

The evolution of correlations between behavioural and morphological defence in Alaskan threespine stickleback fish (*Gasterosteus aculeatus*): evidence for trait compensation and co-specialization

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ABSTRACT

Background: Prey often make behavioural and morphological adaptations to avoid predation, and these alternative defence mechanisms may either compensate for, or reinforce, one another.

Objective: We examined correlations between anti-predator behaviour and morphology in threespine stickleback fish (*Gasterosteus aculeatus*) in four environments distinguished by their predation histories: marine/anadromous populations co-existing with native predatory fish (representative of the ancestral environment), and three freshwater environments with native, introduced, and no predatory fish, respectively. To determine whether morphological and behavioural defences reinforce one another (trait co-specialization) or whether they represent alternative strategies for defence (trait compensation), we related morphology of laboratory-reared stickleback to the intensity of their responses to a simulated predator attack, which had previously been assessed (Wund *et al.*, 2015).

Methods: Eight aspects of stickleback morphology were measured, all of which loaded heavily and positively on a single principal component that accounted for 61.3% of the variation in traits. Pearson correlation was used to determine whether PC1 was associated with the intensity of anti-predator response.

Results: A weak negative correlation was observed between anti-predator behaviour and morphology overall. However, considering each predation environment separately revealed that populations from marine and freshwater environments containing native predatory fish displayed trait co-specialization (positive correlation) between armour and behaviour, while those from environments with recently introduced predatory trout displayed trait compensation (negative correlation). No correlation was observed in populations lacking these predators. Not all populations within the ‘introduced predatory fish’ category showed the same pattern of relationship, however, indicating that additional factors mediate the co-evolutionary dynamics of anti-predator behaviour and morphology. Results were similar whether we considered size-standardized or unstandardized morphological traits.

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Conclusion: Our results suggest that over the long term, the two types of defence co-evolve to reinforce one another, but within the first few decades of exposure to predatory fish, anti-predator behaviour compensates for diminished morphological defences.

Keywords: anti-predator, behaviour, compensation, co-specialization, morphology, threespine stickleback.

INTRODUCTION

The influence of selective agents on the co-evolution of more than one trait in an organism is of great interest to many biologists. DeWitt *et al.* (1999) outlined four functional relationships that can exist between two traits: (1) co-dependence, (2) complementation, (3) co-specialization, and (4) compensation. A relationship of co-dependence can be considered when traits are mechanically linked and one of complementation when traits are mechanically independent, but the behaviour of the individual depends on morphology. Traits such as behaviour and morphology can each be effective on their own. In such an instance, we can focus on trait compensation and co-specialization as a means to describe the potential relationship that exists between these two types of traits (DeWitt *et al.*, 1999; Mikolajewski and Johansson, 2004). Compensation describes a negative correlation in which the intensity of one trait compensates for a reduction or lack of the other. In contrast, trait co-specialization represents a positive correlation in which behaviour and morphology reinforce one another (DeWitt *et al.*, 1999; Grand, 2000).

Compensation encompasses the idea that morphological traits require considerable energy to be both produced and maintained. Likewise, an opportunity cost can be associated with displaying intense fearful behaviour, such as hiding from predators, as less time is spent foraging or focusing on reproduction (Mikolajewski and Johansson, 2004). Therefore, it is presumed that the energetic cost requirements for morphology and behaviour will create an inverse correlation due to a trade-off between traits such as those involved in anti-predator defences (Grand, 2000). For example, an individual with robust armour would tend to be less fearful since their morphology has evolved to maximize their survival rate, meaning an increase in fear response is relatively unnecessary. However, in other instances, behaviour and morphology can each prove to be effective to the individual independently of the other. In such a case, prey can then evolve to enhance both aspects of their anti-predator defence (DeWitt *et al.*, 1999). Although this can be extremely costly, the benefits of survival will outweigh the costs of increased energy demands.

Phenotypes, including the morphology and behaviour of many organisms, are strongly influenced by their exposure to predators (Relyea, 2001b). The predation regime of a given environment can either enhance or reduce the intensity of an animal's anti-predator response via adaptive phenotypic plasticity (Relyea, 2001a, 2001b; Huizinga *et al.*, 2009; Aubret *et al.*, 2010; Dennis *et al.*, 2011; Herzog *et al.*, 2016) and/or through adaptive evolution (McPeck, 1999; O'Steen *et al.*, 2002; Fullard and Ratcliffe, 2006; Kishida *et al.*, 2007; Huizinga *et al.*, 2009; Langerhans, 2009; Nunes *et al.*, 2014). When populations of the same species occupy ecologically varied habitats, we can begin to observe changes that occur between and within these populations over developmental and evolutionary time scales.

On an individual level, the risk of predation to an animal can be reduced dramatically by enhancing both aspects of their anti-predator defence mechanisms. Most prey utilize various behavioural defences to reduce their chances of being spotted and captured by predators

(Huntingford and Wright, 1993; Magurran, 1999; Lind and Cresswell, 2005). The evolution of morphological defences, in contrast, allows for the animal to be difficult to grab hold of and ingest in the event that they are captured (Reimchen, 1992, 2000; Grand, 2000; Mikolajewski and Rolff, 2004). Thus, both behaviour and morphology play important roles in the overall survival of any prey.

