ORIGINAL ARTICLE

PREDATION'S ROLE IN REPEATED PHENOTYPIC AND GENETIC DIVERGENCE OF ARMOR IN THREESPINE STICKLEBACK

Kerry B. Marchinko

Department of Zoology, 6270 University Blvd., University of British Columbia, Vancouver, BC V6T 1Z4, Canada
E-mail: kmarchin@zoology.ubc.ca

Predator-driven divergent selection may cause differentiation in defensive armor in threespine stickleback: (1) predatory fish and birds favor robust armor, whereas (2) predaceous aquatic insects favor armor reduction. Although (1) is well established, no direct experimental evidence exists for (2). I examined the phenotypic and genetic consequences of insect predation using F₂ families from crosses between freshwater and marine stickleback populations. I measured selection on body size, and size-adjusted spine (dorsal and pelvic) and pelvic girdle length, by splitting juvenile F₂ families between control and insect predation treatments, set in pond enclosures. I also examined the effect of insect predation on *Ectodysplasin* (*Eda*), a gene physically linked to quantitative trait loci for lateral plate number, spine length, and body shape. Insect predation resulted in: (1) significant selection for larger juvenile size, and shorter dorsal spine and pelvic girdle length, (2) higher mortality of individuals missing the pelvic girdle, and (3) selection in favor of the low armor *Eda* allele. Predatory insects favor less stickleback armor, likely contributing to the widespread reduction of armor in freshwater populations. Because size strongly influences mate choice, predator-driven divergent selection on size may play a substantial role in byproduct reproductive isolation and speciation in threespine stickleback.

KEY WORDS: Adaptation, diversification, divergent selection, *Ectodysplasin*, speciation.

According to the ecological theory of adaptive radiation, the main driver of phenotypic differentiation is divergent natural selection between environments stemming from differences in resources, habitat structure, and predator or competitor composition (Schluter 2000). Theory and research has focused mainly on the role of competition and differential resource use in phenotypic and genetic diversification in a wide range of organisms (Schluter 2000; Chase et al. 2002; Vamosi 2005; Langerhans 2006). More recently, there has been an increase in theoretical and empirical studies aimed at determining the role of predation in generating biological diversity (Reznick and Endler 1982; Vermeij 1987; Endler 1995; Abrams 2000; Nosil and Crespi 2006).

Empirical evidence of predation's role in diversification remains mostly indirect and limited to changes in phenotype between environments having different predators. The majority

of evidence comes from studies on bacteria and invertebrates (McPeek 1997; Mikolajewski et al. 2006; Nosil and Crespi 2006; Meyer and Kassen 2007; Diabate et al. 2008), with few vertebrate examples (Vamosi 2005; Langerhans 2006). Very recently, the evolutionary consequences of predator-driven divergent selection were elegantly demonstrated in Bahamas mosquitofish, *Gambusia hubbsi*, where low and high predation regimes have driven repeated shifts in morphological traits that also form the basis of assortative mating, and therefore reproductive isolation and speciation (Langerhans et al. 2007).
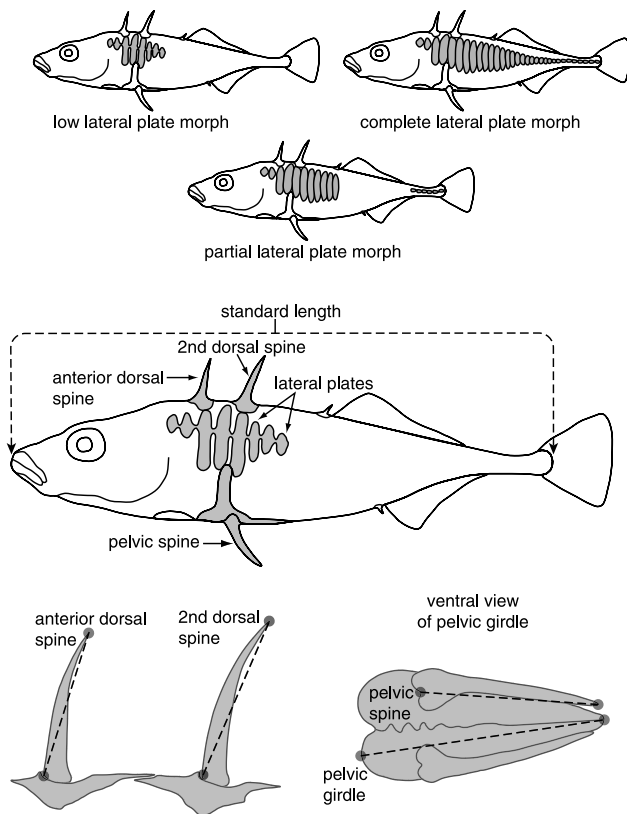
Threespine stickleback, *Gasterosteus aculeatus*, exhibit a correlation between predator community and the amount of external bony armor (Fig. 1). Stickleback from marine and lake habitats with a high abundance of piscivorous fish and birds (Reimchen 1994) are often heavily armored with long spines and more
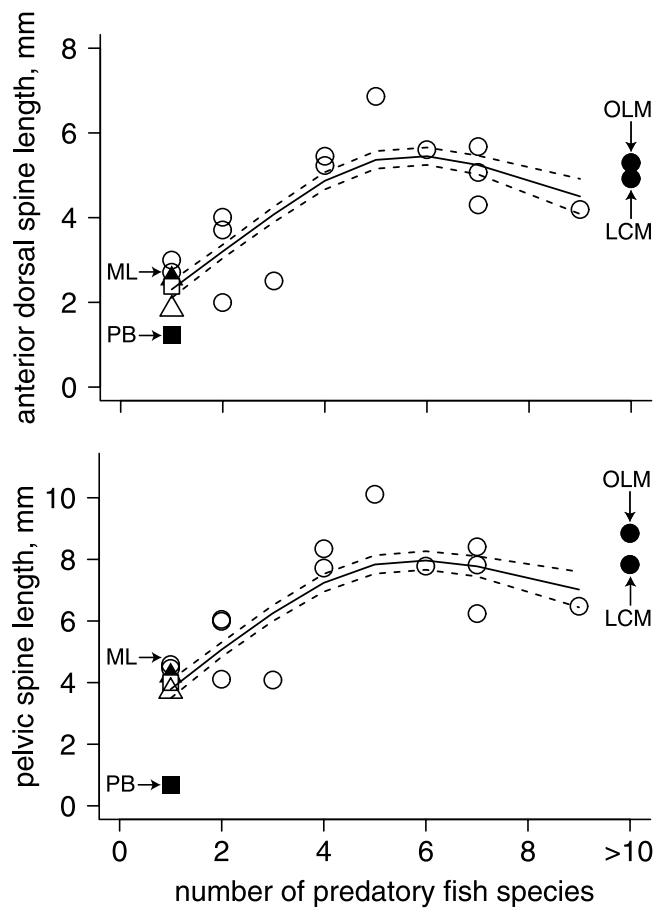
Figure 1. The positive association between spine length and the number of predatory fish species present in natural populations of threespine stickleback. Spearman's Rho = 0.762 and 0.781; *P* < 0.0002 for dorsal (top) and pelvic (bottom) spine length respectively. Each symbol represents the size-adjusted mean of 10 preserved museum specimens from 20 different populations of threespine stickleback from coastal British Columbia, Canada. Spine lengths were adjusted to a standard body length of 51 mm. The curve was estimated using the cubic spline (Schluter 1988); dashed lines represent standard errors generated from 10,000 bootstrap replicates. Open circles represent solitary lake populations, filled circles marine populations, squares represent limnetic (open) and benthic (filled) species from Paxton Lake, and triangles represent limnetic (open) and benthic (filled) species from Priest lake, Texada Island. Letters connected to symbols indicate the populations chosen for this study: Paxton Lake benthic (PB), McKay Lake (ML), Little Campbell marine (LCM), and Oyster lagoon marine (OLM). Data on the number of predatory fish species found in each population were obtained using Fish Wizard (www.fishwizard.com), a database maintained by the provincial government of British Columbia, Canada and the Freshwater Fisheries Society of British Columbia. The two marine populations were excluded from the test of correlation and spline estimation because the number of fish predators is unknown, although likely numerous. See Supporting Figure S1 for the location of each collection site.

