MECHANISMS OF DIVERGENCE IN THREESPINE STICKLEBACK (*GASTEROSTEUS ACULEATUS*)

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ABSTRACT

One of the most impressive examples of parallel divergence in nature is the repeated loss of armour in freshwater threespine stickleback, yet we have little evidence for the mechanisms driving this evolutionary change. I tested two ecological factors hypothesized to drive armour divergence between marine and freshwater populations and examined the mechanisms maintaining armour variation within a polymorphic population of stickleback.

I demonstrate how differences in salinity between marine and freshwater habitats might indirectly drive the reduction of lateral plate number in freshwater. Offspring with a reduced number of armour plates grew much faster than offspring with many plates when raised in fresh water, but not salt water. Larger size is positively associated with two measures of fitness, survival and reproductive potential, suggesting that the parallel loss of plates in fresh water arose through a correlated response to selection for faster growth during plate development.

I show that predation by freshwater aquatic insects favours armour reduction and larger body size. Using an F_2 intercross between divergent marine and freshwater populations, I measured selection on body size, spine traits and *Ectodysplasin*, a gene linked to quantitative trait loci for plate number, spine length, and body shape. Insect predation, when compared to experimental controls, resulted in increased juvenile size, shorter dorsal spine and pelvic girdle length after accounting for size, and favoured the low armor *Ectodysplasin* allele.

My work on an *Ectodysplasin* polymorphism in a freshwater population revealed a rare example of a stable polymorphism with heterozygote disadvantage. Genetic analysis revealed that selection rather than population subdivision or assortative mating accounts for the observed heterozygote deficiency. Differences in carbon isotope signature hint that niche separation exists between *Ectodysplasin* homozygotes, providing an ecological mechanism (frequency-dependent selection) for the maintenance of polymorphism despite heterozygous disadvantage. Overall, my thesis contributes to a growing body of empirical work revealing the powerful effects of divergent selection and highlights the benefits of integrating genetic and ecological approaches for understanding the mechanisms maintaining variation and promoting diversification in wild populations.

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CO-AUTHORSHIP STATEMENT

Chapter 2 was a collaboration between myself and my research supervisor Dolph Schluter. Dolph aided in experimental design, data analysis and helped edit the manuscript. Data was collected, analysed and the manuscript written by myself.

Chapter 4 was a collaboration between myself, Blake Matthews, Sean Rogers and Dolph Schluter. Blake carried out the mass-spec analysis of the stable isotope samples and helped with field collections. Sean helped choose appropriate microsatellite markers, aided in the population genetic analyses and commented on the manuscript. Dolph aided with the development of the selection model, data analysis and manuscript editing. I was responsible for the experimental design, collection of samples, analysis of all data, molecular genetics bench work, and writing the manuscript.

CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

The ecological theory of adaptive radiation states that divergent selection forms the basis of evolutionary diversification and ultimately speciation (Schluter 2000). To properly identify ecology's role in diversification, we must begin by demonstrating that selection is divergent between environments and that ecological mechanisms select against hybrids in natural environments (Schluter 2000). Although populations often diverge in traits associated with habitat differences, the genetic and ecological mechanisms underlying phenotypic divergence are often not well understood in most cases. In this thesis, I experimentally test two of the ecological factors hypothesized to govern armour divergence between marine and freshwater populations of threespine stickleback, *Gasterosteus aculeatus* L, as well as examine the factors maintaining polymorphism with heterozygote disadvantage at a major armour gene. The following chapters are independent manuscripts containing more detailed background than will be covered in this brief introductory chapter.

The extensive data on natural history and the availability of genomic resources have made threespine stickleback a model system in which to ecology's role in adaptive diversification (Gibson 2005). In British Columbia, marine stickleback colonized freshwater lakes and streams recently, roughly 12,000 years ago. Freshwater populations often exhibit parallel divergence in amour morphology from marine ancestors, largely in the reduction of bony armour plates and spines. The multiple, independent colonization of coastal freshwater populations from marine ancestors is supported by sequence variation in mitochondrial DNA and microsatellite loci (Colosimo et al. 2005; McKinnon

et al. 2004; Taylor and McPhail 1999; Taylor and McPhail 2000; Thompson et al. 1997). Marine populations are often heavily armored with a full row of bony plates running down both sides of the body (complete morph) and long dorsal and pelvic spines (Fig. 1.1). Freshwater populations generally have few lateral plates clustered around the pectoral fin (low morph) and short dorsal and pelvic spines (Fig. 1.1). Recently, two major genes were discovered to govern most of the phenotypic variance in pelvic spine length and lateral plate number between marine and freshwater populations. Shapiro et al. (2004) revealed that regulatory changes to the *Pitx1* gene on chromosome 7 are responsible for the majority of phenotypic variance (up to 65%) in the length of the pelvic spine and girdle. Colosimo et al. (2005) identified that the *Ectodysplasin* (*Eda*) gene is the major locus responsible for roughly 75% of the variance in lateral plate number (Colosimo et al. 2004). Interestingly, most freshwater populations examined — 14/15 populations from the Eastern and Western Pacific and Atlantic — share many of same nucleotide changes within and near the *Eda* gene. Colosimo et al. (2005) conclude that the parallel reduction of lateral plate number in freshwater most likely occurred through repeated selection of an ancient low lateral plate *Eda* allele most introduced to freshwater populations from the sea.

Molecular evidence suggests that armour divergence between marine and freshwater habitats is adaptive (Cano et al. 2006; Raeymaekers et al. 2007) and, although similar ecological conditions may have selected for reduction of armour in freshwater threespine stickleback, researchers have had little success explaining the mechanism(s) of armour divergence (Bell 2001; Bertin 1925). Differences in lateral plate number and spine length between marine and freshwater populations are primarily thought to have

arisen from differences in ion concentration (Bertin 1925; Bourgeois et al. 1994; Francis et al. 1986; Giles 1983; Heuts 1947; Klepaker 1995; Klepaker 1996) or differences in predator community and predation rate (Bell 2001; Bergstrom 2002; Reimchen 1980; Reimchen 1994; Reimchen 1995; Reimchen 2000).

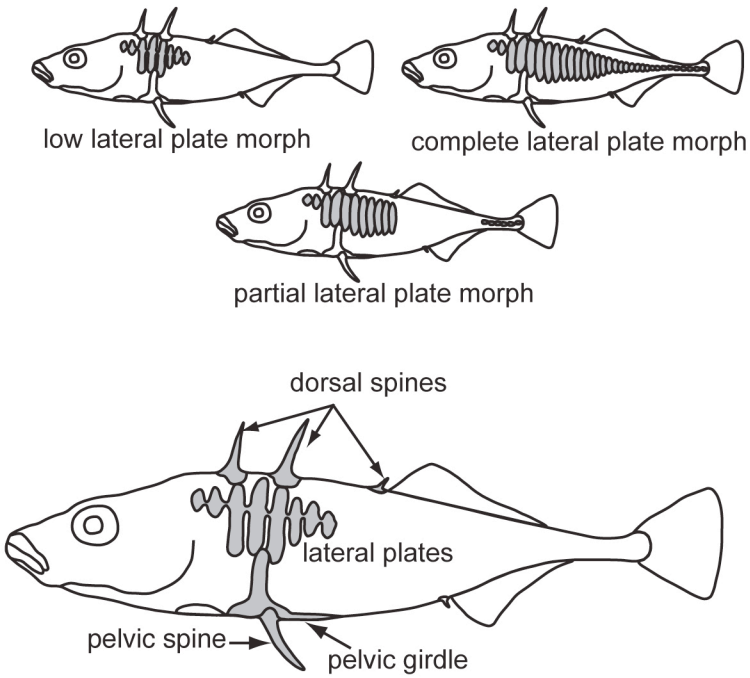

Figure 1.1. Illustration of the major bony armour traits that diverge between marine freswater populations of threespine stickleback, *Gasterosteus aculeatus*, from British Columbia.

Ion Hypothesis

Differences in either ion concentration, specifically salinity and calcium, between marine and freshwater habitats have been suggested as a mechanism driving the divergence stickleback lateral plate number (Bell et al. 1993; Campbell 1985; Giles 1983; Heuts 1947). In accord with one of the fundamental tenets of divergent natural selection (Schluter 2000), this hypothesis posits a trade-off in fitness advantages between fresh water and salt water environments: freshwater fish with few lateral plates (low morph) will have higher fitness than fish with many lateral plates (complete morph) in fresh

water, whereas the complete morph will have higher fitness than the low morph in sea water.

Although both salinity and calcium variants of this hypothesis predict a trade-off in fitness advantages between environments, the mechanism driving phenotypic divergence in lateral plate number differs between them. If the ability of stickleback to maintain ionic homeostasis at different salinities is associated with lateral plate number, then divergent selection could result in the general pattern of the complete morph inhabiting the sea and the low morph inhabiting freshwater lakes. The role of salinity in divergent selection on lateral plate number between marine and freshwater populations was initially supported by Heuts'(1947) study on European threespine stickleback. His work indicated that the complete morph survived better than the low morph in more saline water, whereas the low morph survived better than the complete morph in fresh water. Schaarschmidt et al. (1999) revealed roughly 75% mortality for the complete morph compared to only 5% mortality for the low morph when exposed to fresh water at 4 °C**.** Schaarschmidt et al. (1999) suggest this pattern is based upon differences in the regulation of hormones responsible for water and ion permeability. However, the conclusions of both Heuts (1947) and Schaarschmidt et al. (1999) may be confounded by their collection methods: both studies collected low morphs from freshwater populations and complete morphs from brackish water populations. Thus adaptation to local salinity conditions, rather than lateral plate number, may explain the differences in salinity tolerance observed between plate morphs. Moreover, geographical trends in lateral plate phenotype do not always support a strong association between lateral plate number and water salinity (Baumgartner and Bell 1984; Hagen and Gilbertson 1972) and it remains

unclear whether differences in salinity between marine and fresh water habitats results in selection on lateral plate number (Bell 2001).

Lower levels of calcium in most freshwater habitats compared to marine and brackish habitats should favour the individuals with fewer lateral plates because of an increased cost of building calcium carbonate skeletal components at low calcium levels (Giles 1983). Under this hypothesis of calcium limitation, possessing fewer lateral plates in fresh water of low calcium concentration is advantageous, but not necessarily disadvantageous at higher levels of calcium. Support for this hypothesis is found primarily in the positive correlation between ionic concentration and skeletal armour including lateral plates, dorsal and pelvic spines, and the pelvic girdle among freshwater populations (Bell et al. 1993; Bourgeois et al. 1994; Francis et al. 1986; Heuts 1944). At this point it appears no experimental work has directly tested calcium limitation as a mechanism for the reduction of armour in freshwater stickleback.

Recent molecular evidence suggests that a revisit to the role of salinity in lateral plate reduction is warranted. Colosimo et al. (2005) detailed that *Eda* is closely linked to a gene possibly associated with salt secretion, *Gjb1*. Interestingly, the overall selective advantage of low morphs in fresh water may arise, in part, via selection on differences in salinity tolerance that are correlated with lateral plate phenotypes. In Chapter 2 I test if differences in salinity between marine to freshwater habitats account for the reduction of lateral plate armour in fresh water populations of threespine stickleback. Chapter 2 uses a balanced experimental design to test if estimates for hatching success and juvenile growth rate among lateral plate morphs agree with the geographic patterns in nature: higher performance of individuals with fewer lateral plates than those with many lateral

plates when raised in fresh water, but lower performance of fish with fewer lateral plates than those with many lateral plates when raised in salt water. To minimize the potential artifact of local adaptation salinity among morphs coming from different populations (Heuts 1947), I used morphs from two populations polymorphic for lateral plates (Fig. 1.2, sites 2 & 5).

Predation Hypothesis

Divergent selection is thought to give rise to differences in stickleback armour through a trade-off in the mortality rate associated with differences in the predatory species present between marine and freshwater environments (Reimchen 1980; Reimchen 1994). This hypothesis is rooted in the correlation between predator community composition or seasonality of predator activity and the level of armour found in natural populations. In general, stickleback from marine and lake populations with many predaceous fish and bird species have long dorsal and pelvic spines (Reimchen 1994). Under these conditions lake populations are often completely plated, while nearly all marine populations consist of the complete plate morph only. By contrast, in lakes with few predatory fish and bird species, and where predaceous insects are abundant, stickleback often have short spines and few lateral plates (Bell et al. 1993; Reimchen 1980). Evidence for a trade-off in mortality between habitats differing in predator species was gained through experiments showing that individuals with longer dorsal and pelvic spines suffered lower mortality rates than fish with shorter spines when preyed upon by gape limited fish and birds (Reimchen 1988). Additionally, lateral amour plates increase the chance of survival of stickleback after attack by toothed fish predators and, along with the pelvic girdle, act as buttress to the dorsal and pelvic spines when erect

(Reimchen 1983; Reimchen 1994; Reimchen 2000). In freshwater habitats where non-gape limited aquatic insects are important sources of mortality, longer spines are hypothesized to increase mortality relative to short spines because they provide larger physical structures for insects to grasp while preying upon juvenile stickleback (Reimchen 1980). Furthermore, Chapter 2 revealed that individuals with more lateral plates grew at a slower rate than fish with few lateral plates. Faster growth at the juvenile stage may have severe consequences on juvenile predation rates in fresh water, as predaceous insects feed most frequently on the smallest individuals available (Foster et al. 1988). Thus juveniles of the low morph may exhibit lower mortality than those of the complete morph when exposed to predaceous insects only found in fresh water.

Experimental evidence for differential susceptibility to predators is unequal with respect to the predator species studied. Studies demonstrate the effectiveness of long spines and many lateral plates for reducing mortality due to fish or bird predation (Hoogland et al. 1956; Reimchen 1994; Reimchen 2000). However demonstrations that shorter spines and fewer plates reduce mortality due to aquatic insects are often indirect. Reimchen and Nosil (Reimchen and Nosil 2002; Reimchen and Nosil 2004) revealed a decrease in mean spine number in the summer when predaceous insects are most active, but an increase in mean spine number in the winter when gape limited piscivores were most active. Using benthic-limnetic stickleback species pairs, Vamosi (2002) showed that mortality due to insect predation was greatest in unarmored benthic compared to lightly armored limnetic sticklebacks. Similar to Reimchen and Nosil (Reimchen and Nosil 2002; Reimchen and Nosil 2004), Vamosi's (2002) results also imply a selective advantage to individuals with reduced armor when insect predation is high. However,

Vamosi (2002) is unable to attribute differences in mortality to armor alone because additional traits and behaviors differentiate the wild caught species under investigation. Indeed, a selective disadvantage to individuals with more armour may be unnecessary to explain the divergence between marine and freshwater populations and an alternative hypothesis combines the effects of predation with aspects of the ion hypothesis. In this scenario predators impose directional selection, only favouring more armour under greater levels of predation. Amour is selected against in freshwater habitats with depauperate predator fauna because low calcium concentrations yield a selective disadvantage to individuals required to allocate more energy into building bony armour at a cost to reproductive or somatic tissues (Bell et al. 1993).