Threespine stickleback fish (*Gasterosteus aculeatus*) are one of the most commonly used model systems for analysing the evolution of complex behaviour, as they readily alter their behaviour in response to changes in their surroundings (for reviews, see Huntingford and Wright, 1993; Huntingford *et al.*, 1994; Huntingford and Coyle, 2007; Bell *et al.*, 2013). These fish are ancestrally marine or anadromous, with the latter subset of these fish entering freshwater environments for reproduction (Bell and Foster, 1994; Walker and Bell, 2000; Aguirre *et al.*, 2008). Following the Pleistocene glacial retreats 12,000–20,000 years ago, thousands of freshwater stickleback populations were founded in coastal regions of the high latitudes of the northern hemisphere. Their descendants adapted to their new environments, independently evolving from their marine ancestors, forming one of the most extensively studied adaptive radiations (for reviews, see Bell and Foster, 1994; Gibson, 2005; Cresko *et al.*, 2007).

Numerous morphological differences emerged following freshwater colonization, including changes in armour, trophic morphology, and body shape (Lavin and McPhail, 1985, 1986, 1987; Bell and Ortí, 1994; Bell and Andrews, 1997; Walker and Bell, 2000; Aguirre *et al.*, 2008; Berner *et al.*, 2010). Oceanic stickleback possess lateral plates and dorsal/pelvic spines as a means to defend themselves from predators (Reimchen, 1994). Lateral plates protect them from crushing jaws, whereas dorsal and pelvic spines increase the diameter of the fish's body, making it difficult for them to be swallowed (Reimchen, 1994; Marchinko and Schluter, 2007). In marine environments, stickleback fish are exposed to both predatory birds and fish. While piscivorous birds impact all freshwater environments, variations in fish predators have been observed among freshwater lakes, in which some stickleback populations co-exist with native predators, others experience introduced sport fish for recreational fishing, while still others encounter no predators at all (Reimchen, 1994; Baker *et al.*, 2010). Stickleback from marine environments living with a greater abundance of predators and few areas of refuge tend to have larger bodies, longer spines, and a greater number of lateral plates than those in freshwater environments (Reimchen, 1994; Marchinko and Schluter, 2007). The most extreme reduction in armour, the loss of pelvic spines, only occurs in populations that lack predatory fish (Bell and Ortí, 1994).

Behavioural responses to predators can range from vigilance when encountering a dangerous environment to various evasive manoeuvres as a means of escape (Huntingford and Wright, 1993). All stickleback have the ability to display such responses, but the extent to which each individual does so varies. Some of this variability can be explained by variation in predation environment. Studies have shown that living with abundant predators causes the fish to display stronger fear responses than those from environments that lack predators (for reviews, see Huntingford and Wright, 1993; Huntingford *et al.*, 1994; Huntingford and Coyle, 2007). In previous behavioural experiments conducted in the laboratory, stickleback subjected to piscivorous predators typically darted to the bottom of the tank and assumed a sigmoidal body positioning with spines displayed (Giles and Huntingford, 1984). In general, stickleback from populations that co-exist naturally with predators tend to exhibit stronger responses to predator stimuli than those from environments where predators are less abundant (Giles and Huntingford, 1984; Walling *et al.*, 2004; Messler *et al.*, 2007; Bell *et al.*, 2010). Clearly, stickleback have extensive morphological and behavioural defences, both of which vary with respect to their local predator community. Here, we examine whether these two types of defences co-vary within individuals.

In threespine stickleback, a useful way to investigate the cause of variation (and co-variation) in defence mechanisms is to compare putative anti-predator adaptations displayed by different populations that encounter different types and abundances of predators (Giles and Huntingford, 1984; Messler *et al.*, 2007). Thus, it can be determined whether the nature of an individual's anti-predator defence depends largely upon their predatory environment, which would suggest adaptation to local conditions (Huntingford and Wright, 1993; Huntingford *et al.*, 1994).

Since stickleback exhibit both behavioural and morphological adaptations as a way of avoiding predators, we wished to examine the relationship between these two anti-predator defence mechanisms. This study is an extension of a previous experiment conducted by Wund *et al.* (2015), in which they examined the effects of relaxed and reverse selection on anti-predator behaviour in threespine stickleback from South-Central Alaska. The objective of the current study was to relate this previously quantified behavioural data to armour morphology, and to determine whether the patterns of correlations differ among predation environments. Examining the nature of the correlations allows us to determine which functional relationship outlined by DeWitt *et al.* (1999) best describes the relationship between behavioural and morphological defences in each of the four predatory environments. We considered both raw morphological measurements and measurements standardized for body size, because it is possible that either the absolute or relative size of armour impacts the intensity of behaviour. The absolute size of armour is most important to gape-limited predators, but individual stickleback might adjust their own behaviour with respect to the relative size of their own armour. By using predator-naïve, laboratory-reared fish, we were able to rule out the effects of direct experience and selective predation in establishing any relationships we observed.

METHODS

Populations and field collection

Adult stickleback were caught in June of 2005, 2006, 2007, and 2008. Fish from all twelve populations were collected from the Matanuska-Susitna River Valley of South-Central Alaska, except those obtained from Resurrection Bay and Anchor River, which were from the Kenai Peninsula. The fish were caught with un-baited minnow traps that were deployed in the shallow breeding grounds of each site (for sampling details, see Wund *et al.*, 2015). Three populations from each of four environments were studied, differing in their predator communities (oceanic, freshwater with native predatory fish, freshwater with introduced predatory fish, freshwater with no predatory fish; Table 1). Stickleback collected from the freshwater lakes of Willow, Beverly, and Cornelius contained native rainbow trout (*Oncorhynchus mykiss*). Kalmbach, Boot, and Bear Paw Lakes have no native trout, but do contain trout that were introduced to those lakes over the past three decades as a result of stocking. The stickleback inhabiting Jean, Whale, and Z Lakes did not co-exist with any salmonid predators.