lateral plates. Predaceous fish and birds are often gape-limited, only able to consume prey smaller than the size of their mouth. Longer spines increase the stickleback's effective diameter, cause injury, and make it difficult for gape-limited predators to swallow their prey, whereas lateral plates increase the chance of survival following attack (Hoogland et al. 1956; Reimchen 1992; 1994; 2000). By contrast, aquatic insects become important sources of predation on stickleback from freshwater habitats in which predatory fish and birds are uncommon. Here, stickleback often have short spines, few lateral plates, and some populations have lost the pelvic girdle and associated spines entirely (Reimchen 1980; Bell et al. 1993). Predaceous insects are not gape-limited, instead they use appendages to capture and hold onto prey. However, the small size of many aquatic insects restricts their ability to capture and consume large adult stickleback, constraining them to prey predominantly upon smaller juveniles (Foster et al. 1988). Reimchen (1980) hypothesized that spines provide points of leverage for aquatic insects to hold onto when capturing juvenile stickleback, and any reduction in the length, and number of spines would reduce the predators capture success. Taken all together, the widespread documentation of the relationship between predator regime and stickleback armor has led to the common assertion that differences in armor among populations result from predator-driven divergent selection (Hagen and Gilbertson 1972; Moodie et al. 1973, Gross 1977; Reimchen 1980; Reist 1980; Reimchen 1994; Bell 2001; Reimchen and Nosil 2002; Vamosi 2002; Colosimo et al. 2004; Reimchen and Nosil 2004; Ellegren and Sheldon 2008).

Although several experiments show that predation by gape-limited fish results in selection for longer spines and more lateral plates (Reimchen 1992, 2000; Vamosi and Schluter 2004), evidence that predatory aquatic insects select for armor reduction is scarce and indirect. For example, Reimchen and Nosil (2002; 2004) revealed a correlation between stickleback spine number and season. Spine number was lowest in the summer when predaceous insects are most active, suggesting that individuals missing spines are at a selective advantage when predatory insects predominate. Vamosi (2002) showed experimentally that mortality due to insect predation was greatest in unarmored benthic sticklebacks compared to lightly armored limnetic sticklebacks. His results also imply a selective advantage to individuals with reduced armor when insect predation is significant. In these cases, however, it is impossible to attribute differences in mortality to armor alone when additional traits and behaviors segregate between species under investigation. Alternatively, loss of armor in freshwater stickleback populations might also occur through an energetic trade-off resulting from changes in abiotic conditions. Differences in salinity, calcium, and phosphate concentration among populations may result in faster growth rates of

Figure 2. Illustrations of the threespine stickleback, *Gasterosteus aculeatus*, indicating the bony armor traits examined in this study in gray. Lateral plate morph (top of panel) was assessed by genotyping at an in/del marker (Stn381, Colosimo et al. 2004) within intron six of the *Ectodysplasin* gene. Five traits were quantified phenotypically: standard length, length of the anterior dorsal spine, length of the second dorsal spine, length of the pelvic spine, and length of the pelvic girdle.

individuals with reduced bony armor because more energy can be allocated to somatic and reproductive tissues as opposed to armor plates and spines (Giles 1983). Interactions between ion concentration and predation may also occur (Bell et al. 1993).

