doi:10.1111/j.1558-5646.2009.00762.x

ENVIRONMENT SPECIFIC PLEIOTROPY FACILITATES DIVERGENCE AT THE *ECTODYSPLASIN* LOCUS IN THREESPINE STICKLEBACK

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Received April 14, 2009
Accepted June 1, 2009

Adaptive radiation occurs when divergent natural selection in different environments leads to phenotypic differentiation. The pleiotropic effects of underlying genes can either promote or constrain this diversification. Identifying the pleiotropic effects of genes responsible for divergent traits, and testing how the environment influences these effects, can therefore help to provide an understanding of how ecology drives evolutionary change between populations. Positive selection on low-armor alleles at the *Ectodysplasin* (*Eda*) locus in threespine stickleback has led to the repeated evolution of reduced armor in populations following freshwater colonization by fully armored marine sticklebacks. Here, we demonstrate that *Eda* has environmentally determined pleiotropic effects on armor and growth. When raised in freshwater, reduced armor sticklebacks carrying "low" alleles at *Eda* had increased growth rate relative to fully armored sticklebacks carrying "complete" alleles. In saltwater treatments this growth advantage was present during juvenile growth but lost during adult growth, suggesting that in this environment stickleback are able to develop full armor plates without sacrificing overall growth rate. The environment specific pleiotropic effects of *Eda* demonstrate that ecological factors can mediate the influence of genetic architecture in driving phenotypic evolution. Furthermore, because size is important for mate choice in stickleback, the growth rate differences influenced by *Eda* may have effects on reproductive isolation between marine and freshwater populations.

KEY WORDS: Adaptation, adaptive radiation, morphological evolution, pleiotropy, polymorphism, selection-natural.

A major goal of evolutionary biology is elucidating the mechanisms responsible for patterns of diversity in nature. Divergent natural selection between populations in different environments has been repeatedly demonstrated to be the primary driver of phenotypic variation (Schluter 2000; Benkman 2003; Rundle and Nosil 2005; Grant and Grant 2008). However, the potential for natural selection to shape patterns of variation may be influenced by developmental constraints that prevent all possible phenotypic variants from being produced. One way that these constraints can arise is through pleiotropy, that is, single genes with effects on more than one trait under selection. If there is antagonistic pleiotropy, such that selection on different traits favors different alleles at a gene, then much greater strength of selection is required to produce the same rate and direction of evolution than would be needed if the traits were genetically independent (Lande 1979). Although the importance of pleiotropy for evolutionary processes is well recognized (Barton 1990; Keightley and Hill 1990; Otto 2004), we have little understanding of how these genetic effects are mediated by ecological context. Studies investigating the pleiotropic effects of candidate genes have typically

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been conducted under uniform conditions (Doebley et al. 1997; Nesbitt and Tanksley 2001; Beldade et al. 2002; Bomblies and Doebley 2006; Kronforst et al. 2006; Lattorff et al. 2007; Wagner et al. 2008). The few studies that have tested for pleiotropic effects in varying environments provide evidence that gene by environment interaction can strongly influence the extent of pleiotropy (Lukens and Doebley 1999; Scarcelli et al. 2007; Mensch et al. 2008). Thus, for a comprehensive understanding of the relationship between molecular variation and fitness it is essential that we determine the pleiotropic effects of adaptive genes and the interaction of these effects with the environment.

Genes that produce specific phenotypes in particular environments provide good candidates for studying the genetics of adaptive change. The *Ectodysplasin* (*Eda*) locus controls the majority of variation (∼75%) in bony lateral plates between marine and freshwater threespine stickleback, with the number of lateral plates in freshwater populations greatly reduced relative to marine populations (Colosimo et al. 2005; Makinen et al. 2008). Fish homozygous for "complete" alleles typically possess a row of 30 to 36 plates (complete morph), whereas a majority of homozygotes for "low" alleles possess 0 to 9 plates (low morph). Most heterozygotes possess an intermediate number of plates (partial morph) (Hagen and Gilbertson 1972; Bell 1977; Bell and Foster 1994). Lateral plates play a defensive role in stickleback, not only increasing the difficulty of ingestion by predatory vertebrates (Reimchen 1983), but also improving the probability of escape and survival after capture (Reimchen 1992, 2000). It is thought that the complete allele will be favored in oceanic habitats, where sticklebacks are often far from cover and experience intense vertebrate predation pressure (Reimchen 2000; Bell 2001; Colosimo et al. 2004; Marchinko 2009). In contrast, the presence of lateral plates may be a disadvantage in freshwater, where sticklebacks are closer to cover, and acceleration and maneuverability for escape may be more important than survival after capture (Reimchen 2000; Bell 2001; Bergstrom 2002). Thus, the primary hypothesis for the distribution of *Eda* alleles found between environments is that different predation regimes lead to divergent selection on armor.