Chapter 3 tests the hypothesis that predation by aquatic insects has resulted in selection for reduced stickleback armour in fresh water using F_2 hybrids generated from a cross between freshwater and marine populations divergent in armour (sites 1- 4 in Fig. 1.2). In addition to differences in lateral plate number (low plated fresh water *vs.* completely plated marine), the parental populations chosen to generate F_2 families were divergent in dorsal and pelvic spine length, and pelvic girdle length. The two parental populations represent the extremes of the range of phenotypes observed in natural populations. Two additional benefits are gained by measuring selection on hybrids rather than wild individuals differing in armour. By crossing wild caught parental populations and raising them to the F_2 generation, trait variance is increased beyond that available in a single wild population (Falconer and Mackay 1996), resulting in an increased ability to detect selection (Endler 1986). Additionally, one episode of recombination in the F_2

generation will reduce trait correlations arising from linkage disequilibrium between distant genomic locations, increasing the ability to isolate the effects of predation on armor from those of other traits differing between populations that may also influence predation rate (Bell et al. 2007; Huntingford et al. 1994). However, previous work has shown that several armor traits, including the length of dorsal and pelvic spines, as well as a variety of shape traits map to the same genomic region near *Eda*, the major gene underlying variation in lateral plate number (Albert et al. 2008; Shapiro et al. 2004; S. M. Rogers, unpublished manuscript). This has the advantage in that the effects of armor on mortality via predation will be more apparent if several traits covary together. The disadvantage is that further work will be necessary to distinguish which of the armor traits in this region of the genome are the most important to survival.

Not all freshwater populations in British Columbia exhibit a complete reduction in armour. A few freshwater populations of threespine stickleback remain polymorphic for lateral plate armour, presenting a rare opportunity to investigate the factors maintaining genetic variation for this ecologically important trait.

The maintenance of genetic variation within populations appears paradoxical given that directional and stabilizing selection are common and expected to reduce variation within populations (Fisher 1930; Kingsolver et al. 2001). Indeed, a surprisingly large amount of variation is often found to exist within populations (Mitchell-Olds et al. 2007). Understanding the mechanisms that maintain variation in ecologically important traits is central to our understanding of how populations persist, what generates biodiversity, and how populations may respond to rapid environmental change. Such variation may persist because of balancing selection, recurrent mutation, or gene flow

from nearby populations (Mitchell-Olds et al. 2007). However, few natural systems exist in which to undertake empirical tests of even the most basic question of how variation is maintained within wild populations and species.

In Chapter 4 I examine the possible factors maintaining the polymorphism at the major lateral plate gene, *Eda*, within a wild population of threespine stickleback (Fig. 1.2, site 5). This polymorphism is unusual, however, in I discovered a deficiency of *Eda* heterozygotes in sampled adults. The maintenance of polymorphism with heterozygote deficiency might be due to one of three processes, population subdivision, assortative mating, or frequency dependent selection. Population subdivision could maintain the appearance of *Eda* polymorphisms within a single lake if two reproductively isolated groups were coming into secondary contact (Castric et al. 2002). To test for the presence of population subdivision, the population genetics of six neutral loci were examined for clustering and genetic divergence. Assortative mating in the absence of population structure (Barreto and McCartney 2008) may account for polymorphism maintained in the face of heterozygote deficiency if stickleback within the lake mate according to *Eda* genotype. To test for assortative mating I genotyped the fertilized eggs of wild caught clutches at *Eda* to determine if genotype frequencies agreed with those expected from random mating. Lastly, frequency-dependent selection may maintain a stable polymorphism with heterozygote disadvantage, if it favours each *Eda* homozygote when rare (Wilson and Turelli 1986). I explored one possible ecological factor, niche differentiation (Ayala and Campbell 1974), that could provide such a stabilizing influence by comparing the ratio stable isotopes of carbon and nitrogen in wild caught

adults as indicators of the ultimate source of dietary carbon and trophic position, respectively (Post 2002).

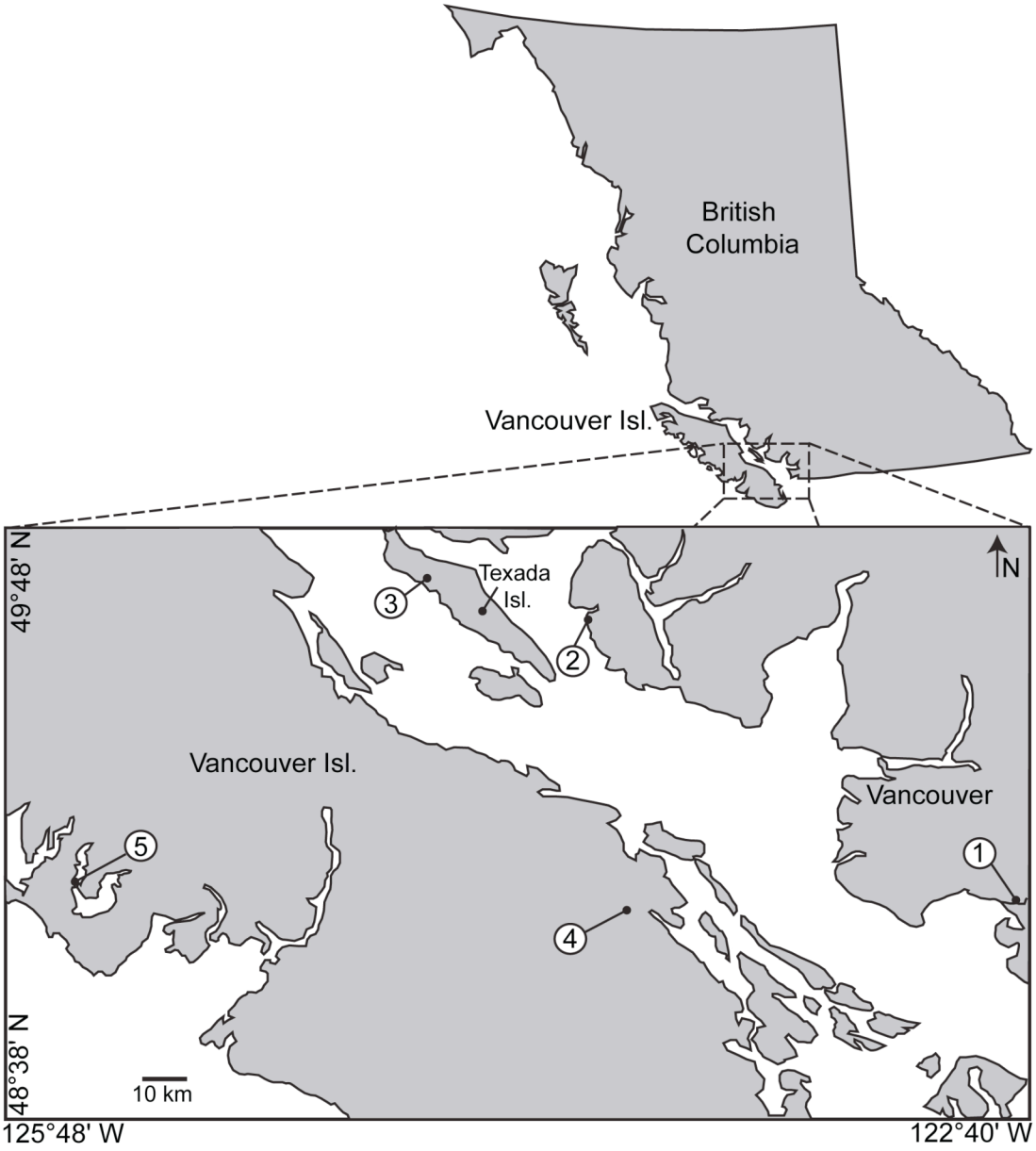

Figure 1.2. Sampling populations in British Columbia. 1. Little Campbell river (49°00´52˝ N, 122°45´33˝ W), 2. Oyster lagoon (49°36´43˝ N,124°01´57˝ W), 3. Paxton Lake, Texada island (49°42´30˝ N, 124°31´28˝ W), 4. McKay Lake, Vancouver Isl. (49°3´23˝ N, 123°58´3˝ W), 5. Kennedy Lake, Vancouver Island (49°5´52˝ N, 125°35´27˝ W). Populations 1 and 2 are marine-anadromous, 3-5 are from freshwater lakes.

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CHAPTER 2. PARALLEL EVOLUTION BY CORRELATED RESPONSE: LATERAL PLATE REDUCTION IN THREESPINE STICKLEBACK*

INTRODUCTION

One of the most impressive examples of parallel evolution in nature is the rapid and repeated loss of lateral plate armour in freshwater populations of threespine stickleback, *Gasterosteus aculeatus* (Colosimo et al. 2005). Most freshwater populations from North America and Western Europe are made up of individuals with relatively few lateral plates clustered near the pectoral fins (< 10 per side = low morph; Fig. 2.1), whereas individuals from marine populations have many lateral plates extending from the head to the tip of the caudal peduncle (> 30 per side = complete morph; Fig. 2.1; Bell 2001; Wooton 1984). A few populations are polymorphic for lateral plate armor, possessing low, complete and partial (11 to 29 lateral plates) plate morphs. Recent molecular evidence (Colosimo et al. 2005; Colosimo et al. 2004) indicates that the recurrent transition from completely plated marine populations to low plated freshwater populations is largely controlled by the effects of a single major gene, *Ectodysplasin* (Eda). The authors suggested that parallel reduction of lateral plate number in fresh water has occurred through repeated selection for low plate morph *Eda* alleles introduced by marine colonists, very few of whom were heterozygous for alleles at the *Eda* locus.

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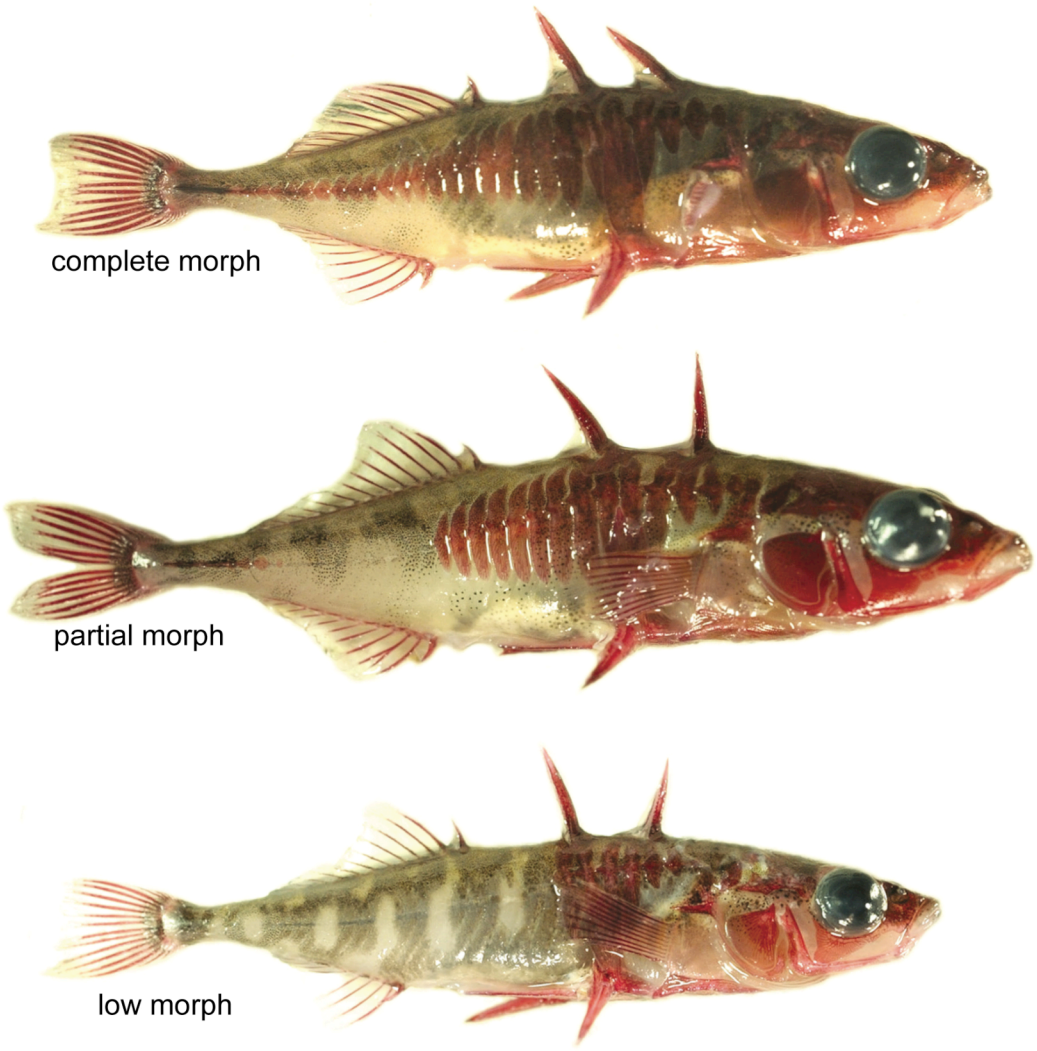

Figure 2.1. The three lateral plate morphotypes of *Gasterosteus aculeatus*. Bony elements were stained red using alazarin red in 1% KOH solution.

Surprisingly, although the molecular mechanisms of lateral plate reduction are becoming established, the ecological mechanisms driving armour evolution remain uncertain. Determining the mechanism responsible has been made difficult by the positive correlation of multiple ecological factors with differences in lateral plate number among populations (reviewed in Bell 2001). One of the oldest and more interesting suggestions was that lateral plate number is correlated with salinity tolerance. Heuts

(1947) found that individuals with greater lateral plate number survive longer and hatch more successfully in salt water, whereas those with reduced lateral plate number survive longer and hatch more successfully in fresh water. Heuts (1947) suggested that differences in salinity tolerance between low and complete lateral plate morphs might explain the pattern of lateral plate divergence between marine and freshwater habitats. Unfortunately Heuts (1947) collected his low morphs from freshwater populations and his complete morphs from brackish water populations introducing a confounding factor: perhaps other adaptations to local salinity conditions, not lateral plates, explain the differences he observed between morphs. Additional criticism for Heuts' hypothesis arises from its failure to account for the presence of completely plated freshwater populations in Eastern Europe and Eastern North America (Banbura 1994; Bell 2001; Hagen and Moodie 1982) and the presence of complete morph populations in lower salinity habitats upstream of polymorphic populations in Northern California (Baumgartner and Bell 1984).

Recent physiological and molecular evidence warrants a revisit to the role of salinity in the evolution of lateral plate number in threespine stickleback. Schaarschmidt et al. (1999) revealed dramatic differences in mortality between low and complete morphs when exposed to fresh water at 4 °C: up to 75% mortality for completely plated morphs compared to only 5% mortality for low morphs**.** They suggest this pattern is based upon differences in the regulation of prolactin, a hormone responsible for water and ion permeability in freshwater fishes. Additionally, Colosimo et al. (2005) revealed that *Eda* is closely linked to three other genes including one possibly associated with salt

secretion, *Gjb1*. Thus, overall selective advantage of low morphs in fresh water may arise, in part, via selection on differences in salinity tolerance that are correlated with lateral plate phenotypes.

In this study, we examine the hypothesis that selection arising from changes in salinity led to the divergence in lateral plate number between marine and freshwater habitats. Using a balanced experimental design, we raised reduced (low and partial) and complete morphs in the lab in either fresh or salt water to test if estimates for hatching success and juvenile growth rate among morphs agree with the geographic patterns in nature: higher performance of reduced morphs than complete morphs in fresh water, but lower performance of reduced morphs than complete morphs in salt water. To minimize the potential differences in adaptation to local salinity among morphs coming from different populations (Heuts 1947), we chose two populations possessing all three lateral plate morphs and only tested for differences in performance between morphs from the same population.

METHODS

Sample Populations, Fertilization And Experimental Rearing

Threespine stickleback were collected in southwestern British Columbia from two populations with a reliable frequency of all three lateral plate morphs: one marine population from Oyster Lagoon, Pender Harbour and one freshwater population from Kennedy Lake, Vancouver Island. Lateral plate morphs within each population differed very little in the external traits (standard length, body depth, spine length; Saimoto 1993; Marchinko unpublished data) known to differentiate anadromous populations from

freshwater populations (McPhail 1994). Thus, completely plated individuals from Kennedy Lake were not anadromous migrants and low plate morphs in Oyster lagoon were not accidental lake or stream migrants.