The recurrent evolution of low morph freshwater populations from completely plated marine ancestors has occurred through the fixation of a low morph allele at the *Ectodysplasin*, *Eda*, gene (Colosimo et al. 2004; 2005). Individuals with two low Eda^L alleles will typically be of the low morph, whereas those with two complete Eda^C alleles will most often be classified as the complete morph (Fig. 2; Colosimo et al. 2004). Depending on the alleles present at modifier loci, heterozygous individuals with one low Eda^L and one complete Eda^C allele may end up as a complete, a partial, or very rarely as a low morph (Colosimo et al. 2004; Creskso et al. 2004). In addition to the strong association of *Eda* with lateral plate phenotype, additional quantitative trait loci (QTL) are now known to be located near the *Eda* gene. QTL associated with *Eda* include body shape (Albert et al. 2008), the

length of the anterior dorsal spine (S. M. Rogers, unpubl. ms.), pelvic spine length (Shapiro et al. 2004), and possibly growth rate (Marchinko and Schluter 2007). Each of these traits show divergence between derived fresh water and ancestral marine populations.

I experimentally tested if predation by aquatic insects results in selection for reduced stickleback armor and changes in *Eda* allele frequency using the F_2 generation from a cross between divergent freshwater (reduced armor) and marine (robust armor) populations. The wealth of natural history and experimental data provide distinct predicted outcomes. Specifically, surviving juveniles from F_2 families exposed to insect predation should have larger mean body size (Foster et al. 1988), shorter mean dorsal spine, pelvic spine and pelvic girdle length (Reimchen 1980; Vamosi 2002), and a higher proportion of individuals missing the pelvic girdle (Reimchen and Nosil 2002), than families not exposed to predation. In addition, the association of *Eda* with several armor traits (Colosimo et al. 2004; Shapiro et al. 2004), suggests that exposure to insect predation should result in F_2 families with a higher proportion of the freshwater, low morph Eda^L allele.

Methods
FISH POPULATIONS
I collected threespine stickleback from four populations in southwestern British Columbia. The two marine populations, Oyster Lagoon (49°36′43″N, 124°01′57″W) on the Sechelt peninsula and Little Campbell River (49°00′52″N, 122°45′33″W) 45 km south of Vancouver, were characterized by long spines (Fig. 1), and a large, robust pelvic girdle. All individuals sampled for crossing were of the complete lateral plate morph (Fig. 2). The two freshwater populations, Paxton Lake (49°36′43″N, 124°01′57″W) on Texada Island and McKay Lake (49°36′43″N, 124°01′57″W) on Vancouver Island, were characterize by short dorsal spines (Fig. 1), and were of the low lateral plate morph (Fig. 2). In Paxton Lake, I only collected individuals of the benthic species (Schluter and McPhail 1992), of which the vast majority of individuals are missing the pelvic girdle and pelvic spines (McPhail 1994). All individuals from McKay Lake possessed a pelvic girdle and pelvic spines, but both traits were greatly reduced compared to the marine population (Fig. 1).

EXPERIMENTAL PROTOCOL
I created two sets of crosses between freshwater and marine populations. The details of crossing, fertilization, and fish husbandry can be found in the Supplementary methods in Supporting Information. The first set began with Paxton benthic males and Oyster Lagoon marine females to establish six separate F_1 lines (Paxton line). A single brother-sister pair from each F_1 Paxton line was crossed, establishing six separate Paxton line F_2 families

for study. The second set of crosses was started from a single McKay Lake male and a single Little Campbell marine female to establish one F_1 line (McKay line). Ten brother-sister pairs were then crossed to establish 10 F_2 families from the McKay line for study. Sample sizes before and after predation trials for each family from each set of crosses can be found in Supporting Table S1. Variation for dorsal and pelvic spine lengths, pelvic girdle length and the number of lateral plates was present in the F_2 progeny of both sets of crosses. Therefore, I examined the effect of predation on dorsal and pelvic spine lengths, pelvic girdle length, and *Eda* allele frequency in both crosses. Although using F_2 families generated from six pairs (Paxton line) or one pair (McKay lines) of wild-caught parents limits the amount genetic variation available in experimental families to that present in the original parents, juvenile F_2 families of both crosses exhibited similar levels of morphological variation upon which selection could act. The coefficient of variation for body size was approximately 10% in each F_2 family and the standard deviation of spine and girdle length residuals was similar in both crosses (Supporting Table S1). Only Paxton line F_2 families exhibited segregation variance in the presence and absence of the pelvic girdle and spine.

Predation trials were conducted in 20 wooden framed enclosures built into the shallow slopes of one experimental pond (23 m by 23 m) on the University of British Columbia campus. Five enclosures were placed on each side of the pond with the long axis perpendicular to the shoreline and sloping toward the 3-m deep center of the pond. The wooden frame of each enclosure measured 1.83 m long, 0.91 m wide, 0.91 m tall on its deepest side and 0.46 m tall on its shallowest side. The vertical sides were covered with 1-mm fine meshed door screen, sealed with silicon and buried into the sandy substrate of the pond. The top was covered with door screen to prevent adult dragonflies from laying eggs in the enclosures. Within each enclosure, I buried 16 floating artificial plants made from shredded green plastic bags to provide refuge for both predators and prey. The pond was then filled so that the water level sat just above the bottom of the shallow end of the enclosure, yielding a maximum water depth of approximately 0.5 m and a final volume of approximately 380 L in each enclosure. I seeded each enclosure with zooplankton captured by plankton tow in adjacent ponds immediately prior to introducing F_2 families.