Crosses and laboratory rearing

Each year, crosses were made from a subset of the twelve lakes sampled (see detailed methods in Wund *et al.*, 2015). Crosses were made by gently extruding eggs from gravid females. The females were then euthanized from an overdose of MS-222 and frozen for future analysis. The males

Table 1. Populations and sample sizes

Predation environment	Population	Coordinates	<i>N</i>
Oceanic (ancestral), native piscivorous fish	Anchor River	59.77444, -151.86530	31
Oceanic (ancestral), native piscivorous fish	Rabbit Slough	61.53606, -149.25381	48
Oceanic (ancestral), native piscivorous fish	Resurrection Bay	60.12622, -149.42050	22
Freshwater, native piscivorous fish	Beverly Lake	61.61508, -149.56567	57
Freshwater, native piscivorous fish	Cornelius Lake	61.37410, -149.15216	50
Freshwater, native piscivorous fish	Willow Lake	61.74875, -150.05539	22
Freshwater, no piscivorous fish	Jean Lake	61.72377, -150.06238	19
Freshwater, no piscivorous fish	Whale Lake	61.54395, -149.74781	59
Freshwater, no piscivorous fish	Z Lake	62.28530, -150.06411	57
Freshwater, introduced piscivorous fish	Bear Paw Lake	61.61390, -149.75325	48
Freshwater, introduced piscivorous fish	Boot Lake	61.72035, -150.12863	47
Freshwater, introduced piscivorous fish	Kalmbach Lake	61.60757, -149.56765	20

were also euthanized with MS-222. Fertilization took place after their testes were removed and macerated, allowing sperm to be spread over the eggs. The males were then frozen. Each day, the embryos were washed with a clean embryo medium consisting of distilled water at 6 ppt Instant Ocean® sea salt. The clutches were disinfected in a 1% iodine solution within 2–3 days after fertilization. Within 5 days post-fertilization, the clutches were shipped overnight to Clark University in Worcester, Massachusetts. They were stored in petri dishes and the embryo medium was changed each day. Any dead or abnormal embryos were removed.

The newly hatched F1 fish (fry) were kept in petri dishes until they absorbed their yolk sac and ate fortified ‘Platinum’ grade brine shrimp (Argent Co.). At this point, they were moved into pint-size and subsequently quart-size jars. After several weeks, they were transferred to 37-litre aquaria in one of six recirculating aquarium systems. The fish were then separated into full-sibling family groups. Here, they were fed brine shrimp nauplii until they were large enough to consume frozen adult brine shrimp and redworms (chironomid larvae). While in the aquaria, they were kept on an 8-hour light/16-hour dark cycle.

Behavioural experiments

The data from the behavioural trials were taken from a previous experiment, and the associated methods are detailed in Wund *et al.* (2015). Briefly, the tests were conducted in the following spring after the fish were collected. Each fish experienced one of two behavioural assays: an attacking trout model or a tern model. The trout model was used to simulate a chasing attack, while the tern model simulated an attack from overhead. The effectiveness of these methods in observing a stickleback’s behavioural response to both types of predators has been demonstrated previously (Messler *et al.*, 2007).

The responses of the fish were recorded using J Watcher v.1.0. When exposed to the predator, the fish typically darted away from the trout (often on a downward trajectory) and froze for a period of time. The freeze time as well as the latency to recover from the attack (when the fish began exploring the tank again and/or eating as before the attack) was recorded. Freeze and recovery times were highly correlated; moreover, not every fish

exhibited a freeze response. Thus, here we used recovery time to represent the behavioural defence of the fish. Once the behavioural assays were completed, the fish were euthanized in an overdose of MS-222 and measured for standard length. Their bodies were then preserved in 10% formaldehyde. They were then stained with Alizarin red solution in order to enhance visualization of the bony tissue. Once stained, they were stored in 70% isopropanol.

Morphological measurements

Nine aspects of armour in 480 fish were measured, including standard length (tip of premaxilla to end of vertebral column), lengths of the left and right pelvic spines, lengths of three dorsal spines, number of lateral plates, pelvic girdle score (Bell and Ortí, 1994), and body depth. All measurements were taken using a digital calliper measuring to the nearest one-thousandth of a millimetre. To ensure consistency, the measurements of body plates were taken from the left side of the fish each time. The traditional method was used for determining the pelvic girdle score, whereby for each component present, a score of '1' was given. Therefore, if there was no girdle, the fish received a score of 0, whereas if it possessed a fully expressed girdle, it received a score of 8 (Bell and Ortí, 1994; Grand, 2000). In determining body depth, the measurements were taken from the pelvic girdle to an area between the first and second dorsal spines. Precision was assessed by re-measuring 20 randomly selected fish and comparing the armour measurements using Pearson correlation. No significant differences were observed for any sets of measurements (left pelvic spine, right pelvic spine, plate count, pelvic score: $r = 0.999$; dorsal spine 1: $r = 0.989$; dorsal spine 2: $r = 0.974$; dorsal spine 3: $r = 0.931$; body depth: $r = 0.962$; all $P \ll 0.001$).

