Current Biology, Volume *24*
Supplemental Information

Maintenance of a Genetic Polymorphism with Disruptive Natural Selection in Stickleback

Kerry B. Marchinko, Blake Matthews, Matthew E. Arnegard, Sean M. Rogers, and Dolph Schluter

Fig. S1, related to Figure 2. *Eda* genotype frequencies in embryos (top row) and estimated fitness of *Eda* genotypes relative to $Eda^{L/L}$ for viability (middle row) and reproductive success (bottom row). Vertical bars indicate Hardy-Weinberg expected genotype proportions in embryos. Embryo proportions were calculated from 341 embryos sampled from 71 egg clutches in 2008, and 303 embryos sampled from 68 clutches in 2010. Proportions here were calculated for all embryos in each year combined, and are for illustrative purposes only. Relative viabilities were based on the genotype frequency shift observed between the embryos and adult stages for 2008 and 2010 separately. Relative reproductive success was based on the genotype frequency shift from adults to embryos, assuming random mating. Error bars for genotype proportions and relative fitness are standard errors estimated from 10,000 bootstrap re-samples.

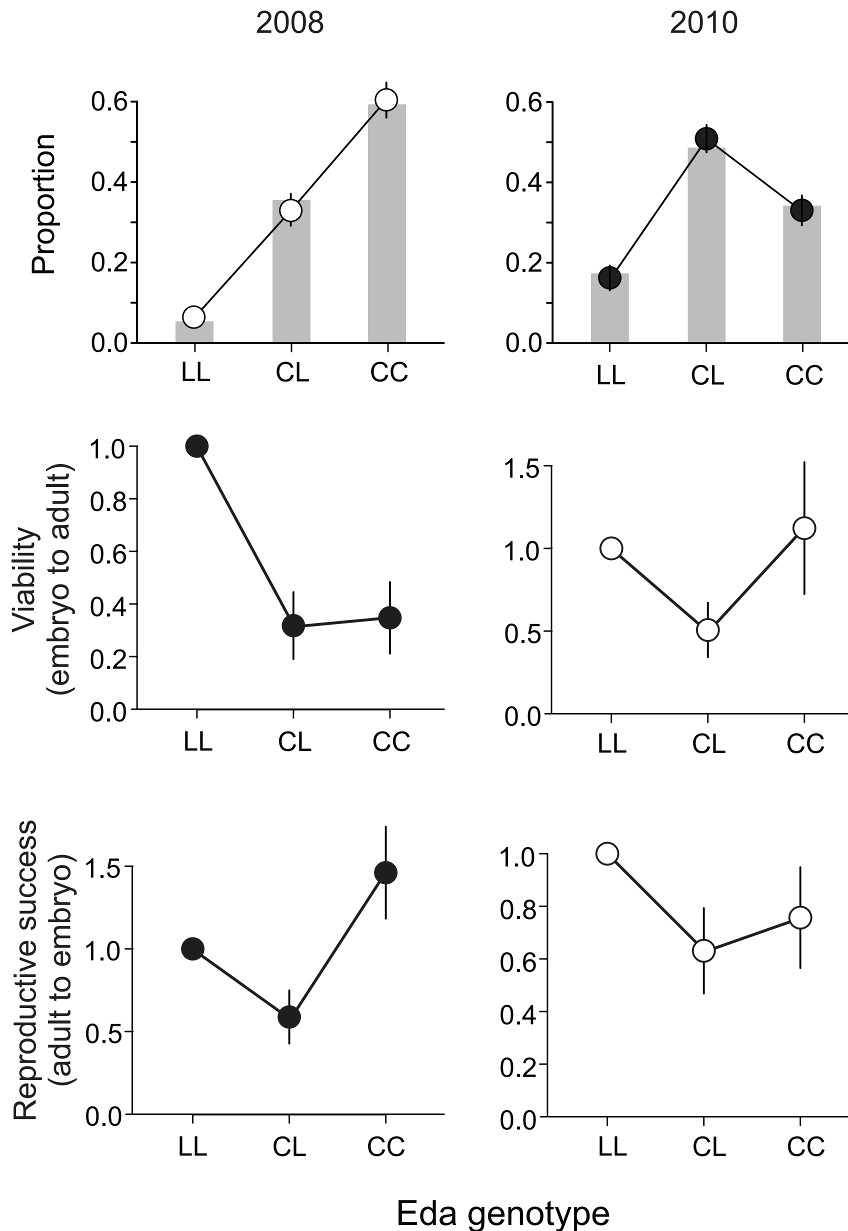

Table S1, related to Figure 1. Morphological and isotope ratio differences between lateral plate morphs, based on 45 completely plated (21 females, 24 males) and 44 low plated (18 females, 26 males) adults sampled in 2004. Morphological traits except gill raker number and standard length were size adjusted to a standard length of 52.33 mm (the mean standard length of the sample) and are given in mm. Means shown are the un-weighted averages of male and female means. The standardized difference, Cohen's *d*, measures the difference between means in units of standard deviations [S1]. *P*-values were obtained from fitting a linear model with sex, morph, and in the case of morphological traits standard length (excepting gill raker number), and all interactions using Type II sums of squares [S2]. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Trait	Complete morph	Low morph	Pooled standard deviation	Cohen's *d*
Morphology				
