki)* more frequently

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*Corresponding author. E-mail: miller@zoology.ubc.ca

(Reimchen, 1994). Consequently, many of the phenotypic differences between the species are thought to be the result of differential predation on limnetics by trout (Vamosi & Schluter, 2002).

Indirect evidence from observational or comparative studies is insufficient to determine if a trait is the target of divergent selection (Schluter, 2009). The presence of aquatic predators can co-vary with environmental factors (e.g. abiotic conditions, food resources) (Jackson, Peres-Neto & Olden, 2001). Controlled experiments manipulating the presence/absence of predators are necessary to confirm that trait shifts are caused by divergent selection from predation. Comparing trait shifts between species is also problematic because species have fixed differences in many traits. As a result, it is difficult to separate the trait(s) that are the target of divergent selection from those traits that are genetically linked but not under direct selection. Predation may also select for correlations between advantageous combinations of behaviour and defence morphology (Sinervo & Svensson, 2002; Murren, 2012). The creation of advanced generation crosses between species with divergent phenotypes can create trait combinations not normally seen in the wild. When such crosses are combined with predator exposure, it is possible to test if predation is responsible for changes in traits and trait correlations.

We experimentally tested the hypothesis that differences in behaviour between benthic and limnetic stickleback are the result of divergent selection from cutthroat trout predation. Benthic–limnetic hybrid families were introduced into large, naturalistic experimental ponds in the presence/absence of trout predation. Experimental stickleback reproduced annually in the ponds and underwent two generations of differential selection prior to measurement in behavioural assays. We measured two putative antipredator behaviours that have been shown previously to differ between the two species – preferred position in the water column and shoaling preference (Larson, 1976; Vamosi, 2002; Kozak & Boughman, 2008; Wark *et al.*, 2011). Behaviours that differ consistently between control and predation ponds can be interpreted to arise in response to trout predation. We then tested for correlations between behaviour and defensive armour, and compared the strength of these correlations between treatments. If trout predation selects for combinations of behaviour and defensive armour, trait correlation will be greater in the predation treatment.

METHODS

Experimental design

In May 2011, four F1 crosses were made between wild-caught benthic females and limnetic males from Paxton Lake, Texada Island. F1 crosses were reared in 300 L tanks in the laboratory without predators for 1 year until adulthood. In May 2012, adult stickleback were collected from First Lake, an advanced generation hybrid population. First Lake is a small shallow lake on Texada Island that was founded in 1981 with Paxton Lake benthic × limnetic F1 stickleback (McPhail, 1993). We consider this population to be a single family of ~F29 benthic–limnetic hybrids at the time of sampling. The First Lake population was included in the study because the greater number of recombination events that this population has undergone affords us the opportunity to investigate the effect of linkage on adaptation.

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

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

Behavioural assays

Behavioural assays were conducted from 8 November to 14 November 2013, in tanks adjacent to the experimental ponds. Twelve randomly chosen stickleback were collected from each pond with unbaited minnow traps (N = 120 total). Paired ponds were tested sequentially, alternating between treatments. Sticklebacks were transferred in a bucket from the pond to the behavioural assay area for a 15-min acclimation period prior to the start of the behavioural trials. At that time, each stickleback was placed into an individual mesh basket inside a larger aquarium so that we could follow the behaviour of individuals across assays. Behavioural tests were conducted in the following order: stickleback were tested in the novel tank test, returned to the holding basket for 15 min, and then tested in the shoaling assay.