Using artificial fertilization, we made 12 low morph families and 14 complete morph families from the freshwater population, and 12 partial morph and 10 complete morph families from the marine population by crossing males and females of the same morph. Because the frequency of low morphs in Oyster lagoon was approximately 0.1%, partial morphs were used in place of low morphs when making crosses from the marine population. Partial-by-partial crosses will generate offspring with a range of lateral plate morphotypes. The ratio of morphs should be approximately 3:1, reduced:complete morph respectively (Colosimo et al. 2004). Our use of partial parents may obscure the comparison between morphs from the marine population, but in a conservative manner as 25% of each family will likely be completely plated as adults. Thus, differences in performance between reduced and complete morph families may be even larger than if only low and complete marine morphs were compared. Freshwater crosses will not be affected.

Crossing was accomplished by first stripping a female's eggs into a single Petri dish, counting the eggs and then splitting the clutch in half. One half of the eggs were placed in a Petri dish containing fresh water (0 ppt) and the other half were placed into artificial salt water (30 ppt; Instant Ocean synthetic seasalt, Aquarium Systems Inc., Mentor, OH, USA) both at a pH of 7.6 (+/- 0.1). A male of the same morph and population was anaesthetized in clove oil and both testes were removed. One testis was

placed into the freshwater Petri dish, the other in the saltwater Petri dish, and then both were crushed to release sperm. The half clutch of eggs along with crushed testes were kept in Petri dishes for 20 minutes, then placed into separate plastic egg-cups (pint cup with fine fiberglass mesh lining the bottom) and each submerged in a separate egg-tank (20 L) according to salinity treatment. Methylene blue was added to egg-tanks to reduce fungal growth. Eggs remained in aerated egg-tanks for eight days, after which they were transferred to one side of a divided 102 L tank of the same salinity and pH, respective to salinity treatment, for the remainder of the experiment.

After the eggs hatched and the larvae dropped into the tanks, egg-cups were removed and the number of unhatched eggs remaining was counted. Hatching success was measured as the proportion of eggs hatched from each half clutch, one in fresh water and one in salt water. The surviving larvae were fed live brine shrimp twice per day for four weeks, after which the number of individuals in each half tank was reduced to 15. Thereafter fish were fed 3.5 oz of frozen *Daphnia* cubes once per day. After feeding stopped any remaining food was removed by filtration or manual siphoning. Thus each individual fed to satiation once per day.

Measurements of growth rate began five weeks after hatching, when individuals reached about 12 mm from snout to the tip of the caudal peduncle (standard length), the size at which lateral plates begin to form. Standard length was measured on every individual once every two weeks for ten weeks during the period of lateral plate development. After an individual reaches about 30 mm standard length the number of plates no longer changes (Bell 1981; Igarashi 1970). The standard lengths of all

individuals from the same family in the same salinity treatment were then averaged, yielding a mean standard length for each family in each treatment. After ten weeks of measurement, the growth rate of each family in each treatment was calculated as the slope of the ordinary least squares regression of standard length on the number of days since hatching. Thus, for each family (the unit of replication) we obtained two measures of growth rate, one in fresh water and one in salt water. The relationship between standard length and the number of days since hatching was linear and highly significant in all replicates. Unfortunately, we were unable to test for differences in growth rate between low and complete morphs from the freshwater population in the saltwater treatment because of very low replication: only two families from each morph survived long enough to obtain growth rate measurements in salt water.

Not all families survived for the entire ten weeks of the experiment (see Fig. 2.3B for sample sizes) and there were families in which only six weeks, or eight weeks of growth rate measurements were available for a particular treatment. However, there was no effect of experiment length (6, 8, or 10 weeks) on the linear relationship between standard length and the number of days since hatching (separate two-factor analysis of variance tests were completed on each morph from each habitat; dependent variable = growth rate, factors = experiment length and treatment; $P_{\text{experiment length}} > 0.16$ in all tests). We have included all available data in the analyses reported in the results. Additionally, not all families maintained 15 individuals in each treatment and any differences in growth rate may be confounded by differences in density and the influence of crowding or food availability. However, the influence of density on growth rate appears minimal and all

data were used for two reasons. First, the effect of food availability was minimized through feeding protocol; individuals were fed to satiation only once per day and there was no other opportunity for feeding. Second, using ordinary least squares regression of the number of individuals per family on mean family growth rate, we found no significant relationship between density and growth rate in seven of eight tests ($P > 0.14$ except for freshwater low morphs raised in 0 ppt where $P = 0.03$; separate regressions of mean family growth rate on mean number of individuals in family were completed for each morph from each population in each treatment using all data).

Analyses

Our main objective was to determine if the performance of lateral plate morphs differed within each salinity treatment in the direction predicted by geographical patterns of plate morph distributions in nature. We employed Student's *t*-test to test for differences between plate morphs in mean hatching success and juvenile growth rate separately for each salinity treatment and each population (Fig. 2.2 comparisons *i* and *ii*). According to Heuts' (1947) hypothesis that differences in salinity drive patterns of armor loss in fresh water and armor maintenance in the sea, we expect: 1) in fresh water, reduced (low and partial) morphs exhibit greater mean hatching success and growth rate than the complete morph and 2) in salt water, the complete morph exhibits greater mean hatching success and growth rate than reduced morphs (see Fig. 2.2). Because of split-family design, evaluating the interaction between lateral plate morphotype and salinity was complicated by a lack of independence between treatments (a single family was raised in both treatments). To test if differences in hatching and growth rate between

morphs depended on salinity treatment, we subtracted the values of hatching success and juvenile growth rate in fresh water from those measured in salt water yielding a single measure of mean hatching success and growth rate for each family in salt water relative to fresh water (Fig. 2.2 comparison *iii*). We then tested for differences in mean relative hatching success and growth rate on each population separately, using Student's *t*-test. Arcsine transformation was performed on proportional hatching data and alpha values were adjusted to 0.0167 to correct for the three tests completed on each performance measure (one in fresh water, one in salt water, one on performance in salt water relative to fresh water) using a Bonferroni correction. All statistics were analyzed using StatView 5.0 (1998 SAS Institute Inc., Cary, NC).

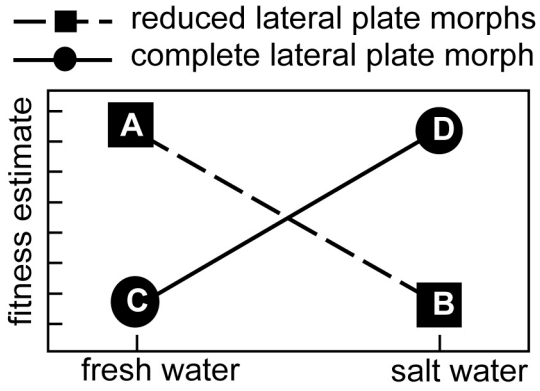

Figure 2.2. Expected patterns of fitness among lateral plate morphs of *Gasterosteus aculeatus* when hatched and raised in fresh water and salt water. According to Heuts (1947): i) reduced morphs should exhibit greater fitness than the complete morph when raised in fresh water, A > C; ii) the complete morph should exhibit greater fitness than reduced morphs when raised in salt water, D > B; and iii) reduced morphs should exhibit lower fitness in salt water relative to fresh water, B - A < 0, whereas the complete morph should exhibit higher fitness in salt water relative to fresh water, D - C > 0, therefore (B - A) < (D - C).

RESULTS

Hatching Success

Contrary to expectation (Fig. 2.2), complete morph embryos from the marine population appeared slightly more tolerant of fresh water than embryos from reduced morph families. Although the effect was small (4%), a significantly greater proportion of marine embryos from complete morph families hatched in fresh water than did embryos from reduced morph families ($t_{[18]} = 2.962$; $P = 0.008$; Fig. 2.3A). In salt water, embryos from marine reduced and complete morph families exhibited similar abilities to hatch ($t_{[18]} = -0.160$; $P = 0.874$; Fig. 2.3A). Complete and reduced morph marine families did not differ significantly in their ability to hatch in fresh water relative to salt water ($t_{[18]} = -2.488$; $P = 0.023$; nonsignificant after Bonferroni correction). Both morphs hatched proportionally more larvae in fresh water than in salt water (Fig. 2.3A).

In the freshwater population there were no significant differences in hatching success between reduced and complete morph families in either treatment ($t_{[24]} > -1.181$; $P > 0.081$; Fig. 2.3A), or in the abilities of each morph to hatch in fresh water relative to salt water ($t_{[24]} = -1.132$; $P = 0.269$). Hatching success was low in the saltwater treatment for both plate morphs from the freshwater population. Furthermore, no embryo development was observed in the unhatched eggs from all of the freshwater reduced morph families raised in salt water and more than 85% of the eggs that died in the complete morph families showed no signs of embryo development. Egg mortality may be due to low success of fertilization in both freshwater morphs in salt water.

Juvenile Growth Rate

Mean juvenile growth rate in the marine population was significantly different between reduced and complete morph families when raised in fresh water ($t_{[12]}$ = -3.06; P= 0.001), but not in salt water ($t_{[12]}$ = -0.382; P = 0.709; Fig. 2.3B). When raised in fresh water, marine families from reduced morph parents grew on average 65% faster than those from complete morph parents. Mean growth rate in salt water relative to fresh water also differed significantly between lateral plate morphs from the marine population ($t_{[12]}$ = 3.286; P= 0.0065; Fig. 2.3B). Reduced morph families grew faster in fresh water relative to salt water, whereas complete morph families grew faster in salt water relative to fresh water (Fig. 2.3B).

Similarly, in the freshwater population low morph families grew significantly faster than complete morphs in the freshwater treatment ($t_{[12]}$ = -2.203; P = 0.0478, Fig. 2.3B). Low sample sizes due to low levels of fertilization and hatching success prevented any comparison among freshwater plate morphs in the saltwater treatment.

DISCUSSION

Our results on hatching success and juvenile growth rate contradict Heuts' (1947) hypothesis that differences in salinity drive the pattern of lateral plate divergence between marine and freshwater populations. Unlike Heuts (1947), we found that lateral plate morphs exhibited little difference in their ability to hatch in fresh water and salt water. This discrepancy likely arises because Heuts' different plate morphs were collected from separate locations: reduced morph fish came from fresh water, whereas his complete morph fish were collected from brackish water. We restricted our tests to comparisons

between morphs collected from the same population, likely minimizing any additional adaptations to local salinity that may have confounded Heuts' (1947) results.

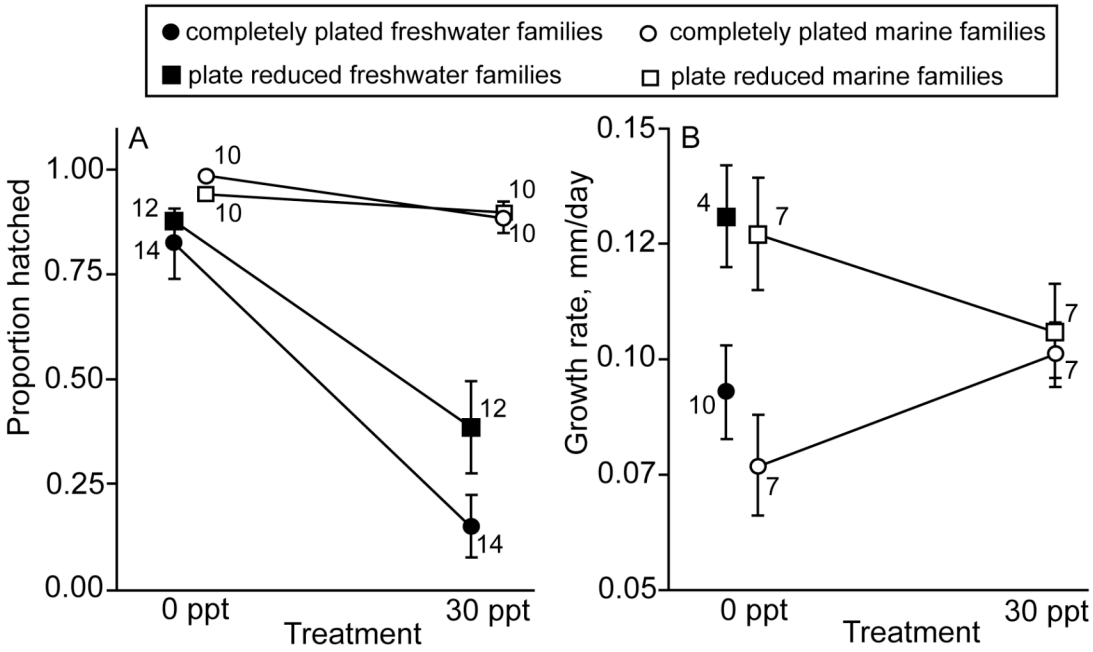

Figure 2.3. Hatching success (A) and growth rate (B) of reduced (low and partial) and completely plated families of *Gasterosteus aculeatus* fertilized and raised in fresh water (0 ppt) or artificial salt water (30 ppt). Symbols and error bars represent mean ± 1 SE. Error bars are less than symbol size where absent and populations are offset for clarity. Numbers nearest points indicate the number of families tested. Growth data from the freshwater population in the saltwater treatment are not shown because of low sample size (N = 2 families for both morphs) due to low hatching success and survivorship.

We also documented significant differences in juvenile growth rate between reduced and complete morph families from both freshwater and marine populations when raised in fresh water (Fig. 2.3B). These differences in growth rate have two important implications for the evolution of lateral plate morphology in populations of threespine stickleback. First, the advantage of the low lateral plate morph in fresh water may stem from the association between juvenile growth rate and lateral plate morphology. Second,

the ecological mechanism of selection on lateral plate morphology likely differs between freshwater and marine habitats. In freshwater littoral habitats, reduction of lateral plate armor may occur as a correlated response to selection for faster juvenile growth rate of individuals with lower lateral plate number. In marine habitats and lakes with large pelagic zones, lateral plate number remains high due to selection by pelagic fish predation (Reimchen, 2000). It is important to note that our conclusions are based on results from laboratory experiment using artificial salt water and a restricted diet. Our results may not reflect the complete suite of differences in salinity between all marine and freshwater habitats nor the differences in growth rate found in the wild, where food may or may not be limited. Additionally, we measured growth rate on juveniles only, leaving the possibility for compensatory growth to occur at later life stages. Lastly, because the marine reduced morph families were created using a partial-by-partial cross, roughly 25% of the offspring will be completely plated as adults. Despite the presence of plate morph variation within marine reduced morph families, the differences in growth rate between marine reduced and complete morphs were similar to the differences observed between true low and complete morphs from the freshwater population (Fig. 2.3B).