Size standardization method

All continuous morphological measurements (spine lengths and body depth) were size-standardized using the mean standard length of 41.33, following the allometric size adjustment described in Reist (1986) and employed by other studies of variation in fishes (Hendry and Taylor, 2004; Paterson *et al.*, 2010; Wund *et al.*, 2016). Standard length (SL) was measured with digital callipers as the distance between the anterior tip of the premaxilla and the end of the vertebral column. All linear traits were log (base 10) transformed, and then regression was used to compute either the pooled slope or common-within-groups slope coefficient for each linear trait, as appropriate (Reist, 1986). To determine which slope to use for standardization, log (trait value) was the dependent variable, population type was a fixed effect, and log SL was a covariate. The initial models included a population type $\times$ log SL interaction term. If the interaction term was non-significant (which only applied to dorsal spine 3), it was removed and the regression was re-computed to obtain the pooled slope coefficient. If the interaction term was significant (all traits except dorsal spine 3), then the common-within-groups slope coefficient was used. This coefficient (b) was then used in the following standardization equation for each trait: $T_{\text{adj}} = T_{\text{obs}} * (SL_{\text{av}}/SL_{\text{obs}})^b$, where T_{adj} = the adjusted trait value (not log-transformed), T_{obs} = the observed trait value (not log-transformed), and $SL_{\text{av}} = 41.33$ and SL_{obs} = the observed SL (not log-transformed) of each specimen.

Data analysis: determining correlations

All statistical analyses were conducted using STATISTICA v.12.0 for Windows, with the exception of Fisher's r to z transformation for comparing correlation coefficients (see below), which was performed with Vassarstats. We performed all analyses on both size-standardized and unstandardized data, because it is possible that the intensity of an anti-predator response is related to the absolute size of armour (i.e. correlated with unstandardized values) or instead to the relative size of armour (i.e. correlated to the size of armour relative to the size of the fish). Because armour characteristics were highly correlated, a principal component analysis (PCA) was used to identify major axes of variation, thus reducing the dimensionality of the dataset. Separate PCAs were performed for unstandardized and standardized measurements, respectively. The first two principal components from each analysis were log-transformed to better conform to the assumption of normality. In order to make all PC scores positive prior to $\log_{10}$ transformation, a value of 2 was added to all PC1 scores and a value of 4 was added to all PC2 scores. Pearson correlations were used to determine whether log PC scores and latency to recover from a simulated attack were correlated, with all fish pooled and for each predation environment separately. We repeated these analyses using PC scores generated from both unstandardized and standardized measurements. We used Fisher's r to z transformation to compare the correlation coefficients between each pair of population types. To account for increased family-wise Type I error due to multiple comparisons, we applied Holm's sequential Bonferroni correction to adjust α (Holm, 1979; Quinn and Keough, 2002).

We also applied a general linear modelling approach to explain variation in morphology (log PC scores). We considered population type (marine, freshwater/native predatory fish, freshwater/introduced predatory fish, freshwater/no predatory fish) as a fixed factor, population as a random factor nested within population type, and log recovery time as a covariate. We included a log recovery time $\times$ population type interaction to determine whether the relationship between PC scores and recovery time differed among population types.

RESULTS

Unstandardized morphology

All characteristics of armour were included in the principal component analysis using a correlation matrix (Table 2). All traits loaded heavily on PC1, which accounted for 61.3% of variation in armour traits. Thus, PC1 was used to represent the overall armour morphology of the fish. Positive values of PC2 (12.8% variance explained) were associated with deeper bodies and longer third dorsal spines (Table 2).

Behaviour and morphology correlations

Log standard length and log latency to recover were weakly but significantly correlated ($r = 0.109$, $P = 0.018$), indicating that larger fish had a tendency to perform more intense anti-predator responses than smaller fish. Considering all individuals together, a negative correlation was observed between log latency to recover from an attack and log PC1 ($r = -0.136$, $P = 0.003$; Fig. 1). However, separating individuals by their predatory environment revealed divergent relationships between the two defence mechanisms (Fig. 2). A

Table 2. Results of principal component analysis in which all eight aspects of armour characteristics for *Gasterosteus aculeatus* are represented

Armour type	PC1	PC2
Left pelvic spine	0.952	-0.222
Right pelvic spine	0.916	-0.263
Dorsal spine 1	0.802	0.032
Dorsal spine 2	0.857	0.203
Dorsal spine 3	0.517	0.595
Body depth	0.565	0.597
Pelvic girdle score	0.811	-0.378
Body plates	0.732	-0.118
Eigenvalue	4.902	1.028
Prp. Totl	0.613	0.128

Note: Armour characteristics were strongly correlated with PC1. Dorsal spine 3 and body depth were most strongly correlated with PC2.