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

The second assay assesses shoaling preference by measuring the time that the focal stickleback spends near a stimulus shoal (Vamosi, 2002; Kozak & Boughman, 2008; Wark *et al.*, 2011). Assay tanks were 75 × 30 × 46 cm with two 10-cm end compartments on either side of the tank that were separated from a large centre arena with window screen (Supporting Information, Figure S1). Ten stimulus stickleback (shoal) were added to one end compartment and two stimulus stickleback (distractor) were added to the other end compartment (Wark *et al.*, 2011). The stimulus sticklebacks were limnetic stickleback from Priest Lake reared at the experimental pond facility. This population was unrelated and unfamiliar to the experimental stickleback and was chosen because individuals have a high shoaling tendency (Wark *et al.*, 2011) and were similar in size to the experimental stickleback. At the start of the shoaling assay, the focal stickleback was gently introduced into the centre arena and was allowed to move for 630 s. We measured the x and y coordinates of the focal fish every 2 s following the method used in the novel tank test. We used two metrics to assess shoaling behaviour: the mean horizontal position in the tank (shoaling position), and the time that the focal fish spends within one body length of the experimental shoal (shoaling preference).

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

ARMOUR

Immediately following the shoaling assay, stickleback were euthanized in MS-222 and fixed in 10% formalin. Specimens were later stained with alizarin red to highlight bony structures following established protocols (Peichel *et al.*, 2001). On the left side of each stained specimen we measured the length of length of the first and second dorsal spines, pelvic spine, pelvic girdle, the number of lateral plates and standard length. Specimens lacking an armour component were assigned a value of zero. Lateral plate number and standard length were not significantly correlated. All other armour traits correlated positively with standard length and were size corrected to the average length (43.82 mm) 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 trait, β is the regression coefficient of the original trait values on standard length, L_i is the standard length of the individual and $\bar{L}$ is the average length (Vamosi, 2002). For the second dorsal spine, the pond had a significant effect on β and thus this trait was size corrected independently for each pond (pond did not have a significant effect for other traits). Principal component analysis (PCA) of the correlation matrix of size-corrected armour traits was used to visualize the overall defensive armour of each stickleback. The first principal component (PC1) accounted for 40.9% of the variation in stickleback armour and primarily describes the pelvic spine and pelvic girdle (Supporting Information, Table S1). The second principal component (PC2) accounted for 25.8% of the variation and describes the length of the first and second dorsal spine.

STATISTICAL ANALYSIS

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

All traits were not normally distributed. Therefore, Spearman's rank correlations were used to evaluate the correlations between armour and behavioural measurements. Confidence intervals for trait correlations were calculated by bootstrapping (1000 replicates) with RVAideMemoire (Hervé, 2014). For traits with significant correlations, we compared the magnitude of the correlations between treatments using the Wilcoxon signed-rank test on Spearman rank correlations calculated separately for each pond. All statistical analysis were conducted using R programming language (version 3.1) (R Core Team, 2014).

RESULTS

The presence of trout did not have a measurable effect upon stickleback behaviour (Table 1; Supporting Information, Figure S2). Predation and control ponds did not differ in vertical position in the water column, the latency to enter the upper half of the tank, or distance travelled during the novel tank assay. Fish from all ponds spent more time shoaling than the random expectation, regardless of treatment (one sample t-test: $t = 9.29$, $P < 0.0001$, d.f. = 10). In the shoaling assay, we observed a trend of increased time spent with the shoal (shoaling preference) in the control ponds for four of the five families (Treatment: $F_{1,4} = 3.24$, $P < 0.2$), and focal fish from control ponds travelled more during the assay (Fig. 1; Treatment: $F_{1,4} = 5.69$, $P < 0.1$), although these results were not significant.

We observed variation in armour traits among experimental families (Supporting Information, Table S2). PC1 differentiated stickleback with robust pelvic armour (limnetic-like) and stickleback with reduced pelvic armour (benthic-like), while PC2 separated individuals with longer dorsal spines (limnetic-like) from those with reduced dorsal spines (benthic-like). Predation and control ponds did not differ in PC1 (Treatment: $F_{1,4} = 0.43$, $P < 0.6$), PC2 (Treatment: $F_{1,4} = 2.5$, $P < 0.2$), or standard length (Treatment: $F_{1,4}$=0.19, $P < 0.7$).