Correlated Response to Selection for Faster Growth Rate

Faster growth at the juvenile stage has three, potentially large, consequences for individual fitness: higher overwinter survival, greater reproductive potential, and decreased predation (Arendt 1997). Firstly, larger, faster growing juveniles posses greater lipid stores and lower metabolism, buffering individuals from lower food availability in winter (Ludsin and DeVries 1997). Because mass specific metabolism and

lipid accumulation favour juvenile fish that attain a larger size at the onset of fall (Thompson et al. 1991), faster growing, reduced lateral plate morphs may suffer less overwinter mortality. Secondly, body length is strongly positively correlated with reproductive output (clutch size and mass) in threespine stickleback (Schluter 1995; Wooton 1984) and earlier breeding in other fish species (Bagenal 1978; Schultz et al. 1991). If differences in growth rate and body size observed here persist into the reproductive season the following spring, the higher growth rate of low and partially plated individuals may result in greater reproductive potential relative to slower growing, completely plated individuals. Thirdly, cannibalistic adult stickleback and three major freshwater invertebrate predators feed mainly on juveniles below 20 mm standard length, and most frequently on the smallest individuals available (Foster et al. 1988). The 65% increase in juvenile growth rate of reduced morphs over the complete morph in fresh water may translate into a substantial decrease in the risk of predation experienced by low and partially plated individuals. In littoral freshwater habitats where juvenile predation is a considerable selection pressure (Foster et al. 1988), passing more quickly through the most vulnerable size range may be extremely advantageous and a substantial reduction in predation alone may contribute significantly to the predominance of the low morph in independently isolated freshwater populations.

Ecological and Evolutionary Mechanisms of Lateral Plate Divergence

The absence of clear reciprocal differences in hatching and growth rate among lateral plate morphs when raised in fresh water vs. salt water (Fig. 2.3), suggests that a single ecological mechanism, like that of salinity tolerance (Heuts 1947; Fig. 2.2), cannot

fully explain the pattern of lateral plate divergence between freshwater and marine habitats. The absence of differences between morphs when raised in salt water suggests that the predominance of complete morphs in marine habitats is not an outcome of differences in hatching or growth rate. We suggest that divergent natural selection on differences in lateral plate morphology between marine and freshwater habitats likely occurs via two mechanisms. Selection first operates on differences in juvenile growth rate between lateral plate morphs, and subsequently on differential mortality of lateral plate morphs as adults due to pelagic fish predation. In adult threespine stickleback, greater lateral plate number is correlated with a greater chance of escape and survival from pelagic fish predation (Bergstrom 2002; Reimchen 2000). The potential fitness advantages of higher juvenile growth rate in fresh water may be beneficial to reduced plate morphs only when levels of pelagic fish predation as adults are relatively low. When pelagic predation intensity becomes too high, completely plated individuals may be favored by selection. Thus, the pattern of lateral plate differentiation may be generated by a correlated response to selection for higher juvenile growth rate in freshwater littoral habitats, but direct selection for reduced mortality from pelagic predation as adults in marine and freshwater habitats with large pelagic zones.

Although our results suggest that a correlated response to selection on differences in growth rate may drive patterns of lateral plate evolution in fresh water, the mechanisms responsible for the association between salinity, juvenile growth rate and lateral plate morphology remain unclear. One possibility is that calcium limitation drives differences in growth rate between lateral plate morphs in fresh water. Calcium is often

limiting in fresh water, and correlational studies have found a reduction in plate and spine number (Giles 1983) and pelvic girdle dimensions (Bell et al. 1993) along a gradient of lakes from high to low calcium concentration. This hypothesis may explain the absence of a difference in juvenile growth rate in the saltwater treatment (Fig. 2.3B) where calcium concentrations were roughly 300 times greater than in the freshwater treatment. The energetic cost associated with producing more bony tissue during plate development in calcium limited freshwater habitats may result in completely plated individuals growing more slowly than individuals with reduced plate number. Bell et al. (1993) noted, however, that separating the effects of a reduction in calcium on bone growth in fresh water from the concomitant reduction of other ions in fresh water is difficult. The low level of ions such as phosphate may also contribute to reduction in bony lateral plates as well as the pelvic girdle in fresh water (Bell et al. 1993).

Although calcium limitation may explain why the growth rate of reduced and complete morphs are similar in salt water, it cannot explain why marine reduced morphs grow faster in fresh water than their native salt water. The differences in growth rate we observed between salinity treatments may arise from unknown pleiotropic effects of low *Eda* alleles reintroduced to salt water, or they could arise by correlated selection for unknown phenotypes produced by genes closely linked to the freshwater low *Eda* alleles (Colosimo et al., 2005). Indeed, not all the functions of *Eda* are known in stickleback and low *Eda* alleles may exhibit deleterious effects on unknown phenotypic traits in salt water only. In addition, if changes in the regulation or structure of a gene implicated in salt secretion, *Gjb1*, that is closely linked to the freshwater low *Eda* alleles have

deleterious effects in salt water, then linkage may also explain why marine reduced morphs grew faster in fresh rather than salt water. In contrast to our results on growth, Colosimo et al. (2004) found no association between quantitative trait loci determining adult body length and lateral plate morph in the F_2 generation between Japanese marine and Paxton lake cross raised in fresh water. This lack of an association between body length and lateral plate morph in adult F_2 may result from breakdown of linkage disequilibrium, possibly refuting the role of pleiotropy. However, their results could also be due to compensatory growth of completely plated individuals after lateral plate development, or the presence of separate genes for juvenile growth rate and final adult size. Disentangling the roles of pleiotropy and genetic linkage in maintaining the association between juvenile growth rate and lateral plate morphology in our populations requires further study.

Lastly, the absence of the low morph from freshwater habitats in Eastern Europe and Eastern North America is puzzling in light of our results. Exceptions to the pattern of reduced morphs occurring in fresh water and complete morphs in salt water can also be found on the West Coast of North America. Baumgartner and Bell (1984) observed that stickleback populations in lagoons near the mouths of streams tend to contain all three plate morphs, but the frequency of complete morphs increased further inland in lower salinity, high gradient stream habitats. Hagen and Moodie (1982) suggested that the global distribution of lateral plate morphs was determined by temperature rather than salinity: areas with lower winter temperatures and large annual fluctuations most often support the complete morph, whereas areas with mild winter temperatures and low

annual fluctuations support the low morph. Unfortunately this association also breaks down when examining morph distributions on a local scale (for example see Coad 1983). Although numerous exceptions to the large scale trends in the distribution of lateral plate morphs exist, these exceptions may indicate the repetition of a common theme: selection for armor reduction in fresh water may not involve selection on the function of lateral plates. Indeed, the parallel recurrence of low *Eda* alleles in fresh water occurs as part of a common haplotype, which includes genes implicated in salt secretion (*Gjb1*) and parasite load (*Tnfs13b*), among others with unknown functions (Colosimo et al., 2005). The likelihood for evolutionary change in lateral plate armor to occur via a correlated response to selection for other phenotypes appears quite high. Selection for lateral plate reduction in fresh water through correlated response to changes in growth rate may be only one of many ways to reduce lateral plate armor in freshwater populations of threespine stickleback.

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CHAPTER 3. PREDATION'S ROLE IN REPEATED PHENOTYPIC AND GENETIC DIVERGENCE OF ARMOR IN THREESPINE STICKLEBACK*

INTRODUCTION

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 (Chase et al. 2002; Langerhans 2006; Schluter 2000; Vamosi 2005). More recently, there has been an increase in theoretical and empirical studies aimed at determining the role of predation in generating biological diversity (Abrams 2000; Endler 1995; Nosil and Crespi 2006; Reznick and Endler 1982; Vermeij 1987).

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 (Diabate et al. 2008; McPeek 1997; Meyer and Kassen 2007; Mikolajewski et al. 2006; Nosil and Crespi 2006), with few vertebrate examples (Langerhans 2006; Vamosi 2005). 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

*A version of this chapter has been published. Marchinko, K. B. 2009. Predation's role in repeated phenotypic and genetic divergence of armor in threespine stickleback. Evolution 63: 127–138.

assortative mating, and therefore reproductive isolation and speciation (Langerhans et al. 2007).

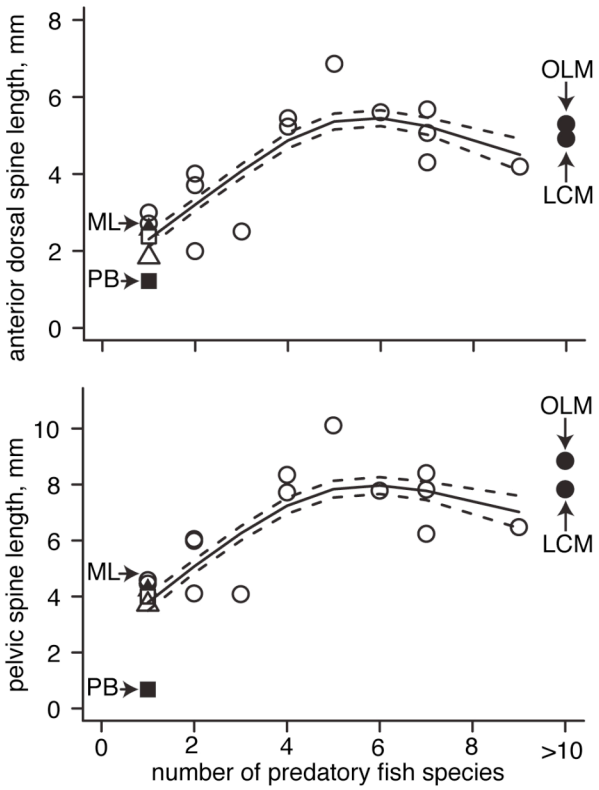

Figure 3.1. The positive association between spine length and the number of predatory fish species present in natural populations of threespine stickleback. Spearman's Rho = 0.762 and 0.781; $P < 0.0002$ for dorsal (top) and pelvic (bottom) spine length respectively. Each symbol represents the size adjusted mean of ten 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 generate 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 Appendix 1 for the location of each collection site.

Threespine stickleback, *Gasterosteus aculeatus*, exhibit a correlation between predator community and the amount of external bony armor (Fig. 3.1). 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 lateral plates. Fish and bird predators 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, while 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 (Bell et al. 1993; Reimchen 1980). 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 (Bell 2001; Colosimo et al. 2004; Ellegren and Sheldon 2008; Gross

1977; Hagen and Gilbertson 1972; Moodie et al. 1973, Reimchen 1980; 1994; Reimchen and Nosil 2002; 2004; Reist 1980; Vamosi 2002).

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 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, while those with two complete Eda^C alleles will most often be classified as the complete morph (Fig. 3.2; 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 unpublished manuscript), 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 in 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. 3.1), and a large, robust pelvic girdle. All individuals sampled for crossing were of the complete lateral plate morph (Fig. 3.2). The two freshwater populations, Paxton Lake (49°42´30˝ N, 124°31´28˝ W) on Texada Island and McKay Lake (49°3´23˝ N, 123°58´3˝ W) on Vancouver Island, were characterize by short dorsal spines (Fig. 3.1), and were of the low lateral plate morph (Fig. 3.2). 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. 3.1).

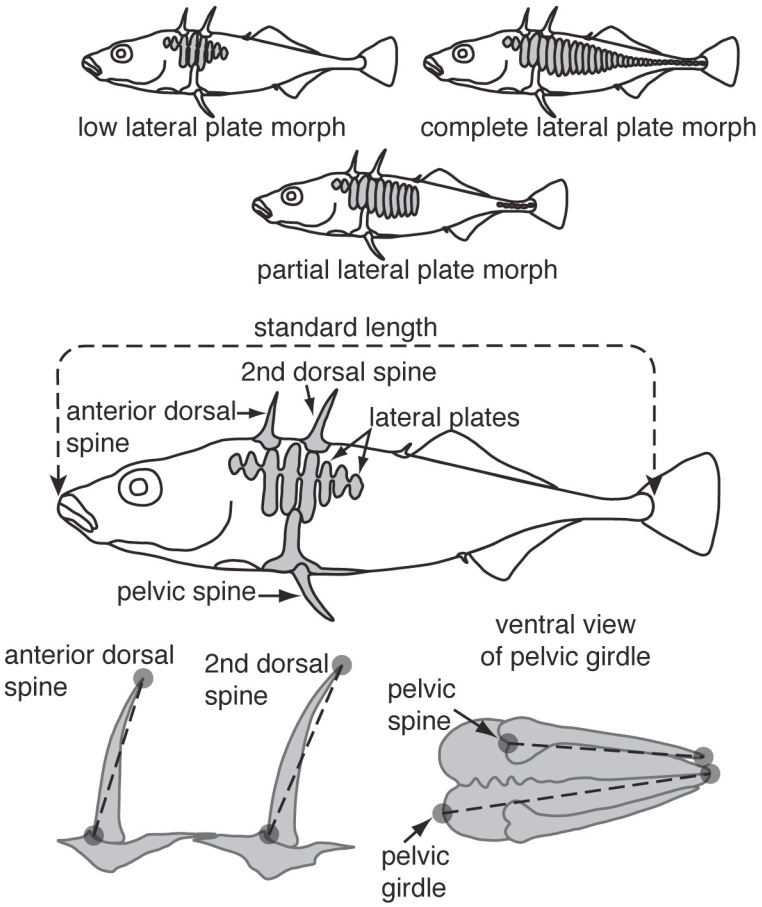

Figure 3.2. Illustrations of the threespine stickleback, *Gasterosteus aculeatus*, indicating the bony armor traits examined in this study in grey. Lateral plate morph (top of panel) was assessed by genotyping at an in/del marker (Stn381, Colosimo et al. 2004) within intron six of the *Ectodysplasin* gene. Five were traits 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.

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 appendix 2. 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 ten 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 appendix 3. 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 (appendix 4). 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 towards 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. *Notonecta* 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 to1, 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), preserved in 95% ethanol, and brought back to the lab.

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 v1.37 (Rasband 2007). I measured five traits (Fig. 3.2): 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 sub-adults, 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 to 15 ng genomic DNA, 1μM 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). Cycling conditions were as follows: 93 °C for 3 min, 95 °C 30 s, 59 °C 30s, 72 °C 30 s, 5 cycles of 94 °C 30

s, 59 °C 30 s, 72 °C 30 s, 35 cycles of 90 °C 30 s, 60 °C 30 s, 72 °C 30 s, followed by 72 °C 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),

$$i = \frac{\overline{X}_a - \overline{X}_b}{\sqrt{var_b}}$$

where $\overline{X}_a$ and $\overline{X}_b$ 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 appendix 4. Selection on each trait was visualized using the relative survival function, $f(z)$ (eqn. 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 non-parametric 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 re-sampled 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 and combining fish from both treatments.