Body depth	11.43	11.51	0.54	–0.15
Head length	16.83	16.41	0.55	0.76***
Head depth	7.10	6.84	0.30	0.87***
Eye diameter	4.68	4.47	0.25	0.81***
Snout length	4.92	4.72	0.32	0.64**
Jaw length	4.67	4.56	0.27	0.39
Gape width	2.56	2.48	0.27	0.32
Pelvic girdle length	12.68	12.79	0.68	–0.16
Pelvic spine length	9.65	9.74	0.67	–0.14
First dorsal spine length	6.63	6.47	0.64	0.25
Second dorsal spine length	7.23	7.13	0.61	0.17
Gill raker number	23.06	22.65	1.36	0.30
Gill raker length	1.41	1.43	0.13	–0.16
Standard length	51.45	53.10	4.09	–0.40*
Isotope ratios				
$\delta^{13}C$	–26.62	–27.83	1.32	0.91***
$\delta^{15}N$	8.94	8.96	0.39	–0.04

Supplemental experimental procedures

Sampling Location and Collection Details

Kennedy Lake is a large (6475 ha), deep (145 m) and extremely oligotrophic lake [S3] lying six meters above sea level and approximately six km from the west coast of Vancouver Island. Fish were collected by minnow trapping each year at the same location, a large breeding site where stickleback congregate during the breeding season near the southern tip of the Clayoqout arm (49° 5'52.41"N, 125°35'34.79"W). Sampling was performed across all available nesting microhabitats, including submerged terrestrial vegetation, aquatic macrophytes, sand, soft bottom and bedrock substrates. Fish were given a lethal dose of tricaine methanesulfonate (MS-222, Syndel Laboratories, Qualicum Beach, BC, Canada) before preservation. The 2006, 2008 and 2010 samples were preserved in 95% ethanol. Specimens from the May 11, 2004 collection were stored on dry ice and transported to the University of British Columbia for storage at –80 °C. These samples were thawed briefly for morphological measurements and then stored at –80 °C until processed for stable isotope analysis.

We collected embryos from all habitat types (indicated above) by removing whole nests while snorkeling June 9-12, 2008 and June 7-11, 2010. 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 and 2010 adult collections. Formalin-preserved specimens from a sample obtained on Aug 2, 1965, by G. Haythorne and D. Hagen were obtained from the University of British Columbia (UBC 65-0506).