An alternative hypothesis is that when marine sticklebacks invade freshwater environments natural selection favors the low allele because it confers an advantage in growth rate. Marchinko and Schluter (2007) found that reduced armor phenotypes grew more quickly than fully armoured phenotypes when raised in freshwater in a laboratory experiment, although the specific genotypes at *Eda* were unknown. A recent field study has found evidence that fish carrying the *Eda* low allele gain a growth advantage that leads to higher overwinter survival and reproductive success (Barrett et al. 2008). These findings suggest that *Eda* may have pleiotropic effects on armor and growth in freshwater. These effects might be antagonistic if reduced ion concentrations in freshwater create a developmental constraint that prevents maximizing both armor and growth (Giles 1983; Bell et al. 1993; Arendt et al. 2001). Under this hypothesis, the evolution of low-plated populations in freshwater environments may be the result of a correlated response to positive selection for increased growth rate, rather than negative selection on armor (Marchinko and Schluter 2007).

We tested these hypotheses by comparing pleiotropy of *Eda* in both salt and fresh water in a laboratory experiment. We measured growth rate and armor phenotype of F1 offspring produced from crosses of wild marine fish that are heterozygous at the *Eda* locus, having one complete and one low allele. Because these fish are otherwise marine in their genetic makeup, our crosses minimize the influence of other genes that differentiate marine and freshwater populations, with the possible exception of genes closely linked to *Eda* (Colosimo et al. 2005; Miller et al. 2007). Antagonistic pleiotropy is central to the study of life history evolution (Leroi 2001) and when combined with gene by environment interaction can lead to the maintenance of genetic diversity (Barton 1990). The presence of environment specific pleiotropic effects may help to provide an explanation for the maintenance of standing variation at the *Eda* locus (Colosimo et al. 2005).

Materials and Methods

SAMPLE POPULATIONS

We collected marine sticklebacks in April and May of 2006 from Oyster Lagoon on the Sechelt peninsula in western British Columbia (49°36′48.6″N, 124°1′46.88″W). Oyster lagoon is a saltwater inlet with salinity ranging from 28–32 ppt, in which phenotypically partial fish occur at an approximate frequency of 0.01. We sampled approximately 10,000 fish using minnow traps. We kept partially plated fish, including those missing only one or two lateral plates, and returned all other captured fish to the lagoon. To allow individual identification, we injected each fish subepidermally with a fluorescent visible implant elastomer tag (Northwest Marine Technology) using a 29 gauge syringe.

GENOTYPING

We genotyped fish at a diagnostic locus within the *Eda* gene that distinguishes between low and complete-morph alleles. We isolated total genomic DNA from small caudal fin clips using a standard proteinase K phenol chloroform protocol (Sambrook et al. 1989). We quantified DNA yield using spectrophotometry and then preserved DNA samples at −20°C. We used diagnostic indel loci to identify low and complete *Eda* alleles (isolated from loci Stn381 within intron six of the *Eda* gene (Colosimo et al. 2005). *Eda* alleles were amplified by PCR using a DNA Engine® Peltier Thermal Cycler (MJ research, Inc., Waltham, MA) in 10 ul reactions containing 5 to 15 ng of genomic DNA, 1 uM of each forward and reverse primer, 1X PCR buffer, 0.25 mM of each

dNTP, 1.5 mM $MgCl_2$, and 0.25U of AmpliTaq Gold polymerase (Applied Biosystems, Carlsbad, CA). Cycling conditions were standardized over all loci as follows: 93°C for 3 min, 95°C 30 s, 59°C 30 s, 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 for 10 min and then cooled to 4°C. Electrophoresis consisted of pooling PCR products with an internal size standard (LIZ 500 bp, Applied Biosystems) and loading onto the Applied Biosystems 3730S Automated Sequencer. Allelic sizes (in base pairs) were determined by reference to the internal sizing standard in the software GENEMAPPER (Applied Biosystems).