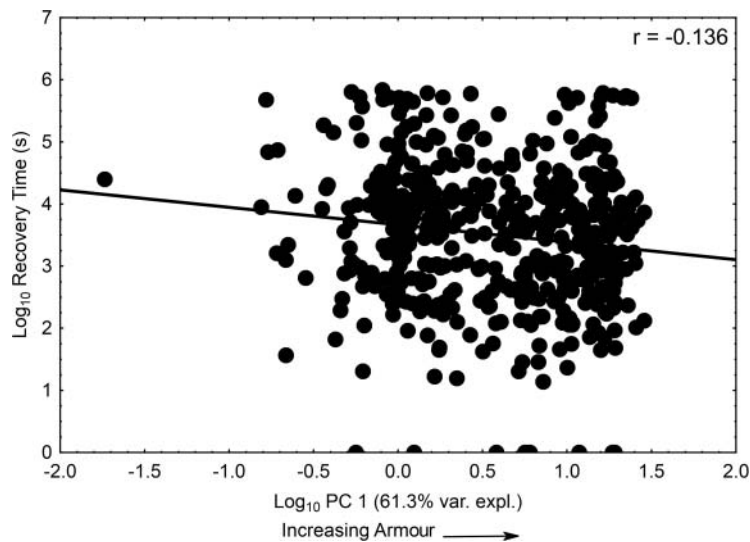


Fig. 1. Scatterplot depicting the relationship between $\log_{10}$ PC1 (armour) and $\log_{10}$ latency to recover from a simulated predator attack for all fish examined. Shown is the best-fit line and correlation coefficient from a Pearson correlation analysis. $N = 480$.

marginally non-significant positive correlation was observed in stickleback from the marine environment ($r = 0.191$, $P = 0.058$) and freshwater populations co-existing with native trout predators ($r = 0.169$, $P = 0.056$). These two correlation coefficients were statistically similar ($z = 0.17$, $P = 0.865$). Stickleback populations co-existing with introduced trout exhibited a negative correlation between $\log$ PC1 and $\log$ recovery time ($r = -0.226$, $P = 0.0156$). This correlation differed significantly from both marine ($z = 3.06$, $P = 0.001$) and freshwater with

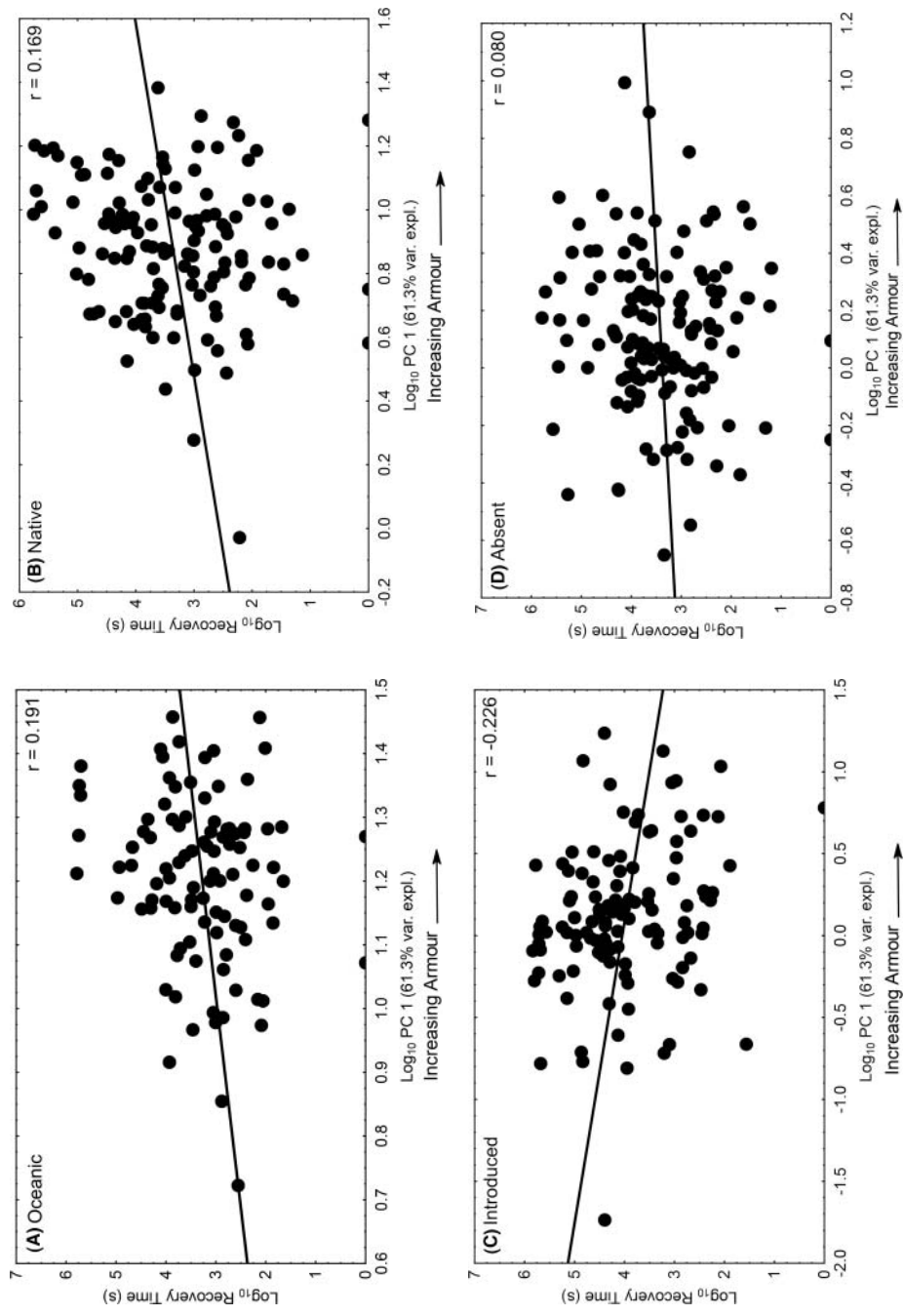


Fig. 2. Scatterplot depicting the relationship between $\log_{10}$ PC1 (armour) and $\log_{10}$ latency to recover from a simulated predator attack for fish separated by their predatory environments: (A) oceanic, (B) native, (C) introduced, and (D) no predatory fish. Each panel shows the best-fit line and correlation coefficient from a Pearson correlation analysis. Note the difference in x-axis scale for each panel.