Lateral plate measurements

Plate morph was assessed by examining the number of plates on the left side of the body on live fish and on the 1965 preserved sample in all years by the same person (K.M.). Low-plated individuals possess a cluster of 1-10 anterior plates along the lateral line near the pectoral fin. Partially plated individuals had 11-29 plates and were recognized by the presence of a gap of 2 or more plates between the caudal and mid-body regions. Completely plated individuals had 30 or more plates and a gap of at most one plate near the caudal region.

Morphological measurements

We measured morphological differences between the complete and low lateral plate morphs in the 2004 sample (Table S1). The following traits were measured in imageJ [S4] on digital photographs of the left side of the body. Standard length is the distance from the tip of the snout to the end of the caudal peduncle. Body depth is the distance from anterior insertion of the first dorsal spine to the posterior extent of ectocoracoid. Head length is the distance from anterior tip of upper lip to the posteriodorsal extent of operculum. Head depth is the distance from the posterior extent of supraoccipital to the anterior extent of ectocoracoid. Eye diameter is the anterior to posterior extent of the orbit. Snout length is the distance from the anterior tip of upper lip to the anterior extent of the orbit. Jaw length is the distance from the anterior tip of upper lip to the posterior extent of maxilla. Pelvic girdle length is the linear distance from the posterior tip of the ectocoracoid to the caudal tip of the posterior process of the pelvic girdle. Pelvic spine length is the distance from the insertion point of the pelvic spine in the pelvic girdle to the tip. First (and second) dorsal spine length is the distance from its anterior insertion to the tip. Gape width was measured from a digital photo of the dorsal side of the body, as the distance between left and right posterior extent of maxilla. Gill raker number is the total number of long gill rakers alone the entire first gill arch on the left side. Gill raker length is the mean of the three longest gill rakers, measured using a dissecting scope and an ocular micrometer.

To compare means, traits except gill raker length were size-adjusted to a standard length of 52.33 mm, the mean standard length of the sample. We regressed each trait against standard length in a linear model that included sex and morph as factors, and assuming equal slopes in all groups. Adjusted trait values were obtained by adding within-group residuals to model predicted values at the mean standard length. We also fitted each trait to a more comprehensive linear model that included all possible interactions among standard length, size and lateral plate morph. We detected three relatively weak but statistically significant interactions: in body depth between sex and standard length; in eye diameter between morph and standard length; and for eye diameter a three-way interaction involving sex, morph, and standard length. However, taking account of these interactions did not substantially change the means and effect sizes and we do not present them here.

Standardized mean differences between morphs (Cohen's d [S1]) used the pooled standard deviations, calculated as the square root of the error mean square from a linear model that fitted each size-adjusted trait to a single factor representing all four combinations of morph

and sex. Standardized mean difference was calculated separately for each sex and then averaged (Table S1).

Stable Isotopes

We measured ratios of stable isotopes of carbon (^{12}C and ^{13}C) and nitrogen (^{14}N and ^{15}N) (table S1) in the 2004 sample. Ratios are expressed as scaled isotope ratios in parts per thousand (‰) relative to Pee Dee Belemnite (for $\delta^{13}C$) and atmospheric nitrogen (for $\delta^{15}N$) [S5, S6]. In lakes, $\delta^{13}C$ indicates the habitat from which dietary carbon was ultimately obtained (low values indicate a pelagic diet and high values a littoral diet); $\delta^{15}N$ estimates trophic position (higher values indicate a higher trophic level) [S6].

