

PARALLEL EVOLUTION BY CORRELATED RESPONSE: LATERAL PLATE REDUCTION IN THREESPINE STICKLEBACK

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Recent work has revealed the molecular mechanisms governing one of the most dramatic examples of parallel evolution in nature: the repeated loss of lateral plate armor in freshwater populations of threespine stickleback. Yet, the ecological mechanisms responsible for armor loss remain unclear. Using a balanced experimental design, we examined Heuts' (1947) hypothesis that selection due to differences in salinity indirectly drive the reduction of lateral plate armor in fresh water while maintaining armor in the sea. We measured two fitness-related traits, hatching success and juvenile growth rate, in offspring of reduced (low and partial) and complete lateral plate morphs from two polymorphic populations when raised in either fresh water or salt water. In contrast to Heuts' results, there was little difference among morphs in hatching success. However, salinity strongly influenced juvenile growth: offspring of reduced lateral plate morphs grew substantially faster (up to 65%) than offspring of completely plated morphs in fresh water, but there was little difference in salt water. We suggest that the parallel loss of lateral plates in fresh water has arisen through a correlated response to selection for faster growth during lateral plate development, but the effect of salinity on hatching success and juvenile growth rate cannot explain the predominance of completely plated morphs in marine populations.

KEY WORDS: Correlated response, growth rate, pleiotropy, polymorphism, stickleback, trade-off.

One of the most impressive examples of parallel evolution in nature is the rapid and repeated loss of lateral plate armor 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. 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. 1; Wootton 1984; Bell 2001). A few populations are polymorphic for lateral plate armor, possessing low, complete, and partial (11–29 lateral plates) plate morphs. Recent molecular evidence (Colosimo et al. 2004, 2005) indicates that the recurrent transition from completely plated marine populations to low-plated fresh-

water 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.

Surprisingly, although the molecular mechanisms of lateral plate reduction are becoming established, the ecological mechanisms driving armor 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

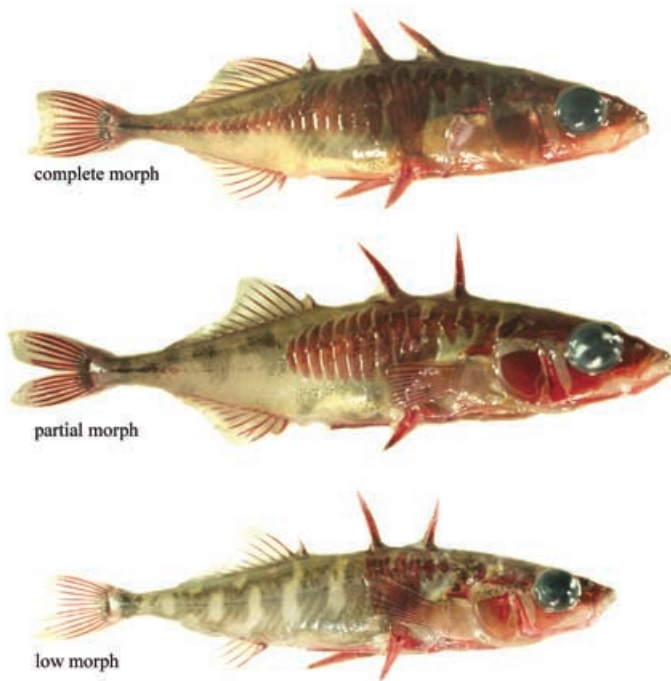


Figure 1. The three lateral plate morphotypes of *Gasterosteus aculeatus*. Bony elements were stained red using alizarin red in 1% KOH solution.

(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; Hagen and Moodie 1982; Bell 2001) 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

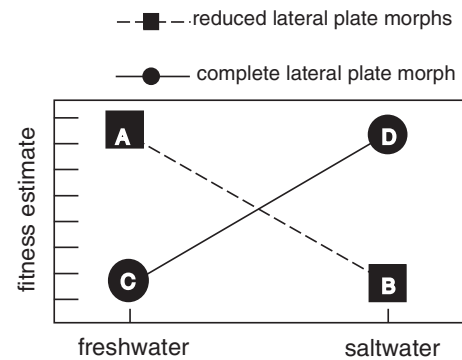


Figure 2. Expected patterns of fitness among lateral plate morphs of *Gasterosteus aculeatus* when hatched and raised in fresh water and salt water. According to Heuts (1947) (i) reduced morphs should exhibit greater fitness than the complete morph when raised in fresh water, $A > C$; (ii) the complete 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 $(B - A) < (D - C)$.