native predator populations ($z = 3.08$, $P = 0.001$). Finally, in populations where the stickleback lived without any trout predators, no significant correlation in the two aspects of defence was observed ($r = 0.080$, $P = 0.359$). This correlation was statistically similar to the marine ($z = 0.85$, $P = 0.395$) and freshwater with native predator populations ($z = 0.72$, $P = 0.472$), and showed a marginally non-significant difference from populations with introduced predators ($z = 2.41$, $P = 0.016$; evaluated at $\alpha = 0.0125$ following sequential Bonferroni adjustment). When considering the relationship between log PC1 and log recovery time in individual populations, no significant correlations were found. All populations with native predatory fish showed positive (albeit non-significant) correlations, while the sign of the relationship varied among populations within the non-native and absent predator categories (Table 3).

The GLM results indicated strong influences of population type ($F_{3,460} = 12.08$, $P < 0.001$) and populations nested within type ($F_{8,460} = 25.54$, $P < 0.001$), and no significant influence of log recovery time ($F_{1,460} = 1.72$, $P = 0.190$) or a population type $\times$ recovery time interaction ($F_{3,460} = 0.41$, $P = 0.743$). The oceanic populations exhibited a greater robustness in armour over the freshwater populations. Freshwater stickleback co-existing with native trout possessed more armour than those co-existing with introduced predators or no predators at all (Fig. 3). The freshwater populations without native predatory fish had the most reduced armour. In a reduced model that excluded the population term, the interaction between population type and log recovery time was significant ($F_{3,460} = 12.08$, $P < 0.001$). Taken together, these results indicate that the substantial variation among populations overwhelms the signal of how the relationship between armour and behaviour is mediated by population type.

Log PC2 scores (which most strongly reflect the length of dorsal spine 3 and body depth; see above) and log recovery time were positively correlated when all fish were considered together ($r = 0.162$, $P < 0.001$). When separated by population, all correlations were positive, but none were significant (all $P > 0.08$). GLM indicated that population nested within population type explained a significant amount of variation in log PC2 ($F_{8,460} = 7.67$, $P < 0.001$), whereas population type ($F_{3,460} = 1.24$, $P = 0.307$), log recovery time ($F_{1,460} = 3.05$, $P = 0.082$), and their interaction ($F_{3,460} = 0.478$, $P = 0.698$) were not significant.

Table 3. Results of correlation analysis between log PC1 and log recovery time in individual populations

Predation environment	Population	<i>r</i>	<i>P</i>
Oceanic (ancestral), native piscivorous fish	Anchor River	0.1178	0.5280
Oceanic (ancestral), native piscivorous fish	Rabbit Slough	0.2131	0.1503
Oceanic (ancestral), native piscivorous fish	Resurrection Bay	0.1357	0.5574
Freshwater, native piscivorous fish	Beverly Lake	0.1624	0.2275
Freshwater, native piscivorous fish	Cornelius Lake	0.0877	0.5449
Freshwater, native piscivorous fish	Willow Lake	0.2648	0.2336
Freshwater, no piscivorous fish	Jean Lake	0.2655	0.2869
Freshwater, no piscivorous fish	Whale Lake	0.1420	0.2835
Freshwater, no piscivorous fish	Z Lake	-0.1066	0.4301
Freshwater, introduced piscivorous fish	Bear Paw Lake	0.0431	0.7712
Freshwater, introduced piscivorous fish	Boot Lake	-0.1143	0.4444
Freshwater, introduced piscivorous fish	Kalmbach Lake	0.0169	0.9453

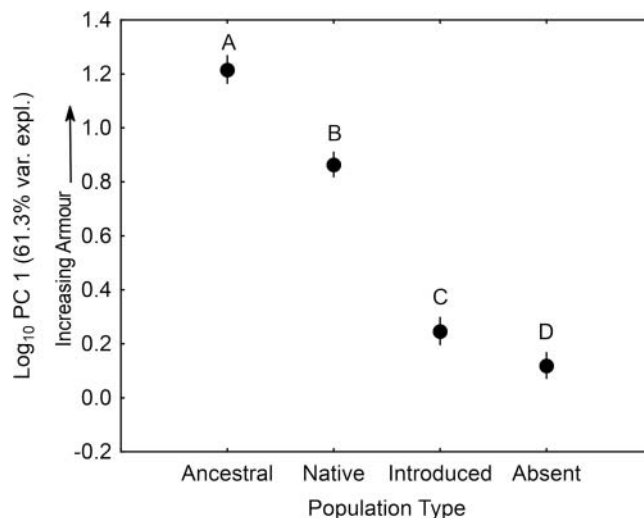


Fig. 3. Mean (and 95% CI) morphology ($\log_{10}$ PC1) of stickleback from the four different predation environments studied. Letters indicate significantly different groups based upon Tukey's HSD *post-hoc* test (all $P < 0.001$).