Experimental trials began by splitting each F_2 family in half by randomly assigning individuals into one of two treatment enclosures: one predation treatment with two common aquatic insect predators of juvenile stickleback (Reimchen 1994), backswimmers (*Notonecta* sp.) and dragonfly naiads (*Aeshna* sp.), and one control treatment containing no predators. Although both *Notonecta* and *Aeshna* prefer to feed on the smallest fish available (Foster et al. 1988), these predatory species differ in their ability to capture and consume stickleback prey of different sizes. *No-tonecta* appear constrained to feed upon juvenile stickleback less than 15 mm in standard length, whereas *Aeshna* have been noted to capture and consume juvenile stickleback up to 25 mm long (Foster et al. 1988). Thus, the survival of juvenile stickleback exposed to predaceous insects in this experiment may occur through predator avoidance (avoiding detection or capture), as well as escape after capture. Juvenile F_2 families were acclimated overnight before predators were introduced. The following day *Notonecta* and *Aeshna* predators were caught in adjacent experimental ponds and added to the enclosure designated for the predator treatment. For each predator trial, the proportion of predatory insects to stickleback prey was 0.6 to 1, and the relative proportion of each predatory species was 0.62 *Notonecta* to 0.38 *Aeshna*, similar to the relative proportion of each species found in a natural stickleback lake with no natural predatory fish (Foster et al. 1988). Initial F_2 family size ranged from 62 to 98 fish in the Paxton lines and from 41 to 140 fish in the McKay lines.

The first trial began on June 1, 2006 and the last trial on September 11, 2006. Every two days, the remaining number of stickleback and predatory insects in each enclosure were counted after slightly disturbing the artificial plants and enclosure sides. Any predators found missing were replaced at each census. Trials were stopped when roughly 50% of the stickleback introduced to the predation treatment were found missing. The mean trial length was nine days, but ranged from six to 11. At the end of each trial, individuals from both treatments were exposed to a lethal concentration of tricaine methanesulfonate (MS-222, Syndel Laboratories Ltd., Qualicum Beach, BC, Canada), preserved in 95% ethanol, and brought back to the laboratory.

The caudal fin of each individual was removed, placed in a labeled 1.5 µL centrifuge tube, and preserved in 95% ethanol for genetic analysis. The remaining body of each individual was placed in a labeled 1.5 µL centrifuge tube and then fixed in 10% formalin for two weeks. I then stained all bony elements using Alizarin red, after which I preserved each individual separately in 40% isopropyl alcohol in preparation for morphological analysis.

Each stained individual was photographed using a Nikon DH1 digital camera. Morphological measurements were made on digital JPEG files using ImageJ version 1.37 (Rasband 2007). I measured five traits (Fig. 2): standard length, anterior dorsal spine length, second dorsal spine length, pelvic spine length, pelvic girdle length, and counted the number of lateral plates. In threespine stickleback, the total number of lateral plates is not determined until fish are subadults, roughly 30 mm in standard length (Bell 2001; Igarashi 1970). All of the juveniles introduced to the enclosures before trials began resembled the low plate morph and lateral plate morph could not be resolved from plate counts after the experiment ended. Instead, I genotyped a molecular marker diagnostic for lateral plate morph (Colosimo et al. 2005) to determine the most likely lateral plate phenotype of individuals.

GENETIC ANALYSIS OF THE ECTODYSPLASIN GENE

I isolated total genomic DNA from caudal fin clips of all six Paxton line F_2 families using standard phenol-chloroform extraction methods. The in/del locus, Stn381, within intron six of the *Ectodysplasin* gene (Colosimo et al. 2005) was used to identify the genotype corresponding to lateral plate phenotype of F_2 juveniles. *Ectodysplasin* alleles were amplified in 10 µl PCR reactions containing 5–15 ng genomic DNA, 1 µM of each forward and reverse primer, 1X PCR buffer, 0.25–0.125 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.25U AmpliTaq polymerase (Applied Biosystems, Foster City, CA). Cycling conditions were as follows: 93°C for 3 min, 95°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec, 5 cycles of 94°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec, 35 cycles of 90°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, followed by 72°C for 10 min, then cooled to 4°C. Amplified PCR products were separated by gel electrophoresis on an ABI 3733 automated sequencer using the GS500 Size Standard (Applied Biosystems) and freshwater low morph Eda^L and marine complete morph Eda^C alleles were scored using GENEMAPPER software (Applied Biosystems).

STATISTICAL ANALYSES

Standardized selection differentials (i) were calculated according to equation (6.1) in Endler (1986),

$$i = \frac{\bar{X}_a - \bar{X}_b}{\sqrt{\text{var}_b}},$$

where $\bar{X}_a$ and $\bar{X}_b$ were the mean trait values of fish from a single family measured at the end of the predation and control treatments, respectively, and var_b is trait variance in the control treatment. Selection differentials were calculated for each family separately and may be found in the Supporting Table S1. Selection on each trait was visualized using the relative survival function, $f(z)$ (eq. 8 in Anderson 1995),

$$f(z) = \frac{S_1}{S_2} \frac{h(z)}{1 - h(z)},$$

where S_1 and S_2 are the total sample sizes at the end of the experiment for the control and predation treatments, respectively. $h(z)$ is the conditional probability that a fish of phenotype z was present in the predation treatment at the end of the experiment given that it was present in the control treatment at the end of the experiment (Anderson 1995). $h(z)$ was estimated for each trait using nonparametric cubic spline (Schluter 1988) with family as a nominal covariate, individual phenotypes present in the control treatment set to zero (survival = 0), and phenotypes of those individuals remaining at the end of the predation treatment set to 1 (survival = 1). The standard errors of the relative survival function $f(z)$ were based on $h(z)$. To calculated standard error, S_1 and S_2 were fixed at observed values, and not resampled for each bootstrap sample. I assume that the phenotypic distributions of two

halves of an F_2 family divided randomly between treatments were roughly the same prior to selection and any difference between treatments after exposure to predation results from selection.

Paired *t*-tests were performed separately for Paxton and McKay lines, and separately for each trait, using family as the replicate to determine the significance of selection differentials (differences in means between treatments). Because spine and girdle lengths grow with body size, I corrected these traits for size using residuals from an ordinary least squares regressions of each trait on standard length. Standard length, the distance from the tip of the snout to the end of the vertebral column, is highly correlated with, and serves as a common measure of body size in fish (Baumgartner et al. 1988). Regressions were carried out separately on each family by combining fish from both treatments.