There was a positive correlation between PC1 score and mean vertical position during the novel tank test (Fig. 1A; Spearman's rank correlation coefficient, $\rho = 0.261$, $P < 0.007$, 95% CI: 0.068–0.442). Individuals with increased pelvic armour preferred a higher vertical position in the water column (PC1: $F_{4,97} = 4.10$, $P < 0.05$). There was a negative correlation between PC2 and distance travelled during the novel tank test [Fig. 1C; $\rho = -0.260$, $P < 0.01$, 95% CI: −(0.428–0.071)]. PC2 and distance travelled during the shoaling assay were not correlated (Supporting Information, Table S3), but there was a significant Treatment × PC2 interaction ($F_{1,95} = 4.52$, $P < 0.05$). One individual had an extreme value for PC2. However, the correlation between these traits remained significant when this point was removed (without point, $\rho = -0.245$, $P < 0.02$). Behaviour did not correlate with standard length (Supporting Information, Table S3). All other armour and behaviour correlations were non-significant (Table 1, Supporting Information, Table S3).

Trout predation did not change the strength of the correlations between PC1 and water column position (Fig. 1B; Wilcoxon signed-rank test, $z = 9$, $N = 5$, $P < 1$), or PC2 and distance travelled during the water column assay (Fig. 1D; $z = 5$, $N = 5$, $P < 0.7$).

DISCUSSION

Divergent selection from trout predation has been hypothesized to be an important driver of behavioural differences between benthic and limnetic stickleback (Larson, 1976; Vamosi, 2002; Vamosi & Schluter, 2004; Wark *et al.*, 2011). To test this hypothesis, we reared families of benthic–limnetic hybrids in naturalistic experimental ponds in the presence or absence of trout predation. Contrary to predictions, there was no significant difference in behaviour between predation and control ponds. Instead, armour morphology was a stronger predictor of behaviour than trout predation.

STICKLEBACK BEHAVIOUR

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

Table 1. Results from the linear mixed effects model comparing the effect of the presence of trout (Treatment), armour principal component score and their interactions with stickleback behaviours

	Treatment				PC1				Treatment × PC1			
	d.f.	*F*	*P*	95% CI	d.f.	*F*	*P*	95% CI	d.f.	*F*	*P*	95% CI
Novel tank test												
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 travelled	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 travelled	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 × PC2			
	d.f.	*F*	*P*	95% CI	d.f.	*F*	*P*	95% CI	d.f.	*F*	*P*	95% CI
Novel tank test												
Mean vertical position	1,4	1.33	0.31	–34.2 to 15.7	1,97	0.49	0.49	–16.4 to 6.0	1,97	0.38	0.54	–11.0 to 20.8
Latency to upper tank	1,4	0.85	0.41	–44.8 to 86.6	1,97	0.02	0.88	–39.6 to 12.5	1,97	3.6	0.06	–1.9 to 75.6
Distance travelled	1,4	0.90	0.40	–1838 to 1585	1,97	1.69	0.20	–1696 to 609	1,97	0.02	0.88	–1838 to 1585
Shoaling assay												
Mean horizontal position	1,4	0.75	0.44	–1.2 to 0.6	1,95	1.78	0.19	–0.6 to 0.5	1,95	2.3	0.13	–1.4 to 0.2
Shoaling preference	1,4	3.27	0.15	–82.7 to 12.8	1,95	0.81	0.37	–38.0 to 11.1	1,95	0.39	0.54	–26.1 to 49.9
Distance travelled	1,4	5.88	0.07	–3269 to 189	1,95	0.09	0.77	–176 to 1378	1,95	4.52	**0.036**	–2380 to –82

The 95% confidence intervals are given for each effect. Significant associations are in bold.