CROSSING DESIGN

Using artificial fertilization, we made 24 families from 18 females and 18 males caught from Oyster lagoon. We never crossed a female to the same male twice. Because all parents are heterozygous, the ratio of *Eda* genotypes in the progeny should be 1:2:1 homozygous complete: heterozygous: homozygous low. To make a cross, we first equally distributed a female's eggs into two separate petri dishes. One petri dish contained fresh water (0 ppt) and the other contained artificial salt water (30 ppt; Instant Ocean synthetic seasalt, Aquarium Systems Inc., Mentor, OH) both at a pH of 7. We then sacrificed a male using MS-222 and removed both testes. We divided one testis in half and placed each half in one of the petri dishes. The second testis was preserved in Ginzberg solution (Hart and Messina 1972) for use in a future cross. We crushed the testis placed in petri dishes to release sperm. We left the half clutches of eggs and the sperm for 20 min and then placed them into separate plastic egg cups (pint cups with fine fiberglass mesh lining the bottom) and submerged each into a separate egg tank (20 L) according to salinity treatment. We added methylene blue to egg tanks to reduce fungal growth and removed any eggs that became unviable due to fungal growth. Eggs remained in aerated egg-tanks for eight days, and then we transferred them to 102 L tanks with the appropriate salinity treatment. We placed up to five half clutches from crosses made within three days of each other into each tank and after eggs hatched and larvae dropped into the tanks, we removed the cups and any unhatched eggs.

EXPERIMENTAL REARING

We fed surviving larvae live brine shrimp twice per day for six weeks and then fed each tank one 3.5 oz cube of frozen *Daphnia* once per day until 12 weeks of age, followed by a blood worm diet. After feeding stopped, we removed any remaining food by filtration or manual siphoning, ensuring that each individual was fed to satiation. After five weeks we distributed the individuals in each tank equally among four 102 L tanks connected by a water circulation system, with no tank containing more than 24 individuals. Each group of four interconnected tanks thus contains the progeny of no more than five families with similar hatching dates and constitutes a separate experimental block in our statistical analysis. We gave each block a salinity treatment identical to that in which the corresponding fish introduced to it were previously raised. We conducted the experiment in two environmental chambers, with four blocks (two saltwater and two freshwater) per chamber.

When fish reached a mean length of 25 mm from snout to the tip of the caudal peduncle (standard length), we injected each individual subepidermally with a fluorescent visible implant elastomer tag (Northwest Marine Technology, Shaw Island, WA) using a 29 gauge syringe, and genotyped the fish at Stn381. Each fish was given a tag that distinguished it from all other fish in the same tank. Thus, we were able to record individual survival and growth throughout the experiment. Juvenile growth rate, defined as growth rate before plates were fully developed, was calculated as the standard length divided by the number of days since the mean hatch date of all the fish in the corresponding block (hatch dates within blocks did not vary by more than three days). The average standard length of the juvenile fish over all blocks was 27 mm. Total growth rate was recorded when we observed the first individuals coming into reproductive condition, approximately nine months after the juvenile growth rate measurement was obtained, and was calculated as standard length divided by the number of days since the mean hatch date of all fish in the corresponding block. Adult growth rate was calculated as the difference in standard length between the total and juvenile length measurements, divided by the number of days since the juvenile measurement. Because of mortality during the experiment, we obtained fewer total growth rate measurements than juvenile growth rate measurements. These deaths did not result in any significant change in genotype ratios during the experiment ($\chi^2 = 0.921$, $df = 2$, $P = 0.631$).

ANALYSES

Our main objective was to determine whether there was an association between *Eda* genotype and growth rate and if this association differed between the extremes of salinity experienced by sticklebacks in nature. To test the influence of genotype on growth rates, we used linear models in R 2.7.0 (R Core Development Team 2008) to obtain the slope coefficient from the linear regression of the number of low alleles per individual against growth rate separately for each block. Each linear model contained terms for the number of low alleles (0,1,2) and the heterozygosity (0,1) of individuals in a block (the heterozygosity term was included to represent dominance). We then employed a linear mixed effects model in R 2.7.0 (R Core Development Team 2008) to test for an effect of salinity on the slopes. Blocks were treated as random effects nested within random chamber effects. To investigate the role of ontogeny, we calculated the difference in slope coefficients for juvenile and adult growth rates in each block and

Table 1. Association between *Eda* genotype and armor phenotype. Goodman and Kruskal Υ=0.70, χ^2=226.57, df=4, $P<10^{-15}$, n=313. Individuals pooled from all blocks. There were no significant treatment effects on genotype-phenotype associations (heterogeneity χ^2=8.1, df=4, P=0.09). *Eda* genotypes are based on the Stn381 indel marker: "complete" (C) alleles represent 162 or 171 bp bands, and "low" (L) alleles represent 191 bp bands.