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 whether 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 (Fig. 2). 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 unpubl. 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) both at a pH of 7.6 (+/- 0.1). A male of the same morph and population was anesthetized 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 min., 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 with 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 10 weeks during the period of lateral plate development. After an individual reaches about 30-mm standard length the number of plates no longer changes (Igarashi 1970; Bell 1981). 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 10 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 10 weeks of the experiment (see Fig. 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 (six, eight, 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. In addition, 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

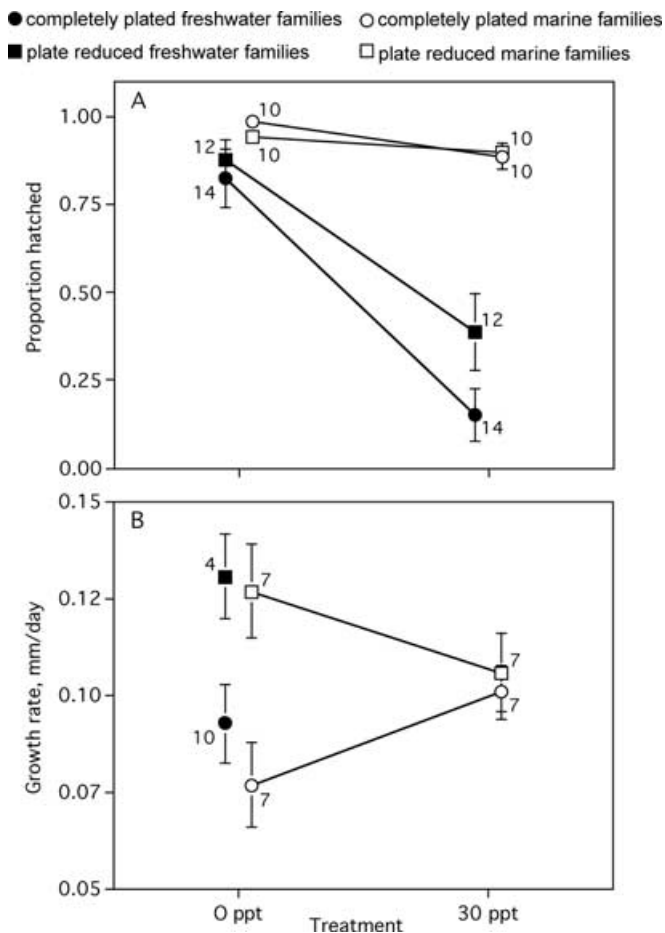


Figure 3. Hatching success (A) and growth rate (B) of reduced (low and partial) and completely plated families of *Gasterosteus aculeatus* fertilized and raised in fresh water (0 ppt) or artificial salt water (30 ppt). Symbols and error bars represent mean \pm 1 SE. Error bars are less than symbol size where absent and populations are offset for clarity. Numbers nearest to the 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.

family were completed for each morph from each population in each treatment using all data).

ANALYSES

Our main objective was to determine whether 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 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). 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 whether 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 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).

Results

HATCHING SUCCESS

Contrary to expectation (Fig. 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. 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. 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. 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. 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. 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. 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. 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. 3B). Low sample sizes due to low levels of fertilization and hatching success prevented any comparison among freshwater plate morphs in the salt water 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.

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. 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 or the differences in growth rate found in the wild, where food may or may not be limited. In addition, we measured growth rate on juveniles only, leaving the possibility for compensatory growth to occur at later life stages. Finally, 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. 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). First, larger, faster-growing juveniles possess 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 favor 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. Second, body length is strongly positively correlated with reproductive output (clutch size and mass) in threespine stickleback (Wootton 1984; Schluter 1995) 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. Third, 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 versus salt water (Fig. 3), suggests that a single ecological mechanism, like that of salinity tolerance (Heuts 1947; Fig. 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 (Reimchen 2000; Bergstrom 2002). 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. 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 in 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 F₂ 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 F₂ 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.

Finally, 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 (e.g., 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 the many ways to reduce lateral plate armor in freshwater populations of threespine stickleback.

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