Size-standardized traits

Following PCA of size-standardized morphological traits, all traits loaded positively on the first principal component, which explained 63.7% of total variance. Only dorsal spine 3 loaded strongly on PC2 (10.7% variance explained; Table 4). The relationships between $\log$ PC1 and $\log$ latency to recover were qualitatively similar to those for unstandardized traits, indicating that the relationships described above did not simply reflect the relationship between body size and intensity of anti-predator response. There was a significant and weakly negative correlation when considering all groups together ($r = -0.161$, $P < 0.001$), a positive but non-significant correlation when considering oceanic fish only ($r = 0.163$,

Table 4. Results of principal component analysis for size-standardized traits

Armour type	PC1	PC2
Adj left pelvic spine	-0.956	0.206
Adj right pelvic spine	-0.959	0.203
Adj dorsal spine 1	-0.801	-0.038
Adj dorsal spine 2	-0.856	-0.080
Adj dorsal spine 3	-0.490	-0.739
Adj body depth	-0.627	-0.318
Adj pelvic girdle score	-0.823	0.346
Adj body plates	-0.758	-0.021
Eigenvalue	5.093	0.859
Prp. Totl	0.637	0.107

$P = 0.110$), a positive correlation in freshwater stickleback sympatric with native trout ($r = 0.186$, $P = 0.034$), a negative and marginally non-significant correlation in freshwater stickleback with introduced trout ($r = -0.181$, $P = 0.056$), and no correlation in stickleback allopatric with predatory fish ($r = 0.011$, $P = 0.905$). Patterns of correlations within individual populations were qualitatively similar to the results for unstandardized traits. Log PC2 and log recovery time were positively correlated when considering all individuals together ($r = 0.108$, $P = 0.020$), and within population types this relationship was also positive, albeit non-significant (all $P > 0.212$).

Likewise, the results of GLM analyses paralleled those for unstandardized morphology. Log PC1 scores differed among population types ($F_{3,450} = 11.65$, $P < 0.001$) and populations within type ($F_{8,450} = 32.30$, $P < 0.001$), whereas the population type $\times$ log recovery time interaction only became significant when the population term was removed from the model ($F_{3,450} = 3.85$, $P = 0.010$). GLM indicated that log PC2 differed among populations nested within population type ($F_{8,450} = 5.21$, $P < 0.001$), whereas population type ($F_{3,450} = 2.53$, $P = 0.066$), log recovery time ($F_{1,450} = 1.65$, $P = 0.199$), and their interaction ($F_{3,450} = 0.933$, $P = 0.425$) were not significant.

DISCUSSION

The primary goal of this study was to determine whether behavioural and morphological defences co-evolve, and if so, whether they exhibit a pattern of co-specialization or compensation. In this experiment, we tested two hypotheses exploring the different ways that threespine stickleback might experience co-evolution of their anti-predator defences. Our results demonstrate that not only do stickleback differ in their individual defence mechanisms according to their predatory environment, but also that differences in predatory environment, and the length of time over which stickleback are exposed to those environments, can lead to trait compensation and trait co-specialization relationships within the same species. While adaptive variation in behavioural and morphological defences in stickleback have often been considered in isolation (e.g. Giles and Huntingford, 1984; Bell *et al.*, 1993; Messler *et al.*, 2007; Wund *et al.*, 2015; Zanella *et al.*, 2015), selection by predators acts upon all aspects of defence simultaneously. While we found support for both the co-specialization and compensation hypotheses overall, not all populations within each predation environment showed consistent relationships between armour and behaviour, indicating that the co-evolutionary dynamics of armour and behaviour are impacted by many more factors than the presence or absence of predatory fish. Our results were consistent whether we did or did not use size-standardized morphological measurements. Thus, we cannot discriminate whether anti-predator behaviour relates to the absolute or relative size of armour. Because all of our fish were laboratory reared and tested within a few months of being the same age, size variation in our sample was much lower than we would observe in the wild. In general, our results support the hypothesis that predation history in threespine stickleback fish in part influences the co-evolution of anti-predator behaviour and morphology, and suggest that adaptive changes in behaviour may precede the evolution of robust morphological defence when novel predators enter the community. Consequently, an initial pattern of trait compensation appears to give way to a longer-term pattern of trait co-specialization.

Following the glacial retreats in South-Central Alaska, freshwater populations diverged substantially from their marine ancestors (Mäkinen *et al.*, 2006; Albert *et al.*, 2008; DeFaveri *et al.*,

2011; Hohenlohe *et al.*, 2012; Jones *et al.*, 2012). Throughout their wide geographic distribution, freshwater stickleback typically have reduced armour relative to oceanic stickleback (Bell and Ortí, 1994; Reimchen, 2000; Marchinko and Schluter, 2007; Zanella *et al.*, 2015). Consistent with other studies (Bell *et al.*, 1993; Reimchen, 1994), we found that stickleback from lakes with native salmonid predators tend to have more robust armour than those living with recently introduced predators or no predators at all, in accordance with many other studies spanning much of the geographic distribution of threespine stickleback. Our previous work (Messler *et al.*, 2007; Wund *et al.*, 2015) has also demonstrated that anti-predator behaviour co-varies with the presence of predatory fish in freshwater environments, whether or not those predators are native to a particular lake. Other authors have also found that stickleback from environments lacking piscivorous fish tend to have reduced anti-predator behaviour relative to populations that experience these predators (Giles and Huntingford, 1984; Huntingford *et al.*, 1994; Bell *et al.*, 2010).