Eda genotype	Armor phenotype		
	Complete	Partial	Low
CC	98	5	0
CL	54	61	3
LL	2	43	47

tested for differences between salinity treatments using the same linear mixed effect model approach as was used for the separate juvenile and total growth analyses. A significant effect of salinity in this model indicates that the effect of salinity on the relationship between genotype and growth differs between ontogenetic stages. We repeated these analyses on the heterozygosity coefficients from the block linear models but do not present them because neither the mean nor the effect of salinity was large or significant for any growth stage.

Results

We found a strong association between plate phenotype and *Eda* genotype in both environments (freshwater: Goodman and Kruskal $\Upsilon = 0.70, n = 187, \chi^2 = 128.7, \text{df} = 4, P < 10^{-15}$, saltwater: $\Upsilon = 0.73, n = 126, \chi^2 = 105.9, \text{df} = 4, P < 10^{-15}$, combined: $\Upsilon = 0.70$, $n = 313$, $\chi^2 = 188.7$, df = 4, $P < 10^{-15}$, Table 1, Table S1). Because *Eda* genotype strongly predicts plate phenotype regardless of environment, we investigated the effect of environment on pleiotropy by testing its effects on the association between *Eda* genotype and growth rate. In each experimental block we obtained the slopes of linear regressions of final growth rate on the number of low alleles possessed by an individual (0 for homozygous complete, 1 for heterozygote, 2 for homozygous low). We found that individual growth rates were positively associated with the number of low alleles at the *Eda* locus in freshwater (Fig. 1A, mean of block slope coefficients = 0.0030; SE = 0.0016). In contrast, there was no association between growth rate and genotype in saltwater (Fig. 1A, mean of block slope coefficients = −0.0010; SE = 0.0015). We detected a marginally significant difference between the mean slope coefficients in freshwater and saltwater treatments (linear mixed effects model on block slope coefficients, salinity: $F_{1,5} = 5.495$, $P = 0.066$).