To analyze the Paxton line F_2 cross, I removed individuals missing the pelvic girdle and spines from the datasets prior to calculating the within family regression of pelvic girdle and pelvic spine on standard length. A separate analysis of the effect of aquatic insect predation on the survival of fish with and without the pelvic girdle was performed using a paired *t*-test between treatments on the difference in the proportion of individuals without a pelvic girdle at the end of trial.

The effect of aquatic insect predation on *Eda* allele frequency in the Paxton line families was analyzed using a paired *t*-test on the difference between treatments in the proportion of the freshwater low morph Eda^L allele (Δp) present at the end of a trial. Because of linkage, any change in the frequency of *Eda* may be due to selection directly on *Eda*, or to selection on traits with genes located near *Eda* on the same chromosome. I examined the association between *Eda* genotype and traits previously shown to be associated with the *Eda* region: the number of lateral plates, anterior dorsal spine length, and pelvic spine length (Colosimo et al. 2004; S. M. Rogers, unpubl. ms.; Shapiro et al. 2004). The association between *Eda* and size-corrected lateral plate number, anterior dorsal spine and pelvic spine length was tested using mixed-model analysis of covariance (ANCOVA; fixed effect: *Eda* genotype, random effect: family, dependent variable: each armor trait). In the analyses, *Eda* genotype was treated as numeric variable based on the number of low morph Eda^L alleles present (0,1, or 2). In addition, because low and complete lateral plate morphs differ in growth rate in fresh water (Marchinko and Schluter 2007), I also tested for and association between *Eda* genotype and body size (standard length), using a similar mixed-model ANCOVA. Note that because selection on *Eda* in the predator treatment could potentially bias the correlation of *Eda* genotype with morphological traits, I only used fish from the control (no predator) treatments in these tests.

Because of the specific direction of predicted outcomes, the significance level of *t*-tests was based on one-tailed probabilities. However, I report all tests in which the statistical significance
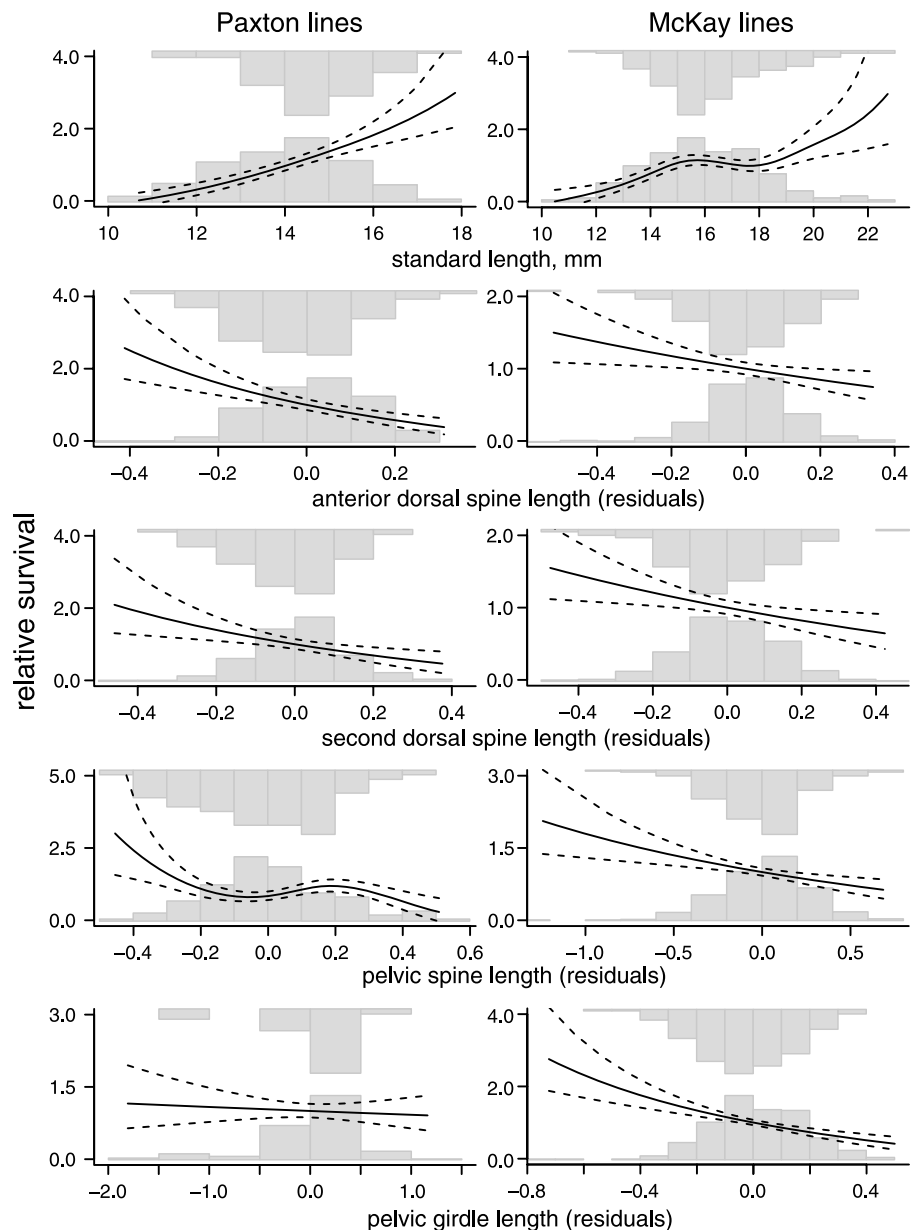
Figure 3. Relative survival function estimates (solid line) based upon the relationship between the probability of surviving aquatic insect predation and phenotype. Each panel is a visualization of the form of selection (Table 1) on stickleback size, spine, and pelvic girdle traits. Dashed lines represent standard errors generated from 10,000 bootstrap replicates. Frequency histograms show the phenotypic distribution of each trait, across all families for both the no-predation control (bottom histogram in each panel) and predation treatment (top histogram inverted in each panel).

of one- and two-tailed tests disagreed. All proportion data were arcsine-square root transformed prior to analysis. All analyses were carried out using R (R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org).