To analyze the Paxton line F_2 cross, I removed individuals missing the pelvic girdle and spines from the data sets 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 paired *t*-tests between treatments on the difference in mean proportion of individuals without a pelvic girdle at the end of at 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 unpublished manuscript; 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 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 resulted in directional selection for larger body size in both crosses (Fig. 3.3): mean standard length was significantly greater in the predator treatment compared to the control (Table 3.1; difference between treatments was 0.7 & 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 3.1; Fig. 3.3). 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 sets of crosses differed with respect to which armor traits experienced significant selection (Table 3.1; Fig. 3.3), and the direction of selection varied among families in some traits

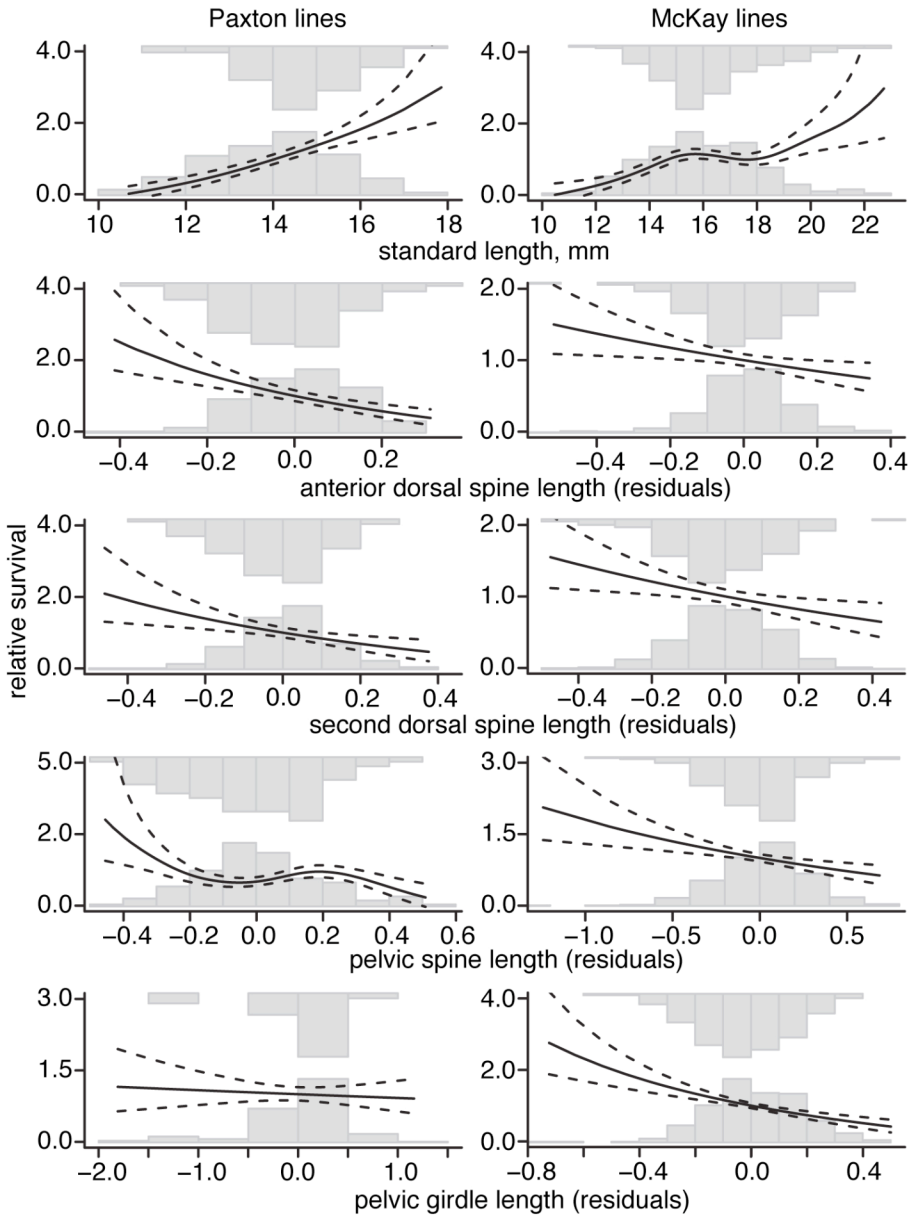

Figure 3.3. Relative survival function estimates (solid line) based upon the relationship between the probability of surviving aquatic insect predation and phenotype. Each panel is a visualization of the form of selection (Table 1) on stickleback size, spine, and pelvic girdle traits. Dashed lines represent 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).

(appendix 4). In McKay lines, mean size-adjusted pelvic girdle length was significantly shorter in predation treatment than in the control group (Table 3.1). Dorsal and pelvic

spines were also shorter in predator compared to control treatments and differences approached significance (Table 3.1). Similarly, in Paxton line F_2 families, predation favored individuals with significantly shorter anterior dorsal spines (Table 3.1; Fig. 3.3).

Table 3.1. 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 appendix 4. † $P < 0.1$, * $P < 0.05$, ** $P < 0.01$ 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 *

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. 3.4). 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. 3.4), 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. 3.3), the complete loss of the pelvic girdle was detrimental to survival in the presence of predatory insects (Fig. 3.4).

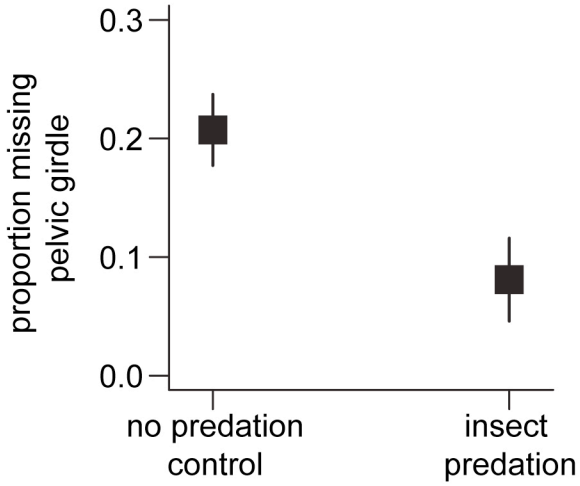

Figure 3.4. Proportion of F2 juvenile stickleback missing the pelvic girdle in no predation control and predation treatments. Points represent the among family mean (± 1 s.e.) from five F2 families generated from the Paxton lines.

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. 3.5). 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; appendix 5). 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. 3.5). 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.

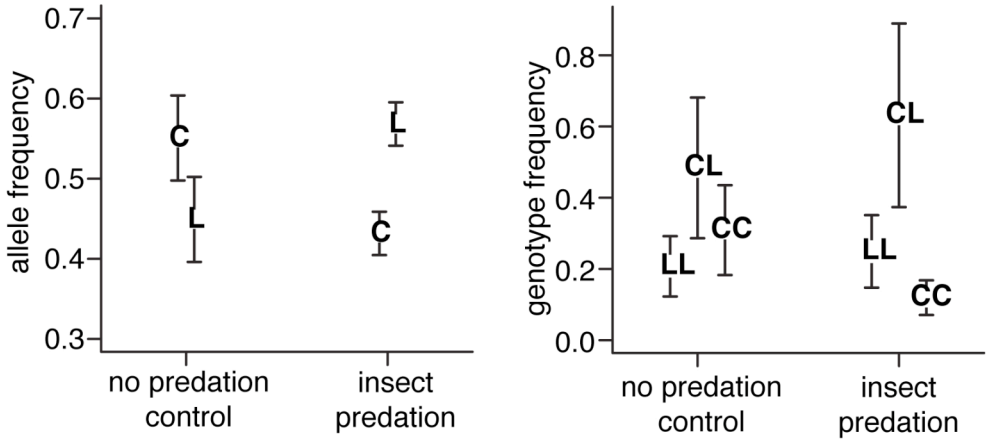

Figure 3.5. Allele frequency (left) and genotype frequency (right) at the *Ectodysplasin* locus (*Eda*) in F2 juveniles from control and predation treatments. Letters indicate among family mean (± 1 s.e.). In the left panel, L corresponds to the freshwater origin, low morph *Eda* allele and C represents the marine origin, complete morph *Eda* allele. In the right 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 F2 families generated from the Paxton lines.

Eda genotype was a significant covariate for size-adjusted anterior dorsal spine length (Table 3.2). 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 3.2). *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 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 for selection for greater levels of armor when fish and bird predation are 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. 3.3). 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

Table 3.2. 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		

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. Firstly, 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 over winter survival are commonly associated with faster growing, larger fish (Ludsin and DeVries 1997; Thompson et al. 1991; Wootton 1984). 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 threespine stickleback. Alternatively,

negative fitness epistasis may be common in crosses between widely divergent populations with different genetic backgrounds (Leips and Mackay 2000; Rogers and Bernatchez 2007; Ungerer et al. 2003). 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 (Arendt 1997; Brown et al. 1993). In addition to influencing susceptibility to predators, size is positively correlated with reproductive output (Wootton 1984) and over winter survival (Ludsin and DeVries 1997; Thompson et al. 1991). Moreover, adult size strongly influences mate choice in reproductively isolated anadromous-stream and benthic-limnetic pairs of threespine stickleback (McKinnon et al. 2004; Nagel and

Schluter 1998; 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 by-product 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 (Mckinnon and Rundle 2002; McPhail 1994; Schluter and McPhail 1992; Taylor and McPhail 2000). 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 by-product reproductive isolation during adaptive radiation in the threespine stickleback species complex.

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CHAPTER 4. POLYMORPHISM MAINTAINED BY NATURAL SELECTION DESPITE HETEROZYGOTE DISADVANTAGE AT A MAJOR GENE

MAIN BODY

Single locus polymorphisms maintained in the face of heterozygote disadvantage (underdominance) are the starting point for multiple evolutionary endpoints (Wilson and Turelli 1986), including dominance and speciation (Durinx and Van Dooren 2009; Otto et al. 2008), yet examples from nature are conspicuously absent. We report the presence of a stable polymorphism with heterozygote disadvantage at a major armour locus, *Ectodysplasin* (*Eda*), in a wild population of threespine stickleback. Armour (lateral plate) morph frequencies in adult fish have remained stable over 40 years with a deficit of intermediate phenotypes. Genetic analysis confirms a deficit of heterozygotes at the *Eda* locus with an absence of population structure at neutral markers. Random (Hardy-Weinberg) genotype frequencies are restored in wild-caught zygotes, suggesting that selection rather than assortative mating is responsible for the observed heterozygote deficiency. Changes in genotype frequencies across a generation reveal that heterozygote disadvantage is the net result of opposing selection between the growth and reproductive phases of the life cycle. Differences in carbon isotope signature expose niche separation between *Eda* homozygotes, providing an ecological mechanism for the maintenance of polymorphism despite heterozygous disadvantage. Our study provides support for the role of disruptive selection in maintaining genetic variation for functionally important traits (Rueffler et al. 2006), and highlights the benefits of integrating genetic and

*A version of this chapter will be submitted. Marchinko, K. B., B. W. Matthews, S. M. Rogers, D.Schluter. Polymorphism maintained by natural selection despite heterozygote disadvantage at a major gene.

ecological approaches for understanding the mechanisms maintaining variation and promoting diversification in wild populations.

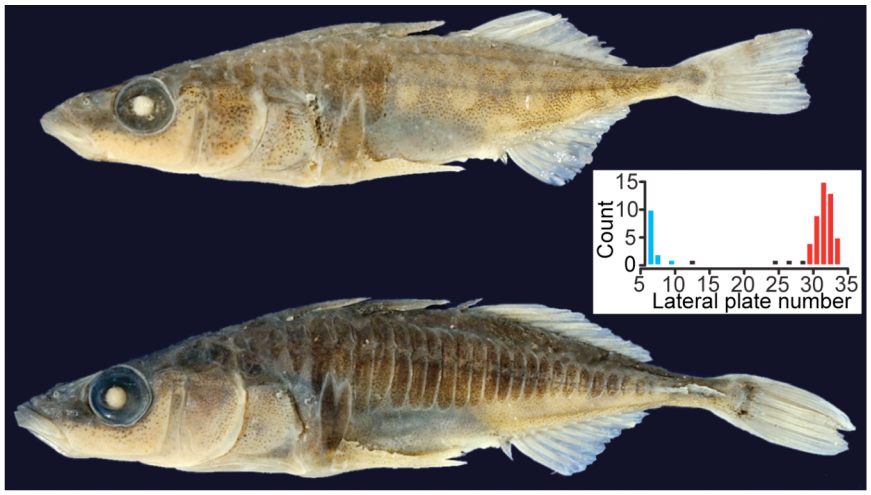

Figure 4.1. Representatives of the low (top) and complete (bottom) lateral plate morphs of the threespine stickleback, *Gasterosteus aculeatus*, from Kennedy Lake. The inset histogram shows the bimodal distribution of the number of lateral plates in 2006 adults (counted on the left side). Red bars indicate the complete morph, black the partial morph and blue the low morph.

We examined the genetic and ecological causes for the occurrence of a lateral plate polymorphism in a population of threespine stickleback inhabiting a large natural lake on Vancouver Island, British Columbia (Fig. 4.1). Kennedy Lake is one of several large lakes in the region in which stickleback exhibit polymorphism in this trait. This lateral plate polymorphism has remained stable over the last 43 years, with a lower frequency of individuals having an intermediate number of plates (partial morph) than individuals having either few (low morph) or many lateral plates (complete morph; Fig. 4.2).

Genotyping of a random sample of adults from Kennedy Lake confirmed that lateral plate polymorphism is associated with variation at the *Ectodysplasin* locus (Cano et al. 2006; Colosimo et al. 2005), *Eda* (Appendix 6). The observed scarcity of the partial

morph corresponded to a significant deficit of *Eda* heterozygotes, as indicated by a Wright's F_{IS} coefficient substantially greater than zero (F_{IS} = 0.44, 95% CI [0.19, 0.67] and F_{IS} = 0.36[0.19, 0.52] for adults in 2006 and 2008, respectively). As a result, genotype frequencies at *Eda* in breeding adults showed a significant deviation from Hardy-Weinberg equilibrium, HWE, in 2006 and 2008 (Fig. 4.2; χ^2 = 12.36 P = 0.002 and χ^2 = 17.96 P = 0.0001, respectively).

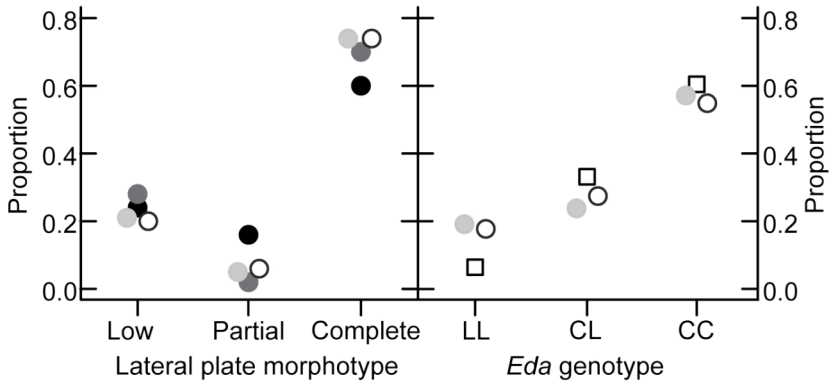

Figure 4.2. The frequencies of lateral plate morphs (left) and Eda genotypes (right) in stickleback samples spanning 44 years. Phenotypic samples (left) represent adults collected in 1965 (black, N=35), 2004 (dark grey, N=329), 2006 (light grey, N=63), and 2008 (white, N=135). Genetic samples (right) were from the 2006 and 2008 collections with circles and squares representing individuals collected as adults and eggs respectively. Points were jittered for clarity.

The heterozygote deficit observed in adults is not associated with genome-wide population structure (Castric et al. 2002). To test this, we genotyped six unlinked neutral microsatellite loci located on linkage groups 2, 6, 8, 9, 18, and 20, in 63 adults collected in the May 2006 breeding season. In sharp contrast to *Eda*, all six neutral loci were in HWE (Appendices 7&8). Individual based population assignment using a Bayesian MCMC approach implemented in STRUCTURE (Pritchard et al. 2000) provided strong support for a single genetic cluster (Pr[K of 1] = 0.99; Fig. 4.3). Moreover, the

differences in allele frequencies at neutral loci contrasted between the low and complete morphs explained less than 1% of the total allelic variation between low and complete morphs (F_{ST}=0.008; P= 0.112), which is similar to estimates of F_{ST} found within solitary stickleback populations (Bolnick et al. 2008). This suggests that the stickleback within Kennedy Lake comprise a single, panmictic population.