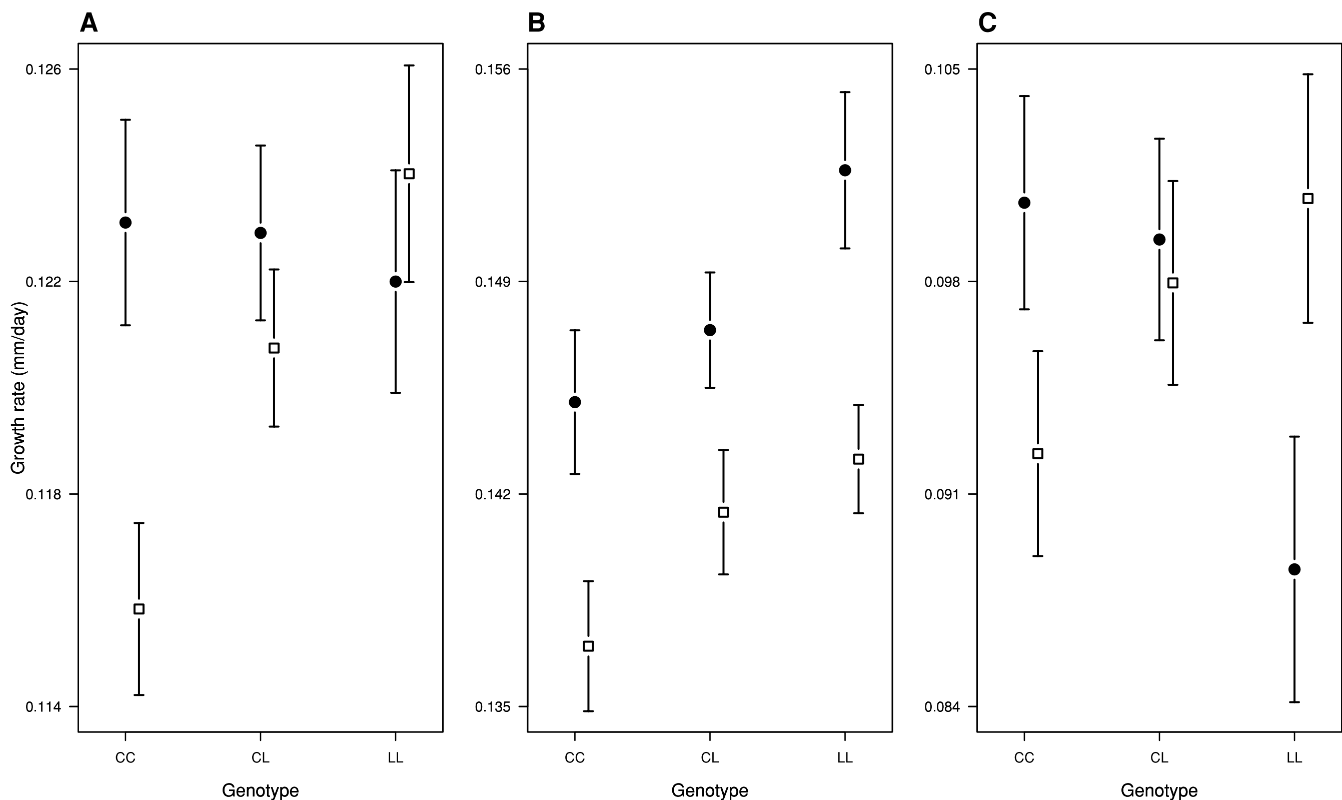

Figure 1. (A) Total growth rate by *Eda* genotype in salt (•) and fresh water (□). (B) Juvenile growth rate by genotype, calculated from birth to the end of adult plate number development at average standard length of 27 mm. Symbols as in (A). (C) Adult growth rate by genotype, calculated between juvenile growth and the end of the experiment at average standard length = 44 mm. Symbols as in (A). Error bars show ± 1 standard error. Note: different scale on vertical axis in each panel.

The contrasting effects of *Eda* on growth in saltwater and freshwater are primarily the result of effects occurring relatively late in development. We repeated our analysis on growth rate during two separate stages of development: juvenile and adult. We define juvenile growth as growth occurring from birth to the time that fish acquired their full adult number of lateral plates (Bell 1981, 2001; Barrett et al. 2008) (mean standard length = 27 mm). Adult growth is that occurring between the end of the juvenile stage and the end of the experiment (mean standard length = 44 mm). We found that the mean slope coefficients were similar in freshwater and saltwater treatments during juvenile growth (Fig. 1B, linear mixed effects model on block slope coefficients, salinity: $F_{1,5} = 0.156$, $P = 0.709$). Juvenile growth rate was positively associated with the number of low alleles at the *Eda* gene in freshwater (mean of block slope coefficients = 0.0033, SE = 0.0015) as well as in saltwater (mean of block slope coefficients = 0.0028, SE = 0.0009). In contrast, there was a significant difference in the effect of *Eda* on adult growth between the treatments (Fig. 1C, linear mixed effects model on block slope coefficients, salinity: $F_{1,5} = 12.675$, $P = 0.016$). In freshwater the relationship between growth and number of low alleles was positive (mean of block slope coefficients = 0.0017, SE = 0.0024) whereas it was negative in saltwater (mean of block slope coefficients = −0.0068, SE = 0.0023). Therefore, in saltwater growth of homozygous complete and heterozygous genotypes caught up to that of the homozygous low genotypes, whereas in freshwater the disadvantage of possessing complete alleles persisted through adulthood. This change in the pattern of slopes, comparing juveniles (Fig. 1B) with adults (Fig. 1C), was significantly different between saltwater and freshwater treatments (linear mixed effects model on the change in block slope coefficients, salinity: $F_{1,5} = 13.43$, $P = 0.015$). Thus, *Eda* has different pleiotropic effects in freshwater and saltwater, but this difference is dependent on ontogeny. Overall, having more low alleles, which reduces number of lateral plates, resulted in faster growth in freshwater but not in saltwater (Fig. 1A,C).

Discussion

Pleiotropy is thought to be one of the most common properties of genes (Dobzhansky and Holz 1943; Barton 1990; Otto 2004), but rarely have the pleiotropic effects of candidate genes been measured in different environments. Testing how pleiotropic effects interact with the environment will greatly facilitate our understanding of the role that genetic architecture can play in promoting or constraining phenotypic diversification. By tracking genotyped individuals throughout life, we were able to identify whether the *Eda* locus has environment-specific pleiotropic effects on two fitness-related traits. Our results show that in freshwater stickleback experience a tradeoff between armor and growth, because fish carrying the complete allele have high armor but reduced growth rate. Given this pleiotropy, the predominance of low-plated populations in freshwater may be due to the growth rate advantage of this allele rather than a disadvantage of armor as such. Depending on the relative strength of selection acting on growth and armor, the low allele may be favored even when armor is under positive selection. A tradeoff between armor and growth also exists in saltwater, but only during juvenile growth. In the absence of an adult-size advantage of the low allele, it will be selected against in the ocean if disadvantages of reduced armor in adult sticklebacks outweigh the advantages of increased growth in juveniles. Thus, environment specific pleiotropic effects may cause the direction of selection on *Eda* to vary across environments, even if the direction of selection on both traits remains constant.