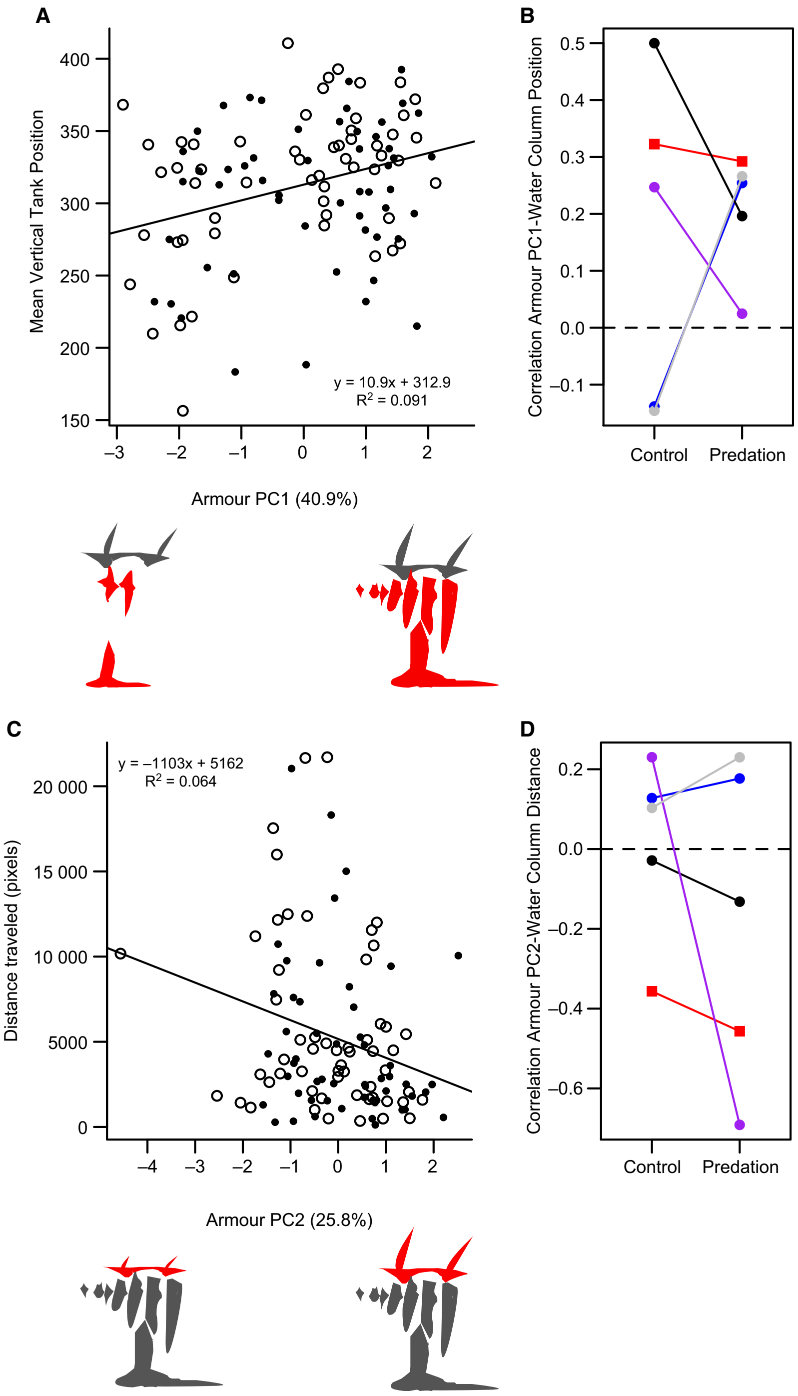

Figure 1. (A) Association between the mean position in the water column and armour PC1 with linear regression line. Trait variation in PC1 (lateral plates and pelvic spines) is shown in red along the *x*-axis. Each point is an individual from either a predation (filled symbols) or control (open symbols) pond. (B) Spearman's correlation coefficient between armour PC1 and mean vertical position in the water column for each pond. Paired ponds are connected with a line. The F3 families are circles and the family from First Lake is a square. (C) Association between armour PC2 and distance travelled during the water column assay with linear regression line. Trait variation in PC2 (first and second dorsal spines) is shown in red along the *x*-axis. Individuals from predation ponds are indicated with filled symbols and individuals from control ponds are shown with open symbols. (D) Spearman's correlation coefficient between armour PC2 and distance in the water column assay. Paired ponds are connected with a line. The F3 families are circles and the First Lake family is a square.

preference. Trout predation may have also led to non-consumptive changes in behaviour by reducing competition and increasing intimidation in the open water environment (Preisser, Bolnick & Benard, 2005). Our findings suggest that differential predation alone is unlikely to explain the differences in shoaling behaviour and water column preference observed in the wild.