Results

SELECTION ON ARMOR TRAITS

Aquatic insects preyed most heavily upon the smallest and most heavily armored individuals within F_2 families. Predation re-

sulted in directional selection for larger body size in both crosses (Fig. 3): mean standard length was significantly greater in the predator treatment compared to the control (Table 1; difference between treatments was 0.7 and 0.4 mm in the Paxton and McKay lines, respectively). Selection resulting from insect predation produced similar patterns of shorter spine and girdle lengths in surviving juveniles from both Paxton and McKay lines (Table 1; Fig. 3). F_2 families split between treatments had shorter size-adjusted dorsal spine and pelvic spine and girdle lengths in the predation treatment compared to the control. However, the two

Table 1. Standardized selection differentials and significance tests for standard length and size-adjusted (residual) spine lengths in F_2 families split between two treatments: one with predatory aquatic insects, one without. The selection differentials shown below are the median of all families within each cross type. Mean trait values, standard deviations and selection differentials for each family can be accessed online in Supporting Table S1. $†P < 0.1$, $* P < 0.05$, $** P < 0.01$ for one-tailed tests. Note that the two-tailed test of standard length in the McKay lines was not significant ($P = 0.0873$).

	Paxton lines			McKay lines		
	Median selection differential (i)	$t_{0.05(1),5}$	P	Median selection differential (i)	$t_{0.05(1),9}$	P
Standard length	0.403	−3.939	0.005**	0.352	−1.918	0.044*
Anterior dorsal spine length	−0.246	4.947	0.002**	−0.103	1.078	0.154
Second dorsal spine length	−0.065	1.012	0.179	−0.121	1.479	0.087†
Pelvic spine length	0.011	0.132	0.450	−0.193	1.414	0.095†
Pelvic girdle length	0.011	0.246	0.408	−0.359	2.290	0.024*

sets of crosses differed with respect to which armor traits experienced significant selection (Table 1; Fig. 3), and the direction of selection varied among families in some traits (Supporting Table S1). In McKay lines, mean size-adjusted pelvic girdle length was significantly shorter in predation treatment than in the control group (Table 1). Dorsal and pelvic spines were also shorter in predator compared to control treatments and differences approached significance (Table 1). Similarly, in Paxton line F_2 families, predation favored individuals with significantly shorter anterior dorsal spines (Table 1; Fig. 3).

Five of the six Paxton line F_2 families exhibited segregation for pelvic girdle loss in a nearly 3:1 ratio of girdle presence to girdle absence (control treatment in Fig. 4). In contrast to expectation, however, the proportion of individuals missing the pelvic girdle was lower in the predator treatment (8%) than in the control (21%; Fig. 4), and this difference between treatments approached significance ($t_{0.05(2)4} = -2.308$; $P = 0.08$). Although insect predation resulted in the higher survival of individuals with shorter pelvic girdles (Fig. 3), the complete loss of the pelvic girdle was detrimental to survival in the presence of predatory insects (Fig. 4).

SELECTION ON THE ECTODYSPLASIN GENE

Eda allele frequency differed significantly between predator and control treatments ($t_{0.05(1)5} = 2.528$; $P = 0.026$), and predation was greatest on individuals with complete morph Eda^C alleles (Fig. 5). The frequency of the low morph Eda^L allele was 12% higher in predation treatments compared to that in the control (within family Δp ranged from -0.002 to 0.25; Supporting Table S2). The higher frequency of the Eda^L allele in predation compared to control treatments was due to an increase in the proportion of individuals with either heterozygous ($Eda^{C/L}$), or homozygous low morph ($Eda^{L/L}$) genotypes at the *Eda* locus (Fig. 5). Thus, in the absence of countervailing selective forces, selection on juvenile stickleback by predatory freshwater insects

will lead to an increase in the frequency of the partial and low lateral plate morphs in adults.

Eda genotype was a significant covariate for size-adjusted anterior dorsal spine length (Table 2). Individuals homozygous for the low morph Eda^L allele had shorter size-adjusted anterior dorsal spines than individuals with two complete morph Eda^C alleles. Size-adjusted lateral plate number did not differ between predation and control treatments ($t_{0.05(2)5} = -0.9538$, $P = 0.384$) and *Eda* genotype was not a significant covariate for lateral plate number in juveniles (Table 2). *Eda* genotype was not a significant covariate for either standard length, or size-adjusted pelvic spine length (Table 2).