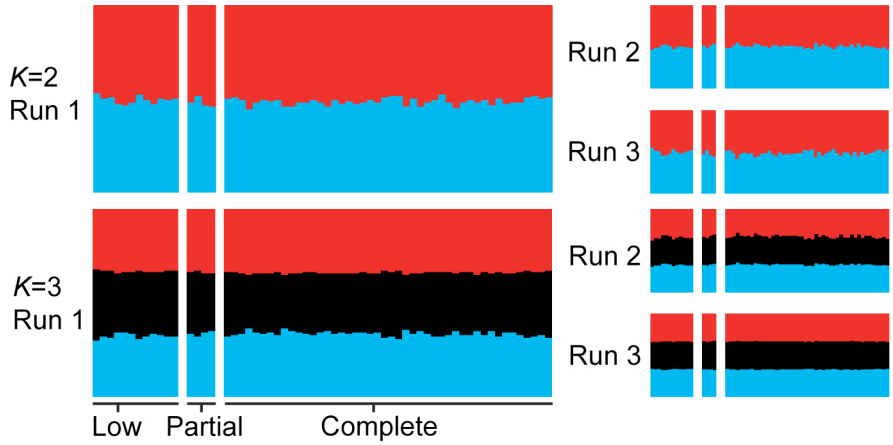

Figure 4.3. Estimated population structure of Kennedy Lake threespine stickleback (N= 62 from the 2006 sample) for three runs of STRUCTURE at K= 2 and K=3 inferred genetic clusters. Each individual is represented by a single, thin column, which is partitioned into K coloured segments that represent that individual's estimated membership fractions in K genetic clusters. For K=2 and K=3, each individual shares an equal likelihood of belonging to all genetic clusters, suggesting that low, partial and complete morphs belong to a single cluster (population). This pattern was statistically supported by individual based Bayesian assignment, implemented in STRUCTURE, which found that one was the most likely number of genetic clusters within this sample (Pr[K=1] =1.0; Pr[K=2]=0, Pr[K=3]=0).

Assortative mating among *Eda* genotypes also fails to account for the observed heterozygote deficiency (Barreto and McCartney 2008). To test this we genotyped the *Eda* locus from six fertilized eggs (zygotes) collected from each of 71 stickleback nests during breeding season in June 2008. If the heterozygote deficiency found in adults at *Eda* reflects assortative mating, then zygotes should show a similar deficiency of *Eda*

heterozygotes. In contrast to adults, zygotes showed no deviation from HWE (χ^2 = 0.433, P = 0.81), nor a significant deficiency in heterozygotes (F_{IS} = 0.061, 95% CI [-0.071, 0.203]), implying that heterozygote deficiency is not generated by assortative mating at the *Eda* locus. Direct comparison shows that adults exhibit significantly more heterozygote deficiency than zygotes (95% CI [0.08 - 0.51] for difference in F_{is} between adults and eggs sampled in 2008). *Eda* allele frequency differed somewhat between zygotes (frequency of low allele = 0.23) and adults (0.31), but the difference was not statistically significant (χ^2 = 0.815; P = 0.37).

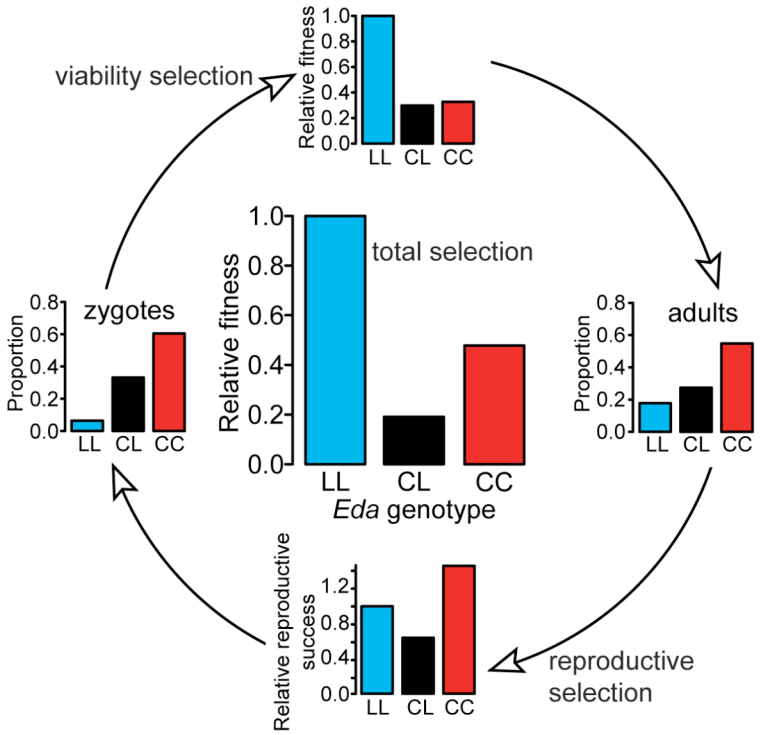

Figure 4.4. Estimated relative fitness components of genotypes at the *Ectodysplasin* locus based on genotype frequencies of zygotes and adults sampled during the breeding season of June 2008.

We estimated the selection pressures necessary to account for the observed change in genotype frequencies between life stages, from zygote to adult and from adult to zygote, across one generation (Fig. 4.4). The frequency of $Eda^{C/C}$ homozygotes and heterozygotes were similar across life stages (Fig. 4.2); however, the proportion of $Eda^{L/L}$ homozygotes was significantly lower in zygotes when compared to adults (proportion $Eda^{L/L}$ in zygotes = 0.06, 95% CI [0.035, 0.098], in adults = 0.18[0.122, 0.251]). On this basis we estimate viability selection (survival from zygote to adult) to strongly favour individuals homozygous for low alleles, $Eda^{L/L}$ (s = 0.67, hs = 0.7). This is consistent with previous studies indicating a growth advantage held by individuals carrying low alleles that likely translates to a survival advantage (Barrett et al. 2008; Marchinko and Schluter 2007). Indeed, in our Kennedy Lake samples, adult individuals of the low morph were slightly larger in standard length than individuals of the complete morph (difference of means = 1.8 mm; P = 0.048, N = 44 and 45 for the low and complete morph respectively; Appendix 9).

In contrast, selection on breeding adults during the reproductive phase favours complete $Eda^{C/C}$ homozygotes (s = -0.46, hs = 0.37; Fig. 4.4), returning the frequency of $Eda^{L/L}$ homozygotes to the lower levels observed in zygotes (see paragraph above). Our model makes no assumption about the type of selection – sexual, fecundity, or gametic – during the reproductive phase. The differences in relative fitness among *Eda* genotypes at this stage may result from mate preferences or differences in clutch size or sperm viability. However, differential survival of zygotes prior to hatching, one form of gametic selection, is unlikely to contribute as lateral plate morphs from Kennedy Lake

differ little in hatching rate in freshwater (Marchinko and Schluter 2007). Finally, the combined effect of viability and mating yielded net selection against heterozygotes at the *Eda* locus ($hs = 0.81$), favouring $Eda^{L/L}$ homozygotes overall ($s = 0.52$; Fig. 4.4).

Theory suggests that frequency-dependent selection is required to maintain a stable polymorphism with heterozygote disadvantage, favouring each homozygote when rare (Wilson and Turelli 1986). Niche differentiation among morphologically divergent individuals naturally gives rise to frequency-dependent selection, although it is not the only potential mechanism (Ayala and Campbell 1974). We explored the possibility of niche differentiation among *Eda* homozygotes by comparing the ratio of stable isotopes of carbon, $\delta^{13}C$, and nitrogen, $\delta^{15}N$, between low and complete morphs. In lakes, $\delta^{13}C$ indicates the ultimate source of dietary carbon (littoral or pelagic), whereas $\delta^{15}N$ estimates trophic position (Post 2002). Our results revealed significant differences in $\delta^{13}C$ between low and complete morphs collected in 2004 (Fig. 4.5; $P < 0.0001$; Appendix 10). $\delta^{15}N$ were similar between morphs and non-significant ($P = 0.8$; Appendix 10). Based upon the $\delta^{13}C$ signatures of sticklebacks and three additional lake species (Fig. 4.5), complete morphs feed more often upon prey with carbon derived from littoral sources than low morphs, although both appear to feed predominantly on prey from pelagic food chains. If these niche differences provide separate density-dependent regulation, then frequency-dependent selection may act to stabilise the observed *Eda* polymorphism even in the presence of underdominance (Schluter 2003).

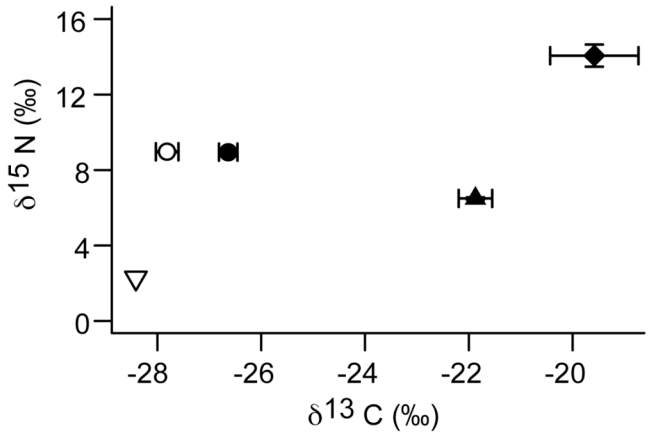

Figure 4.5. Isotopic representation ($\delta^{13}C$ and $\delta^{15}N$) of the long term diet of low morph (open circle) and complete morph stickleback (closed circle) collected from Kennedy Lake in 2004. Low and complete morphs differ significantly in $\delta^{13}C$ signature ($P < 0.0001$; two factor ANOVA, Table S4) suggesting diets are divided along a pelagic-littoral axis. Lower $\delta^{13}C$ values indicate that individuals feed on prey from pelagic food chains14. Additional species within the Kennedy lake food web were collected in 2004 to provide landmarks upon which to compare diet: Coho parr, *Oncorhynchus kisutch* (diamond), juvenile Peamouth Chub, *Mylocheilus caurinus* (closed triangle), mussels *Anodonta sp*. (open triangle). The $\delta^{13}C$ signature of mussels provides a landmark where individuals feed primarily upon pelagic sources of $\delta^{13}C$, whereas juvenile Peamouth Chub, which feed almost exclusively on benthic invertebrates (Gray and Dauble 2001), provide a landmark where individual diets are rich in benthic sources of $\delta^{13}C$. Points and error bars represent the mean ± SE. Error bars are absent where point size exceeds error magnitude. Sample sizes were 45 individuals of the low morph, 45 complete morph, 50 mussels, 15 Coho, 39 Peamouth Chub.

Polymorphisms maintained in the face of low heterozygote fitness satisfy the basic theoretical conditions required to initiate diversification and maintain genetic variation through either the modification of dominance or the evolution of assortative mating (Wilson and Turelli 1986). Strong underdominance at a single locus yields the most likely scenario under which assortative mating should develop (Otto et al. 2008). Subsequent reproductive isolation and speciation is a possible outcome in this population (Dobzhansky 1940; Gavrilets et al. 2007; Otto et al. 2008; Wilson and Turelli 1986). However the strongly dissimilar allele frequencies

observed here may result in the extinction of the rarer allele during the establishment of assortative mating due to the disadvantage that individuals possessing the rare allele face when locating mates that are also rare (Otto et al. 2008). Alternatively, large inequalities in allele frequencies may result in a greater chance that dominance modification will lead to a reduction of the formation maladaptive heterozygotes (Durinx and Van Dooren 2009). At this point we are unable to predict which evolutionary trajectory is most likely proceed.

Our results supply the first example of a single locus polymorphism maintained despite heterozygote disadvantage. The maintenance of this polymorphism supports a growing body of work which suggests that selection maintains ecologically important genetic variation in the wild (Fitzpatrick et al. 2007; Mitchell-Olds et al. 2007; Subramaniam and Rausher 2000). Our ability to detect this process at a single locus highlights the advantage of integrating genetic and ecological tools when studying the mechanisms maintaining genetic variation and promoting diversification in natural populations. As the integration of genetics and ecology becomes more prevalent in studies of evolutionary divergence (e.g., localization of QTL for ecologically relevant traits) a better understanding of the role of population-level variation driving biological diversification may be achieved. If the theory is robust, then patterns of underdominance leading to diversification in natural populations will be fundamental towards explaining these evolutionary patterns, particularly in cases when major genes underlie the genetics of adaptive change (Hori 1993; Smith 1993; Ueshima and Asami 2003).

METHODS

Collections

Kennedy Lake is a large (6475 ha), deep (145 m) and extremely oligotrophic lake (Hyatt and Ringler 1989), lying six meters above sea level and approximately six kilometres from the west coast of Vancouver Island. We collected adult threespine stickleback in minnow traps near the southern tip of the Clayqout arm of Kennedy Lake on the following dates: May 11, July 9 and Aug 17, 2004, June 11, 2006, and June 12, 2008. Specimens from the May 11, 2004 collection were frozen immediately upon capture using dry ice and transported to the University of British Columbia for storage at -80 °C prior to stable isotope analysis. These samples were thawed briefly for morphological measurements and then stored at -80 °C until processed for stable isotope analysis. Specimens collected in July and August of 2004, and in 2006 and 2008 were given a lethal dose of tricaine methanesulfonate (MS-222, Syndel Laboratories, Qualicum Beach, BC, Canada) and then preserved in 95% ethanol. We collected eggs by removing whole nests while snorkeling June 9-12, 2008. Eggs were preserved in 95% ethanol. We also collected the individual male guarding each nest (the putative fathers of clutches within a nest) using a dip net while snorkeling. These guardian males were combined with the minnow trap samples from the same collection date to make up the June 2008 adult collection. Preserved specimens from a sample obtained on Aug 2, 1965, were available from the University of British Columbia Fish Museum (Catalogue No. UBC 65-0506; collectors G. Haythorne and D. Hagen).

Stable Isotope Analysis

For the analysis of stable isotopes of Nitrogen ($\delta^{15}N$) and Carbon ($\delta^{13}C$), we removed tissue from the foot of mussels, and dorsal white muscle from threespine stickleback, peamouth chub and coho parr. All tissue was freeze-dried and ground to a fine powder with a mortar and pestle. Isotope samples were run on a Finnigan Delta Plus Advantage stable isotope ratio mass spectrometer at the University of Victoria. Variation in $\delta^{13}C$ of fish tissue can arise from individual variation in lipid content, because lipids have a lower $\delta^{13}C$ than proteins and carbohydrates (Kiljunen et al. 2006). However, we found no evidence for variation in lipid content among morphs (C:N ratio: $p>0.05$), and following chemical lipid extraction (Ingram et al. 2007), the difference in $\delta^{13}C$ between morphs was significant ($P = 0.0001$), while the factor sex and the interaction between sex and morph were non-significant ($P > 0.19$). This is the same pattern of isotopic differentiation between morphs that we observed with the untreated samples and presented in the text.

Genotyping

Total genomic DNA was isolated using standard phenol-chloroform extraction methods. Six microsatellite loci, isolated and characterized by the Stanford Genome Research Center, were selected for genetic analyses (Genbank Accession numbers and reference in parentheses); Stn301 (BV678111, unpublished), Stn65 (G72254 (Peichel et al. 2001)), Stn216 (BV102494 (Colosimo et al. 2004)), Stn250 (BV678075, unpublished), Stn387 (BV678140, unpublished), and Stn388 (BV678141, unpublished).

A diagnostic in/del locus (isolated from the locus Stn381 within intron six of the *Eda* gene (Colosimo et al. 2005) was used to identify the genotype of lateral plate morphs.

Microsatellite and *Eda* 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). Cycling conditions were standardized over all loci as follows: 93 ^{0}C for 3 min, 95 ^{0}C 30 s, 59 ^{0}C 30s, 72 ^{0}C 30 s, 5 cycles of 94 ^{0}C 30 s, 59 ^{0}C 30 s, 72 ^{0}C 30 s, 35 cycles of 90 ^{0}C 30 s, 60 ^{0}C 30 s, 72 ^{0}C 30 s, followed by 72 ^{0}C 10 min, then cooled to 4 ^{0}C.