We ended the experiment when we observed fish entering reproductive condition because breeding substantially alters how much energy is devoted towards growth (Day and Taylor 1997). It is possible that if the experiment had been continued for longer, the completely plated fish would have eventually reached similar adult sizes to low-plated fish in freshwater. Juvenile growth in saltwater was faster than in freshwater (Fig. 1B) and low-plated fish may have reached an upper size limit earlier, allowing completely plated fish to "catch-up." In contrast, in freshwater, low-plated fish may not have reached their upper size limit during the experiment and therefore maintained a growth-rate advantage throughout the adult stage. Regardless of whether genotypes would have eventually reached equivalent sizes in freshwater, size differences occurring through the developmental stages that we measured in this study would have important fitness consequences in wild fish through effects on overwinter mortality and breeding time (Schultz et al. 1991; Curry et al. 2005; Barrett et al. 2008).

This experiment used offspring of wild-caught individuals rather than near-isogenic experimental lines, which means we cannot rule out the possibility of effects on growth of genes linked to the *Eda* locus. If linked genes are responsible for differences in growth between *Eda* genotypes, then recombination could eventually eliminate the association between the low allele and increased growth rate. Indeed, mapping studies using crosses of individuals from distantly related populations have found no evidence that *Eda* explains any variation in body size (Colosimo et al. 2005; Albert et al. 2008; Marchinko 2009). Regardless of whether the association between armor and growth detected in this study is due to pleiotropic effects of *Eda* alone or the pleiotropic effects of a group of tightly linked genes including *Eda*, the functional result will be the same upon colonization of freshwater; the low allele will increase in frequency due to its associated growth advantage and this will lead to the evolution of reduced armor. However, the association between reduced armor and increased growth will break down over time if linkage is playing a more important role

than pleiotropy. The strength of our approach is that it used naturally occurring standing genetic variation, which was presumably the source of variation available to selection when fish first colonized freshwater at the end of the last ice age.

Our results have implications for the genetics of speciation in stickleback because assortative mating between marine and stream stickleback populations is strongly influenced by body size (Ishikawa and Mori 2000; McKinnon et al. 2004). Marine sticklebacks are consistently larger in size than stream sticklebacks (McKinnon et al. 2004) and size differences are heritable (McPhail 1977; Snyder and Dingle 1989). *Eda* may influence this size difference after colonization of freshwater by completely plated marine sticklebacks. We found that homozygous complete genotypes raised in freshwater were on average 2.6 mm smaller than homozygous complete genotypes raised in saltwater. This difference accounts for approximately 9% of the magnitude of difference typically observed between freshwater and marine populations, suggesting that the slower growth in freshwater caused by *Eda* could have a moderate influence on assortative mating between marine and newly formed stream stickleback. In contrast, the eventual fixation of the low allele in freshwater, which has happened countless times in postglacial streams across the northern hemisphere (Colosimo et al. 2005), will have the opposite effect on assortative mating. In freshwater, the low allele will cause an increase in body size, which will diminish the size difference between marine and stream-resident fish. This effect could serve to increase gene flow between environments and may help to explain how the low allele has been maintained at low frequency in marine environments for over 2 million years (Colosimo et al. 2005). Understanding the overall pleiotropic effects of candidate genes in different environments can provide a more comprehensive view of the mechanisms that drive patterns of phenotypic evolution.

ACKNOWLEDGMENTS

We thank M. Dosani and A. Paccard for assistance with the experiments. P. Nosil, S. Otto, and T. Vines provided useful comments on the manuscript. We are supported by NSERC Discovery and Special Research Opportunity grants (DS), grants from the Canada Foundation for Innovation (DS), and NSERC postdoctoral (SMR) and graduate scholarships (RDHB).

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Associate Editor: C. Peichel

Supporting Information

The following supporting information is available for this article:

Table S1. Association between *Eda* genotype and armor phenotype.

Supporting Information may be found in the online version of this article.
(This link will take you to the article abstract).

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