Discussion

Predation by aquatic insects resulted in higher survival of individuals with shorter spines and pelvic girdles, greater body length, and led to an increase in the frequency of the freshwater, low
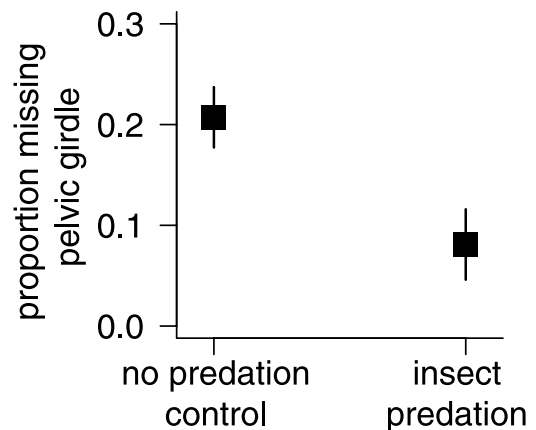
Figure 4. Proportion of F_2 juvenile stickleback missing the pelvic girdle in no−predation control and predation treatments. Points represent the among family mean (± 1 SE) from five F_2 families generated from the Paxton lines.
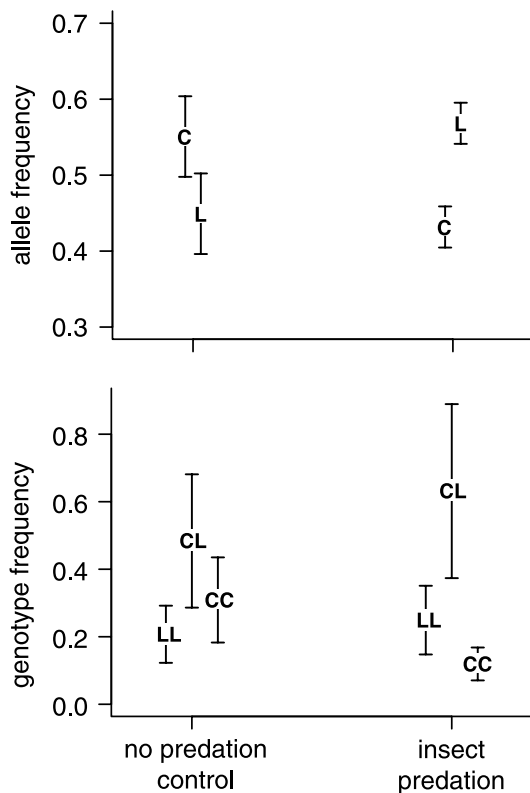
Figure 5. Allele frequency (top) and genotype frequency (bottom) at the *Ectodysplasin* locus in F$_2$ juveniles from control and predation treatments. Letters indicate among family mean (± 1 SE). In the top panel, L corresponds to the freshwater origin, low morph *Eda^L* allele and C represents the marine origin, complete morph *Eda^C* allele. In the bottom panel, LL, CL, and CC represent the low morph *Eda* homozygote, the heterozygote, and complete morph *Eda* homozygote genotypes, respectively. Data are from the six F$_2$ families generated from the Paxton lines.

morph *Eda^L* allele in F$_2$ families. This provides critical evidence that selection resulting from aquatic insect predation contributes to the reduction of stickleback armor during their radiation in freshwater lakes and streams. Moreover, this study, in conjunction with ample evidence of selection for greater levels of armor when fish and bird predation is high (Reimchen 1992; 1994; 2000), strongly indicates that divergent selection, based on differences in predator regime among populations, is one mechanism contributing to the vast morphological diversification observed in threespine stickleback.

Insect predation upon juvenile stickleback clearly results in a reduction of armor traits in the direction predicted from natural populations (Fig. 3). It is important to note though, that selection was demonstrated on juveniles from F$_2$ crosses between divergent populations, rather than wild caught, or pure population lines. As such, recombination will have reduced trait correlations arising from linkage disequilibrium between distant genomic locations. This makes it likely that the effects of predation on armor were

isolated from effects on unlinked behavioral or physiological traits relevant to predator avoidance and escape (Bell et al. 2007; Huntingford et al. 1994). However, several armor traits map to the same genomic region near *Eda*, including lateral plates, dorsal, and pelvic spines. Covariance of several armor traits increases the ability to detect total selection on armor, but results in an inability to distinguish which trait(s) are the target of selection. The application of multiple regression to selection analyses can often distinguish which traits are under the strongest selection, but they assume that all important characters were measured (Lande and Arnold 1983). Because *Eda* genotype was correlated with dorsal spine length, and possibly with unmeasured or unknown traits also affecting fitness (Albert et al. 2008; Barrett et al. 2008), further work is necessary to distinguish which characters are the most important to surviving insect predation.

Predatory insects likely impose selection for reduced armor in freshwater populations of threespine stickleback via two mechanisms. First, insect predation may result in armor reduction through direct selection on specific traits. Longer stickleback spines may increase the ability of predatory insects to hold onto and consume their prey (Reimchen 1980). Whether spine length, or the absence of specific spines, influences the probability that a stickleback escapes after capture by predatory insects remains to be confirmed. A greater number of lateral plates is associated with reduced velocity and displacement during the fast-start escape response (Bergstrom 2002), making it more likely for predaceous insects to capture stickleback with higher lateral plate counts. However, juvenile stickleback differed little in lateral plate number and the number of lateral plates did not differ between predation and control treatments in this experiment. Any direct effect of predation on lateral plate number is likely limited to adults that are largely invulnerable to predatory insects (Foster et al. 1988). Secondly, insect predation may result in reduced armor indirectly through selection on differences in growth rate: growth rate is slower in stickleback with more armor than those with reduced armor (Marchinko and Schluter 2007). In the current study, however, selection on spine and girdle length was independent of body size, suggesting that the reduction in armor in natural populations is not entirely due to the association between growth rate and armor.

The parallel reduction of stickleback armor during the colonization of fresh water is likely due to the action of many selective agents. Although insect predation appears important, my results do not necessarily imply that it is the sole, or even the predominant factor. Ion concentration is often low in freshwater environments. Reduced levels of calcium, phosphate, or other ions, may impose a trade-off in growth rate between heavily and lightly armored fish that is rooted in the development of bony armor versus soft tissues (Giles 1983). Increased reproductive output and higher overwinter survival are commonly associated with faster

Table 2. Mixed-model ANCOVA results testing the effects of genotype at the *Eda* locus, family as a random factor, and their interaction on standard length, and size-adjusted lateral plate number, anterior dorsal spine, and pelvic spine length in F_2 families from the Paxton lines. *Eda* genotype was treated as a numeric variable based on the number of low plate *Eda^L* alleles present in an individual (*Eda^{C/C}* = 0, *Eda^{C/L}* = 1, *Eda^{L/L}* = 2).