Amplified PCR products were separated by gel electrophoresis on an ABI 3733 automated sequencer using the GS500 Size Standard (Applied Biosystems) and scored with the software GENEMAPPER (Applied Biosystems). Tests of genetic diversity and Hardy Weinberg equilibrium were estimated with GENEPOP v.4.0 (Rousset 2008). Genetic differentiation in allele frequencies between low and complete morphs was tested in ARLEQUIN (v2.001). The most likely number of candidate source populations (from *K*=1 to 3) was assessed with a Bayesian individual-based assignment approach implemented in STRUCTURE (Pritchard et al. 2000).

Reproductive Fitness Model

The model of selection during the reproductive phase of the life cycle assumes that breeding is random with respect to *Eda* genotype, and that the genotype frequencies of females and males equals those frequencies observed at *Eda* in randomly sampled adults. Selection in this model results from differences in mate preferences, viability or fecundity

among *Eda* genotypes.

Model calculations:

p_1, p_2, p_3 represent the observed breeding frequencies of male and female $Eda^{L/L}$, $Eda^{C/L}$, and $Eda^{C/C}$ genotypes, respectively.

m, n represent the fraction of $Eda^{C/L}$, and $Eda^{C/C}$ genotypes contributing offspring during the reproductive phase. This is equivalent to the fitness of $Eda^{C/L}$, and $Eda^{C/C}$ genotypes relative to $Eda^{L/L}$ genotypes (set to 1). We then solve for the values of *m* and *n* that generate zygote allele frequencies (p', q') equal to those observed in zygotes sampled from wild clutches.

q_1, q_2, q_3 represent the zygote frequencies of $Eda^{L/L}$, $Eda^{C/L}$, and $Eda^{C/C}$ genotypes, respectively, calculated using the following random mating equations.

Genotype frequencies:

$$q_1 = (p_1 \bullet p_1) + (0.5 \bullet ((p_1 \bullet mp_2) + (mp_2 \bullet p_1)) + (0.25 \bullet (mp_2 \bullet mp_2))$$

$$q_2 = (p_1 \bullet np_3) + (np_3 \bullet p_1) + (0.5 \bullet ((p_1 \bullet mp_2) + (mp_2 \bullet p_1) + (mp_2 \bullet mp_2) + (mp_2 \bullet np_3) + (np_3 \bullet mp_2))$$

$$q_3 = (np_3 \bullet np_3) + (0.5 \bullet ((np_3 \bullet mp_2) + (mp_2 \bullet np_3)) + (0.25 \bullet (mp_2 \bullet mp_2))$$

Allele frequencies:

$$p' = q_1 + (0.5 \bullet q_2)$$

$$q' = q_3 + (0.5 \bullet q_2)$$

Relative Fitness and Selection Coefficients

Relative fitness for each *Eda* genotype was calculated as the change in the observed frequency of a genotype as individuals aged from zygote to adult (adult frequency divided by zygote frequency), standardised to the homozygous low $Eda^{L/L}$ genotype. The total relative fitness (total selection) of each genotype across one generation was calculated as the product of the relative fitness from viability and reproductive phases, standardised to the homozygous low $Eda^{L/L}$ genotype. Selection coefficients were calculated as one minus the relative fitness of $Eda^{C/C}$ homozygote (*s*) and heterozygote (*hs*) (Hedrick 2000).

Statistical Analyses

All statistical analyses were carried out using R version 2.8.0 (R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org). Confidence intervals for the *Eda* genotype frequencies observed in adults were calculated using the Wilson method from the Hmisc package in R. Heterozygote deficiency was quantified using Wright's coefficient of inbreeding (F_{IS}), which was calculated as:

$$F_{IS} = 1 - \frac{H}{2pq}$$

where H is the frequency of the heterozygote class, and p and q represent the allele frequencies (Hedrick 2000). Confidence intervals of F_{is} in adults and zygotes were made using bootstrap re-sampling. Confidence intervals for adult samples from 2006 and 2008

were calculated from a bootstrap distribution of F_{is} for each year, generated by re-sampling individuals 10000 times. Confidence intervals for zygote genotype frequencies and F_{is} were estimated from a bootstrap distribution of either genotype frequency or F_{is} (depending on the analysis), generated from 10000 re-samples of the 71 clutches. We also calculated the 95% confidence interval for the difference in F_{is} between adults and eggs sampled in 2008. This interval did not include zero (0.08 - 0.51) confirming that the heterozgyote deficit found in adults was significantly greater than that observed in zygotes.

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CHAPTER 5. GENERAL CONCLUSIONS

In chapters 2 and 3 I tested two of the ecological factors thought to drive armour divergence between marine and freshwater populations of threespine stickleback: the ion and predation hypotheses. In chapter two I've shown that lateral plate morphs differ little in hatching success, but strongly in juvenile growth rate when raised at different salinities. Offspring of parents with fewer plates grew substantially faster than offspring of parents with many lateral plates in fresh water. Growth rates differed very little in salt water. Results were consistent in offspring from two populations, one marine and one fresh water, strengthening the conclusion that lateral plates are disadvantageous in fresh water habitats. These tests were restricted to comparisons between morphs collected from the same population, minimizing any additional adaptations to local salinity possibly confounding previous work that demonstrated differences in hatching and survival between plate morphs when exposed to water of different salinity (Heuts 1947; Schaarschmidt et al. 1999). The results of Chapter 2 suggest that the repeated loss of lateral plates in fresh water may have arisen through a correlated response to selection for faster growth during lateral plate development. Faster growth rates may translate into higher fitness through the positive effects that body size has on overwinter survival, clutch size and predator vulnerability (Arendt 1997). In this case, the reduction of lateral plates in fresh water could be non-adaptive, with plate number decreasing because of the fitness advantages of larger size.

Chapter 2 does not, however, distinguishing whether a difference between morphs in the ability to maintain ionic homeostasis (regulating Na^+, or Cl^- exchange) or calcium limitation is responsible for differences in growth rate between plate morphs. *Gjb1*, a

gene associated with salt secretion in animals lies in genomic region near *Eda* in the F_2 progeny of a marine by fresh water cross (Colosimo et al. 2005), making it plausible that linkage between *Eda* and *Gjb1* results in differences in ion regulatory ability between plate morphs. However, in the polymorphic Kennedy Lake population, the recombination that has occurred over thousands of generations since being colonized by marine stickleback should have broken down any linkage between *Eda* and *Gjb1*, dissolving the correlation between lateral plates and growth rate. The observed association between growth rate and lateral plate number in the polymorphic Kennedy Lake population might thus indicate that pleiotropic effects of *Eda*, not linkage between *Eda* and other genes, results in growth differences between plate morphs. If pleiotropy is responsible, as suggested by Barrett et al. (Barrett et al. 2008; Cresko 2008), then calcium limitation may be a more likely mechanism by which bony lateral plates are negatively associated with growth rate in freshwater only. Measuring the growth rates of different lateral plate morphs raised at different concentrations of calcium in fresh water will be required to test this alternative.

In Chapter 3 I tested the hypothesis that predator-driven divergent selection causes differentiation in defensive armor between marine and freshwater threespine stickleback. Using F_2 families from crosses between freshwater and marine populations, I demonstrated that predaceous insects impose selection for larger juvenile size, and shorter dorsal spine and pelvic girdle after accounting for size. Targeted genotyping of the gene associated with building armour plates, *Eda*, revealed that freshwater *Eda* alleles are increase relative to marine alleles in the presence of insect predators. Note however, that the experiment was conducted before the number of amour plates had reached its

final number during development. Predaceous aquatic insects likely contribute to the reduction of armour in freshwater populations of threespine stickleback via direct selection on spine length. Insects prey most heavily on the smallest individuals and we have evidence that as juveniles, the complete morph grows more slowly than the low morph. The results from both Chapters 2 and 3 suggest that reduction of lateral plate number in fresh water may result from the indirect effect of insects preying most heavily on smaller juveniles through the association of bony armour and growth rate.

Although Chapter 3 documented selection on armour after adjusting for differences in body size, the correlation between armour traits obscures which trait or traits are the target of selection. Indeed, the correlation of anterior dorsal spine demonstrates our inability to distinguish whether spine length, *Eda*, or both are under selection. This problem also illustrates our inability to attribute whether the selection at *Eda* is due to the effects of *Eda* itself, or whether it results from linkage disequilibrium between *Eda* and additional armour or shape genes, or genes of unknown phenotypic or physiological effects. Distinguishing which trait or set of traits influence predator induced mortality rates could be accomplished with careful trait manipulations, or selection experiments using advanced generation hybrids between marine and freshwater populations, instead of F_2 hybrids used here, in order to breaking down linkage disequilibrium between genes affecting different traits.

One final note on Chapter 3 concerns the level of significance found for the association between pelvic girdle loss and insect predation and between *Eda* allele frequency and insect predation. Tests revealed that pelvic girdle absence and *Eda* allele frequency were nearly significant at the 0.05 alpha level when using a two-tailed *t*-test (*P*

= 0.08 and 0.052 for pelvic girdle and *Eda* respectively). Because of the moderate number of tests performed in this study (16) it is possible that one or both may be nearly significant by random chance. As a result it should be acknowledged that both the association of pelvic girdle absence and insect predation rate, and *Eda* allele frequency and insect predation rate could be specific to this study. Concluding that predaceous aquatic insects have played a significant role in the loss of the pelvic girdle, and the sweep of the low *Eda* allele in fresh water requires further examination.

In Chapter 4 I explored the ecological and genetic mechanisms maintaining phenotypic variation within a wild population. In this chapter I demonstrated an extremely rare example of a single locus polymorphism being maintained despite heterozygote disadvantage. Single locus underdominance is predicted to lead to the evolution of dominance or assortative mating (Durinx and Van Dooren 2009; Otto et al. 2008), yet no evidence of either pattern was found. Monitoring long-term changes in genotype frequencies in this population may eventually capture and identify the genetic and ecological conditions resulting in dominance or possibly the early stages of reproductive isolation. Niche separation between *Eda* homozygotes, observed as differentiation in carbon isotopes, suggests that frequency-dependent selection is involved in the maintenance of polymorphism despite heterozygous disadvantage. Both resource and predator density may drive frequency-dependent selection in this system and independent confirmation that frequency-dependence maintains this polymorphism despite heterozygote disadvantage is needed. This particular system could provide an explicit test of the theoretical prediction that frequency-dependent selection can maintain underdominant polymorphism in both lab and natural settings, through short-term

experimental and long-term field monitoring approaches. For example, experimental manipulations of planktonic *vs*. benthic food availability and its effect on growth rate and mortality of individuals differing in *Eda* alleles would be useful for testing frequency-dependent selection in response to resource availability.

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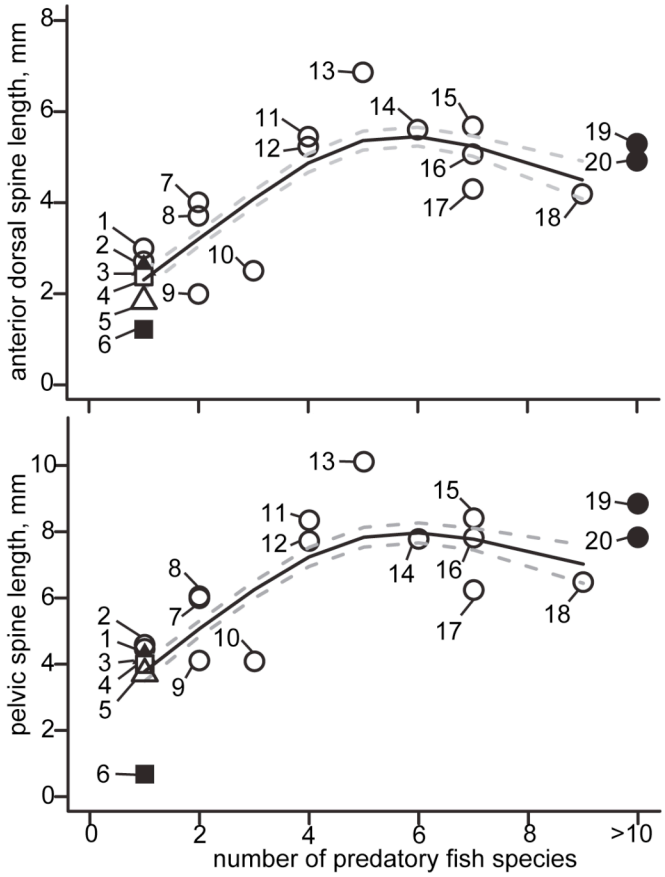
APPENDICES

Appendix 1. Locations of sampling sites associated with chapter three, Figure 3.1.

Numbers connected to symbols indicate the populations used in this chapter three: Klein Lake (1), McKay Lake (2), Priest Lake benthic (3), Paxton Lake limnetic (4), Priest Lake limnetic (5), Paxton Lake benthic (6), Paq Lake (7), North Lake (8), Cranby Lake (9), Dougan Lake (10), Beaver Lake (11), Blackjack Lake (12), Mayer Lake (13), Brannen Lake (14), Erroch Lake (15), Kennedy Lake (16), Fairy Lake (17), Sproat Lake (18), Oyster lagoon marine (19), Little Campbell River marine (20). All sites are located in British Columbia, Canada.



Appendix 2. Supplementary methods from chapter three.

Crossing, fertilization and fish husbandry

The crossing of wild caught individuals from parental populations to generate F_1 hybrid lines was accomplished using standard laboratory protocol. Fertilization was performed by first stripping a female's eggs into a Petri dish containing a small amount of de-chlorinated tap water. Next, a male was anaesthetized in clove oil, both testes were removed and placed into the Petri dish with the eggs, and then crushed to release sperm. The macerated testes were then removed from the Petri dish after 20 minutes, the eggs transferred to plastic egg-cups (pint cup with the bottom replaced with fine fiberglass mesh) and submerged in an egg-tank (20 L) containing methylene blue to reduce fungal growth. Eggs remained in aerated egg-tanks for eight days, after which they were transferred to a 102 L tank. F_1 hybrid lines were raised from hatchlings to adults in 102 L tanks. Hatchlings were fed newly hatched live brine shrimp twice a day for the first six weeks, after which diets were supplemented with chopped frozen blood worms. At about three months post-hatching, F_1 lines were split into two to four 102 L tanks to maintain roughly 20 individuals per tank throughout the following winter, during which time individuals were fed frozen bloodworms once daily.

The following spring, when F_1 males began to show nuptial coloration and females were gravid, brother-sister mating pairs were chosen from each F_1 hybrid line. Using the protocol described above, eggs from an F_1 female were stripped and fertilized with sperm from an F_1 male of the same family to establish an F_2 family to be used in the predation trials. One mating pair from each of the six F_1 hybrid lines was created from

the PBxOLM cross, resulting in six F_2 families. A total of ten brother-sister mating pairs were chosen from the single MLxLCM F_1 hybrid line, resulting in ten F_2 families. F_2 hatchlings were fed newly hatched live brine shrimp twice a day and raised for about three weeks in 102 L tanks until they reached about 11 mm standard length. At this time, hatchlings were transported from the laboratory to the UBC experimental ponds to begin the predation experiment.

Appendix 3. Family sample sizes from each F_2 family in the Paxton and McKay lines found in chapter three.