Trait co-specialization had been observed in stickleback populations prior to this study. Lacasse and Aubin-Horth (2012) examined the coupling of defence mechanisms by quantifying morphological traits in wild-caught adults from two distinct freshwater Alaskan lakes and predator avoidance behaviour in lab-reared juveniles from the same populations. A comparative analysis between the armour of wild-caught individuals and fear responses of lab-reared fish revealed evidence for co-specialization, although armour and morphology were not measured from the same individuals. In addition, Kim and Velando (2015) investigated genetic and phenotypic correlations between anti-predator behaviour and cryptic coloration in lab-reared stickleback from a single population in Spain. They also found evidence of co-specialization, with more camouflaged individuals exhibiting greater boldness towards predators. Our investigation complements these two previous studies by incorporating a paired analysis on populations from two different types of high predation freshwater environments in addition to oceanic and low predation environments. Comparing stickleback from four distinct types of environments differing in their predator regime, we could make inferences about how the dynamics of compensation/co-specialization change over time following the arrival of a new predator to a community.

Most comparative ecomorphological analyses assume that observed patterns of divergence result from variation in one or more selective agents between environments. Such assumptions can be inaccurate if the historical context of the system is unknown (Losos and Miles, 1994). However, given the extensive knowledge of how predation impacts morphology and behaviour in the stickleback radiation, we can be fairly confident that the intraspecific differences in morphological and behavioural defence we observed are the result of divergent predation regimes acting on varying phenotypes. Given that our stickleback were reared in a common laboratory environment, it is also likely that the observed phenotypic variation was the result of underlying genetic variation. We observed significant correlations between the two defence mechanisms in pooled samples from environments with similar predation characteristics and history. This reinforces what we already know about the importance of predators to the evolution of morphology and behaviour in stickleback. With respect to populations lacking predatory fish, reduced armour and behavioural responses, as well as a lack of correlation between the two, suggests a relaxation of selection (Lahti *et al.*, 2009) on both types of defence.

Introducing predators to environments with native prey that have not experienced similar predators could have detrimental effects on the prey's fitness. If a prey does not exhibit the appropriate fear response, it risks the chance of being consumed (Nyström *et al.*, 2001). In

a species-poor environment where populations lack sufficient predators and competitors, the prey species may not recognize introduced inhabitants as a threat to their survival. Prey species subjected to such situations may not have evolved effective means of defence in order to cope with novel predators. If there is extensive variation in fear responses between individuals in a population, selection pressures influencing the evolution of morphological traits could vary tremendously (McPeck, 1999). Indeed, stickleback with reduced armour are most vulnerable to introduced predators (Patankar *et al.*, 2006; Baker *et al.*, 2010), and perhaps reduced anti-predator responses plays a role in their decline as well.

We know that morphology and behaviour can both together and independently play an important role in predator avoidance. However, a long-standing theory in evolutionary biology is that morphological changes might often be preceded by a change in behaviour. When an organism starts doing something new (e.g. using a new habitat, exploiting novel prey), selection pressures on correlated traits change, leading to evolutionary adaptation (Baldwin, 1902; Mayr, 1958, 1982; Weislo, 1989; West-Eberhard, 2003; Corning, 2014; Lister, 2014). This initial behavioural plasticity can itself evolve via genetic accommodation (West-Eberhard, 2003). The evidence we present here is consistent with such a scenario. Fish from environments with a long history of co-existence with piscivorous fish co-evolved robust armour and behavioural responses; however, fish from populations with only a recent history of exposure to these predators appear to compensate for their lack of armour defences by enhancing their fear response. One would predict that in time, more effective armour would evolve, becoming co-specialized with behaviour. The pace of adaptation will of course depend in part upon how much heritable variation in armour is present in these populations. An experiment conducted by Kitano *et al.* (2008) revealed that increased water transparency was correlated with an increase in stickleback armour following cutthroat trout introductions in Lake Washington. Cutthroat trout are visual predators, thus increased water clarity would improve their ability to find prey. Our results suggest that behaviour might be leading the way following the mitigation of eutrophication in Lake Washington, allowing adequate time for armour characteristics to evolve, thereby rescuing the population.

One caveat of using F1 individuals that have not experienced predation in a natural habitat is that the morphological and behavioural traits we observed are likely not entirely representative of wild-caught fish. The effects of learning, in particular, might alter how wild stickleback respond to predators, thereby influencing the relationship between anti-predator behaviour and armour. There might also be the potential of transgenerational maternal effects to influence these results. Previous work in insects (Storm and Lima, 2010) and stickleback (Giesing *et al.*, 2010) suggest that maternal exposure to predators during egg formation can influence both behaviour and development of their offspring. Storm and Lima (2010) found that gravid fall field cricket (*Gryllus pennsylvanicus*) mothers co-existing with an abundance of wolf spiders tend to produce offspring that are more responsive to predator cues, thus exhibiting increased survival. In stickleback, exposure to predators was correlated with larger egg sizes. These offspring exhibited tighter shoaling behaviours, a common anti-predator response that has been shown to also increase survival (Giesing *et al.*, 2010). Given our experimental design, we cannot rule out such non-genetic transgenerational maternal effects in the patterns of co-variation we observed.

The present study not only gives insight into the effects of predation on the co-evolution of morphological and behavioural defences, but also provides evidence to show that the introduction of novel predators can lead to contemporary evolutionary change in behaviour and morphology. We typically consider the effects of anthropogenic threats on

extinction risk, but it is becoming increasingly clear that species that are able to persist often experience shifts in phenotypic diversity as a result of phenotypic plasticity and/or rapid evolutionary change (Hendry *et al.*, 2008, 2010; Candolin, 2009; Sih, 2013).

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