Source of variation	df	MS	F	P
(A) Standard length				
Genotype	1	0.0002	0.00006	0.994
Family	5	17.165	10.165	<0.0001
Genotype × family	5	3.472	2.056	0.074
Residual	150	1.689		
(B) Number of lateral plates				
Genotype	1	12.603	4.081	0.099
Family	5	0.139	0.078	0.995
Genotype × family	5	3.088	1.740	0.129
Residual	150	1.775		
(C) Anterior dorsal spine length				
Genotype	1	0.067	11.634	0.019
Family	5	0.003	0.227	0.951
Genotype × family	5	0.006	0.402	0.847
Residual	148	0.014		
(D) Pelvic spine length				
Genotype	1	0.077	1.610	0.260
Family	5	0.024	0.703	0.622
Genotype × family	5	0.047	1.397	0.231
Residual	148	0.034		

growing, larger fish (Wootton 1984; Thompson et al. 1991; Ludsin and DeVries 1997). If faster growth rates of individuals with less armor yield larger adult stickleback, then body size may also feature as a target of selection during episodes of armor reduction. As such, the relationship between armor, body size, and growth rate warrants further investigation.

Predatory insects preyed more heavily upon stickleback missing, rather than possessing, a pelvic girdle. This result appears to contradict Reimchen's original hypothesis that individuals lacking spine and girdle structures should experience the lowest mortality from predaceous insects (Reimchen 1980). If the pattern I observed reflects mechanisms occurring in wild populations, then predation by insects cannot be the selective agent driving pelvic girdle loss in threespine stickleback. Alternatively, negative fitness epistasis may be common in crosses between widely divergent populations with different genetic backgrounds (Leips and Mackay 2000; Ungerer et al. 2003; Rogers and Bernatchez 2007). Negative fitness epistasis occurs when the insertion of novel alleles into a widely divergent genetic background results in decreased fitness. For epistasis to account for the pattern observed in this study, individuals lacking a pelvic girdle, yet possessing marine alleles at a particular, but different set of loci must suffer the highest predation rates. Only those individuals that lack a

pelvic girdle, but also possess freshwater alleles at other essential loci survive. Intriguingly, such negative epistatic interactions may account for the genetic incompatibility and decreased hybrid fitness that occurs in other wide crosses between parents from strongly divergent threespine stickleback populations (Mckinnon and Rundle 2002).

DIVERGENT SELECTION BY PREDATORS: IMPLICATIONS FOR STICKLEBACK SPECIATION

Divergent selection on body size arising from different predator regimes may feature prominently in the evolution of reproductive isolation in threespine stickleback. In this study, insect predation had a large effect on juvenile body size. Larger, faster-growing individuals survived more often than smaller, slower-growing individuals. Growth rate and body size may influence fitness in a variety of ways (Brown et al. 1993; Arendt 1997). In addition to influencing susceptibility to predators, size is positively correlated with reproductive output (Wootton 1984) and overwinter survival (Thompson et al. 1991; Ludsin and DeVries 1997). Moreover, adult size strongly influences mate choice in reproductively isolated anadromous-stream and benthic-limnetic pairs of threespine stickleback (Nagel and Schluter 1998; McKinnon et al. 2004; Vines and Schluter 2006). Males and females most

often choose to mate with individuals that most closely resemble their own size. If differences in juvenile growth rate persist through to breeding adults, predator-driven divergent selection on juvenile size may be one ecological mechanism by which size-based assortative mating is established.

Predation's role in the evolution of reproductive isolation may also involve *Eda*. Mate choice in stickleback also appears to be based, at least in part, on body shape. Benthic species mate more often with benthic-shaped individuals from allopatric populations, and limnetic species with more limnetic-shaped allopatric individuals (Vines and Schluter 2006). In an F_2 cross generated from highly divergent populations similar to those used here, Albert et al. (2008) found that the genomic region in tight linkage with *Eda* had multiple effects on body shape in the head and pelvic regions. It remains unknown whether changes in stickleback body shape are due to pleiotropic effects of *Eda,* or linkage with other genes for body shape. However, *Eda* appears to have widespread pleiotropic effects on tissue and bone morphology in both mice and humans (Colosimo et al. 2005). My work demonstrated that exposure to insect predation resulted in a higher frequency of individuals possessing the low morph Eda^L allele due to decreased survival of individuals with homozygote $Eda^{C/C}$ genotypes. If changes in stickleback body shape occur through the pleiotropic effects of *Eda*, then predation may influence assortative mating through its effects on *Eda* allele frequency. This would be of particular importance when freshwater populations fixed for the low morph Eda^L allele still experience gene flow from, or come into secondary contact with, anadromous populations fixed for the complete morph Eda^C allele.

Reproductive isolation as a byproduct of adaptive differences in size and shape between geographic pairs of threespine stickleback is traditionally thought to arise via divergent (or disruptive) selection driven by resource competition (Schluter and McPhail 1992; McPhail 1994; Taylor and McPhail 2000; Mckinnon and Rundle 2002). The effect of insect predation on juvenile size and *Eda* allele frequency, suggests that divergent selection arising from dissimilar predator communities may have been an important source of phenotypic differentiation upon which mate choice is based. Along with the diversifying effects of resource competition, predation likely contributes to byproduct reproductive isolation during adaptive radiation in the threespine stickleback species complex.

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Supporting Information

The following supporting information is available for this article:

Table S1. Means, standard deviations and selection differentials for standard length and all size-adjusted armor traits from each F^2 family in the Paxton and McKay lines.

Table S2. Change in frequency (Δp) of the low morph *Ectodysplasin* allele, Eda^L, between no-predator control and predator treatments in F_2 families generated from the Paxton lines.

Figure S1. The positive association between spine length and the number of predatory fish species present in natural populations of threespine stickleback. Spearman's Rho = 0.762 and 0.781; $P < 0.0002$ for dorsal (top) and pelvic (bottom) spine length, respectively.

Supporting Information may be found in the online version of this article.
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