Paxton lines	family	No-predation before(after)	Predation before(after)
sample size	1	49(45)	49(24)
	2	42(42)	43(21)
	3	36(20)	35(7)
	4	31(20)	31(7)
	5	40(11)	39(6)
	6	41(31)	41(20)
McKay lines			
sample size	1	37(31)	37(25)
	2	36(31)	36(14)
	3	32(30)	32(21)
	4	29(29)	28(12)
	5	36(25)	36(15)
	6	43(31)	44(19)
	7	70(66)	70(34)
	8	21(19)	20(9)
	9	32(28)	32(17)
	10	48(40)	48(20)

Appendix 4. 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.

Paxton lines		No-predation		Predation		selection
Trait	family	Mean (X_B)	S.D.	Mean (X_A)	S.D.	differential (i)
standard length	1	14.708	1.390	15.051	1.003	0.247
	2	13.909	1.192	14.217	0.895	0.258
	3	13.556	1.229	14.845	1.061	1.049
	4	12.451	1.130	12.847	0.995	0.351
	5	15.107	0.805	16.323	1.068	1.511
	6	13.967	1.616	14.702	0.944	0.455
anterior dorsal spine length	1	0.023	0.114	-0.043	0.137	-0.577
	2	0.008	0.118	-0.015	0.116	-0.194
	3	0.015	0.116	-0.044	0.128	-0.511
	4	0.006	0.136	-0.017	0.104	-0.164
	5	0.012	0.131	-0.022	0.154	-0.256
	6	0.011	0.116	-0.017	0.140	-0.236
second dorsal spine length	1	0.032	0.124	-0.060	0.137	-0.741
	2	0.006	0.129	-0.011	0.125	-0.132
	3	0.000	0.101	0.000	0.121	0.002
	4	0.007	0.095	-0.019	0.098	-0.271
	5	-0.005	0.093	0.009	0.097	0.145
	6	-0.008	0.157	0.012	0.120	0.128
pelvic spine length	1	0.045	0.249	-0.072	0.205	-0.470
	2	0.021	0.186	-0.037	0.203	-0.313
	3	0.036	0.159	-0.107	0.227	-0.896
	4	-0.025	0.174	0.072	0.141	0.557
	5	-0.054	0.057	0.080	0.195	2.337
	6	-0.020	0.148	0.030	0.181	0.335
pelvic girdle length	1	-0.018	0.776	0.033	0.480	0.065
	2	0.054	0.207	-0.086	0.481	-0.676
	3	0.063	0.479	-0.152	0.698	-0.450
	4	-0.054	0.211	0.153	0.201	0.986
	5	0.013	0.721	-0.018	0.982	-0.043
	6	-0.015	0.120	0.023	0.181	0.314

Appendix 4 continued.

McKay lines		No-predation		Predation		selection
Trait	family	Mean (X_B)	S.D.	Mean (X_A)	S.D.	differential (i)
standard length	1	15.359	1.696	15.092	1.191	-0.157
	2	14.853	2.144	15.520	0.811	0.311
	3	17.443	1.039	17.558	1.206	0.110
	4	15.131	1.452	15.702	1.154	0.393
	5	17.945	0.897	17.544	1.091	-0.447
	6	16.067	1.579	15.553	1.145	-0.325
	7	14.447	1.159	14.903	1.024	0.394
	8	19.369	2.147	20.513	1.462	0.533
	9	17.944	1.934	18.737	1.990	0.410
	10	14.898	1.089	16.021	1.487	1.032
anterior dorsal spine length	1	-0.007	0.161	0.009	0.131	0.099
	2	-0.041	0.221	0.010	0.101	0.230
	3	0.006	0.157	-0.009	0.127	-0.096
	4	0.009	0.104	-0.021	0.087	-0.288
	5	0.023	0.112	-0.038	0.094	-0.542
	6	0.006	0.138	-0.009	0.111	-0.110
	7	0.009	0.111	-0.018	0.137	-0.244
	8	0.018	0.119	-0.037	0.096	-0.460
	9	0.004	0.157	-0.007	0.192	-0.067
	10	-0.009	0.110	0.018	0.119	0.244
second dorsal spine length	1	-0.010	0.163	0.012	0.130	0.132
	2	-0.001	0.115	0.002	0.127	0.028
	3	0.023	0.144	-0.033	0.152	-0.393
	4	0.004	0.106	-0.010	0.107	-0.139
	5	0.034	0.107	-0.057	0.158	-0.852
	6	-0.008	0.137	0.013	0.122	0.155
	7	0.006	0.119	-0.012	0.127	-0.156
	8	-0.006	0.164	0.013	0.140	0.116
	9	0.017	0.147	-0.028	0.179	-0.307
	10	0.004	0.130	-0.009	0.159	-0.103

Appendix 4 continued.

McKay lines		No-predation		Predation		selection
Trait	family	Mean (X_B)	S.D.	Mean (X_A)	S.D.	differential (i)
pelvic spine length	1	-0.016	0.241	0.019	0.262	0.144
	2	-0.010	0.238	0.022	0.215	0.132
	3	-0.010	0.269	0.014	0.284	0.086
	4	0.054	0.212	-0.129	0.228	-0.862
	5	0.034	0.202	-0.056	0.269	-0.446
	6	0.040	0.312	-0.065	0.263	-0.338
	7	0.015	0.231	-0.029	0.202	-0.193
	8	-0.027	0.256	0.058	0.250	0.333
	9	0.053	0.314	-0.087	0.195	-0.445
	10	0.001	0.264	-0.002	0.271	-0.012
pelvic girdle length	1	0.001	0.166	-0.001	0.158	-0.010
	2	-0.005	0.180	0.012	0.175	0.094
	3	0.036	0.169	-0.052	0.119	-0.520
	4	0.015	0.105	-0.036	0.148	-0.488
	5	0.031	0.162	-0.051	0.184	-0.508
	6	-0.024	0.190	0.040	0.188	0.338
	7	0.015	0.161	-0.030	0.175	-0.278
	8	0.063	0.162	-0.133	0.119	-1.211
	9	0.035	0.213	-0.058	0.188	-0.439
	10	0.013	0.185	-0.026	0.181	-0.214

Appendix 5. 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.

family	Δp	$t_{0.05(1)5}$	P
1	0.145	-2.528	0.026
2	0.247		
3	0.025		
4	0.050		
5	0.250		
6	-0.002		

Appendix 6. The association between *Eda* genotype and lateral plate morph phenotype pooled across all sample years. Fishers' exact test $P = 2.2*10^{-16}$.

	Plate morph		
Eda genotype	Complete	Partial	Low
CC	104	3	3
CL	42	7	3
LL	0	2	34

Appendix 7. Locus specific tests for Hardy-Weinberg equilibrium (HWE) and inbreeding coefficients (F_{IS}) at six neutral microsatellite loci genotyped in adult stickleback collected in May 2006 from Kennedy Lake. Each molecular marker used is not known to be linked to quantitative trait loci for any morphological traits. Bold values indicated p-value remained significant after a sequential Bonferroni adjustment.

Locus	Number of alleles	Ho	He	HWE (p-value)	F_{IS}	Linkage group
Stn381	3	0.355	0.624	**< 0.0001**	0.433	4
Stn301	32	0.871	0.956	0.2223	0.0874	18
Stn065	14	0.717	0.664	0.6383	-0.0807	6
Stn216	7	0.452	0.416	0.843	-0.1193	20
Stn250	37	0.919	0.946	0.9958	0.0286	8
Stn387	13	0.435	0.535	0.0423	0.1705	2
Stn388	7	0.71	0.693	0.9196	-0.0363	9

Appendix 8. Pairwise tests for linkage disequilibrium.

Locus#1	Locus#2	p-value	SE
STN381	STN301	1.0000	0.0000
STN381	STN065	0.5210	0.0330
STN301	STN065	1.0000	0.0000
STN381	STN216	0.1815	0.0200
STN301	STN216	0.2193	0.0760
STN065	STN216	0.8353	0.0432
STN381	STN250	1.0000	0.0000
STN301	STN250	1.0000	0.0000
STN065	STN250	1.0000	0.0000
STN216	STN250	0.5715	0.0733
STN381	STN387	0.2998	0.0346
STN301	STN387	0.9879	0.0121
STN065	STN387	0.9253	0.0392
STN216	STN387	0.2925	0.0490
STN250	STN387	0.8391	0.0700
STN381	STN388	0.8859	0.0104
STN301	STN388	1.0000	0.0000
STN065	STN388	0.7840	0.0590
STN216	STN388	0.6856	0.0505
STN250	STN388	1.0000	0.0000
STN387	STN388	0.0929	0.0476

Appendix 9. Results from ANOVA testing for effects of lateral plate morph, sex and their interaction on standard length, a measure of overall body size, from randomly sampled adults trapped in May of 2004.

source of variation	df	mean square	*F*	*P*
lateral plate morph	1	0.02588	4.0392	0.04763
sex	1	0.00137	0.2131	0.64557
morph * sex	1	0.01473	2.2995	0.13313
residuals	85	0.00641		

Appendix 10. Results from two-factor ANOVA testing the effects of lateral plate morphotype and sex on the ratio of stable isotopes $\delta^{13}C$ and $\delta^{15}N$.

source of variation	df	mean square	*F*	*P*
a) $\delta^{13}C$ ‰				
lateral plate morph	1	30.83	17.5637	< 0.0001
sex	1	1.046	0.5957	0.4424
morph * sex	1	2.924	1.6657	0.2003
residuals	85	1.755		
b) $\delta^{15}N$ ‰				
lateral plate morph	1	0.0097	0.0637	0.80128
sex	1	0.4566	3.0054	0.08661
morph * sex	1	0.0174	0.1148	0.73556
residuals	85	0.1519		

Appendix 11. Animal care certificates.

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0293

Investigator or Course Director: Dolph Schluter

Department: Zoology

Animals:

Sticklebacks	Threespine stickleback 4000
Invertebrates	Backswimmers (Notonecta) and dragonfly nymphs (Aeshna) 750
Trout	Cutthroat trout 10
Fish	Prickly sculpin 100

Start Date: April 1, 2006 **Approval Date:** November 17, 2008

Funding Sources:

Funding Agency: Canada Foundation for Innovation
Funding Title: CFI Infrastructure Operating Funds - The Origin and Persistence of Species - Operations

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: The genetics of adaptation to new environments

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: Ecology and genetics of adaptive radiation

Funding Agency: Canada Foundation for Innovation
Funding Title: CFI Infrastructure Operating Funds - The Origin and Persistence of Species - Operations

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: Ecology and genetics of adaptive radiation

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: The genetics of adaptation to new environments

Funding Agency: National Institutes of Health
Funding Title: 1 F32 GM086125-01 Genetics of Behavioral Reproductive Isolation in Threespine Stickleback, Schluter/Arnegard

Unfunded title: Ecology and genetics of adaptive radiation

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration

Appendix 11. continued

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0293

Investigator or Course Director: Dolph Schluter

Department: Zoology

Animals:

Trout Cutthroat trout	10
Fish Prickly sculpin	100
Sticklebacks Threespine stickleback	4000

Start Date: April 1, 2006 **Approval Date:** September 17, 2007

Funding Sources:

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: Ecology and genetics of adaptive radiation

Funding Agency: Canada Foundation for Innovation
Funding Title: CFI Infrastructure Operating Funds - The Origin and Persistence of Species - Operations

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: The genetics of adaptation to new environments

Unfunded title: Ecology and genetics of adaptive radiation

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

Appendix 11. continued

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A04-0208

Investigator or Course Director: Dolph Schluter

Department: Zoology

Animals:
Brienomyrus magnostipes 500
Fish 1000

Start Date: April 28, 2005 **Approval Date:** July 21, 2006

Funding Sources:

Animal Protocol Number: A04-0208
Grant Agency: Centre National De La Recherche Scientifique
Grant Title: Resting the predictions of ecological speciation in sticklebacks (EcoSpec)
Grant Number: 04-5521

Grant Agency: Natural Science Engineering Research Council
Grant Title: Ecology, genetics, and the origin of species
Grant Number: 00-3385

Grant Agency: BC Ministry of Environment, Lands and Parks
Grant Title: Population Assessment for Stickleback Species Pairs
Grant Number: 06-0798

Animal Protocol Number: A04-0208
Grant Agency: Natural Science Engineering Research Council
Grant Title: Ecology, Genetics and the Origin of Species

Grant Agency: Natural Science Engineering Research Council
Grant Title: Ecology and genetics of adaptive radiation

Grant Agency: Natural Science Engineering Research Council
Grant Title: The Genetics of Adaptation to New Environments

Grant Agency: National Geographic Society
Grant Title: National Geographic grant to collect electric fishes in Gabon

Grant Agency: Royal Society of Canada
Grant Title: Ecological Causes of Adaptive Radiation

Unfunded title: n/a

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

Appendix 11. continued

The University of British Columbia

Animal Care Certificate

Application Number: A04-0208

Investigator or Course Director: Dolph Schluter

Department: Zoology

Animals Approved:

Fish 1000
Frogs 500

Start Date: **April 28, 2005** Approval Date: **June 21, 2005**

Funding Sources:

Funding Agency: Centre National De La Recherche Scientifique
Funding Title: Resting the predictions of ecological speciation in sticklebacks (EcoSpec)

Funding Agency: Natural Sciences and Engineering Research Council
Funding Title: Ecology, genetics, and the origin of species

Funding Agency: Natural Sciences and Engineering Research Council
Funding Title: Ecological Causes of Adaptive Radiation

Funding Agency: Royal Society of Canada
Funding Title: Ecological Causes of Adaptive Radiation

Funding Agency: Natural Sciences and Engineering Research Council
Funding Title: Ecology, Genetics and the Origin of Species

Funding Agency: Natural Sciences and Engineering Research Council
Funding Title: The Genetics of Adaptation to New Environments

Unfunded title: n/a

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

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Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

Appendix 11. continued

The University of British Columbia

ANIMAL CARE CERTIFICATE

PROTOCOL NUMBER: A04-0208	
INVESTIGATOR OR COURSE DIRECTOR: Schluter, D.	
DEPARTMENT: Zoology	
PROJECT OR COURSE TITLE: Ecological Causes of Adaptive Radiation	
ANIMALS: Fish 1000 Frogs 500	
START DATE: 04-04-01	APPROVAL DATE: JUL 2 8 2004
FUNDING AGENCY: Natural Science Engineering Research Council	

The Animal Care Committee has examined and approved the use of animals for the above experimental project or teaching course, and have been given an assurance that the animals involved will be cared for in accordance with the principles contained in Care of Experimental Animals - A Guide for Canada, published by the Canadian Council on Animal Care.

Approval of the UBC Committee on Animal Care by one of:

Dr. W.K. [illegible], Chair

Dr. [illegible], Director, Animal Care Centre

Ms. L. Macdonald, Manager, Animal Care Committee

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
[illegible] Road, Vancouver, [illegible]
Phone: [illegible] FAX: [illegible]

Appendix 11. continued

The University of British Columbia

ANIMAL CARE CERTIFICATE

PROTOCOL NUMBER: **A00-0191**

INVESTIGATOR OR COURSE DIRECTOR: **Schluter, D.**

DEPARTMENT: **Zoology**

PROJECT OR COURSE TITLE: **Ecological Causes of Adaptive Radiation**

ANIMALS: **Fish**

START DATE: **00-04-01** APPROVAL DATE: **June 30, 2003**

FUNDING AGENCY: **Natural Science Engineering Research Council**

The Animal Care Committee has examined and approved the use of animals for the above experimental project or teaching course, and have been given an assurance that the animals involved will be cared for in accordance with the principles contained in Care of Experimental Animals - A Guide for Canada, published by the Canadian Council on Animal Care.

Approval of the UBC Committee on Animal Care by one of:
Dr. W.K. Milsom, Chair
Dr. J. Love, Director, Animal Care Centre
Ms. L. Macdonald, Manager, Animal Care Committee

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

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