The experimental ponds provide an improvement over behavioural studies conducted in mesocosms or in the laboratory because experimental subjects can be manipulated in a natural environment. However, the paired design limited the statistical power of this experiment to detect small differences in behaviour between treatments. Additionally, behaviours were assayed at a single end point, therefore if paired ponds did not start at the same trait value this would decrease our ability to detect a treatment effect.

CORRELATION BETWEEN MORPHOLOGY AND BEHAVIOUR

The likelihood that an individual escapes a predation event may be determined by an interaction between behavioural and morphological traits (e.g. Brodie, 1992; Dewitt, Sih & Hucko, 1999; Buskirk, 2000; Relyea, 2001). We found a correlation between behavioural traits and bony armour. Armour PC1 (increased pelvic armour) was associated with a higher position in the water column and armour PC2 (longer dorsal spines) was associated with increased movement during the water column assay. These correlations may be underestimated because behavioural traits have high variance and any measurement error can decrease the correlation between traits (Whitlock & Schluter, 2009). As a result, correlations between these traits in the wild are likely to be greater than reported in this study. Functionally these associations match the greater pelvic armour and preference for a higher water column position found in limnetics (Larson, 1976). A previous study by Grand (2000) found that, within benthic stickleback, those individuals with reduced pelvic armour were less bold than individuals with increased pelvic armour.

The observed correlations between armour morphology and behaviour could result from genetic linkage or pleiotropy (Schlosser & Wagner, 2004). Several inferences can be made regarding the possible genetic basis of the correlations. Recombination events in advanced generation hybrids should uncouple many traits that were genetically linked in limnetics and benthics; yet three generations of recombination were insufficient to break up the association between armour and behaviour in the F3 families and > 30 generations of recombination in First Lake ponds did not decrease the correlation. The maintenance of these correlations in spite of genome-wide recombination indicates that genetic linkage or pleiotropy underlies these associations.

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

When certain trait combinations are preferentially favoured, natural selection may directly or indirectly lead to an increase in the correlation between these traits (Sinervo & Svensson, 2002; Murren, 2012). While we describe a correlation between multiple armour and behavioural traits, the strength of these correlations did not differ between treatments. We were unable, therefore, to support the hypothesis that trout predation is the causal mechanism for the associations. The lack of change in correlation between treatments, however, could be a consequence of the limited power of our experiment, or insufficient variation in correlation for selection to act upon. Trout may have also played an important role during the historical divergence between benthic and limnetic stickleback. While trout predation may not be the proximate cause for the correlation between defence morphology and behaviour, it cannot be ruled out as the ultimate cause for this association. Future work examining the genetic basis of these traits will be required to elucidate the role of pleiotropy and linkage in behaviour and armour morphology in stickleback.

ACKNOWLEDGEMENTS

We would like to thank Kira Delmore for loan of the cameras and Andy LeBlanc for technical assistance. Seth Rudman and Dolph Schluter (D.S.) assisted with the set-up of the predation experiment. S.R., D.S., and two anonymous reviewers provided helpful comments to the manuscript. The experimental ponds were created with a grant from the Canadian Fund for Innovation (D.S.) and this project was funded by an NSERC discovery grant (D.S), and graduate fellowship (D.J.R).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Shoaling Assay Set-up. A standard aquarium tank was separated into a central arena and two end compartments using window mesh (dotted outline). The back and sides of the tanks were covered with white paper to reduce external visual cues. Tanks were backlit to increase the contrast between the focal fish and the background. The tank was filled with water to 32 cm.

Figure S2. Mean value for behavioural traits between control and predation ponds presented as reaction norms. The standard error is given for each pond. Each family is represented with a separate colour with the First Lake family given in red.

Table S1. Trait loadings from the Principle Component Analysis.

SHARED DATA

Data deposited in the Dryad digital repository (Miller SE *et al.*, 2016).