We removed tissue from the foot of mussels, and dorsal white muscle from 89 threespine stickleback, 40 peamouth chub and 15 coho salmon parr (juveniles still in freshwater) collected from Kennedy Lake at the same time in 2004. The $\delta^{13}C$ signature of 53 mussels, which filter feed on phytoplankton, provides a baseline measurement indicating a predominantly pelagic source of dietary carbon and a low trophic level (means, $\delta^{13}C$ -27.57 ± 0.51 SD; $\delta^{15}N$ 2.28 ± 0.28SD) [S6]. We used the $\delta^{13}C$ signature of juvenile peamouth chub, which feed almost exclusively on littoral benthic invertebrates [S7], to represent the baseline for a diet consisting predominantly of littoral carbon sources ($\delta^{13}C$ -21.11 ± 2.04 SD; $\delta^{15}N$ 6.49 ± 0.41SD). Coho parr were included to represent species with a varied diet, often feeding upon terrestrial insects ($\delta^{13}C$ -18.90 ± 3.13SD; $\delta^{15}N$ 14.06 ± 2.27SD) [S8]. 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 [S9]. We repeated the analysis of isotope ratios following chemical lipid extraction [S10], and the results were unchanged. Here we present the analysis only of the lipid extracted samples.

Standardized mean differences between morphs (Cohen's *d* [S1]) used the pooled standard deviation, calculated as the square root of the error mean square from a linear model that fitted each isotope ratio to a single factor representing all four combinations of morph and sex. Standardized mean difference was calculated separately for each sex and then averaged (Table S1).

Genotyping

Total genomic DNA was isolated using standard phenol-chloroform extraction methods. Microsatellite and *Eda* alleles were amplified in 10 µl PCR reactions containing 5 to 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 0C for 3 min, 95 0C 30 s, 59 0C 30s, 72 0C 30 s, 5 cycles of 94 0C 30 s, 59 0C 30 s, 72 0C 30 s, 35 cycles of 90 0C 30 s, 60 0C 30 s, 72 0C 30 s, followed by 72 0C 10 min, then cooled to 4 0C.

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).

Statistical analysis

The association between *Eda* genotype (diagnostic indel Stn381) and lateral plate morph was measured using the Goodman-Kruskal gamma for ordered categorical variable [S11]. The association was measured using data from adults in sample years 2006, 2008 and 2010 combined, using the frequencies of the *CC*, *CL*, and *LL* genotypes in the complete (177, 5, and 6, respectively), partial (89, 8, and 10), and low (2, 35, and 35) morphs.

Tests of genetic diversity and Hardy Weinberg equilibrium were carried out with seven putatively neutral, unlinked microsatellite loci genotyped in 63 adult stickleback collected in May 2006, using GENEPOP v.4.0 [S12]: Stn301 (chr 18, 32 alleles, F_{IS} = 0.078), Stn065 (chr 6, 14 alleles, F_{IS} = -0.057), Stn216 (chr 20, 7 alleles, F_{IS} = -0.109), Stn250 (chr 8, 37 alleles, F_{IS} = 0.013), Stn387 (chr 2, 13 alleles, F_{IS} = 0.010), Stn388 (chr 9, 7 alleles, F_{IS} = -0.027), and Stn51 (chr 5, 15 alleles, F_{IS} = -0.055). Molecular markers used were not closely linked to known quantitative trait loci for morphological traits [S13-S15]. *P*-values were adjusted for false discovery rate using the method of [S16].

Standard errors and confidence intervals for embryo genotype frequencies and F_{IS} used a bootstrap procedure that resampled clutches of embryos, rather than individual embryos, to ensure independence of observations. Statistics were then calculating using the combined pool of embryos. Each bootstrap used 10,000 re-samples of whole clutches. The standard error of each statistic was estimated as the standard deviation of resampled estimates; 95% confidence intervals used the 0.025 and 0.975 percentiles.

In the absence of selection, a slight excess of heterozygotes might be expected at a locus in a random sample of families, because by chance male parents are likely to have slightly different allele frequencies than the female parents [S17, S18]. The effect is independent of the number of offspring sampled per family and is negligible unless the number of families is 10 or fewer [S18], whereas we had 71 and 68 clutches from our two collection years. The effect is small relative to sampling error and has a low probability of detection unless family sizes are large and data from many loci are combined [S18]. We did not detect any excess in heterozygote frequency at the *Eda* locus in our embryo samples (see main text).

Supplemental References

S1. Cohen, J. (1988). Statistical power analysis for the behavioral sciences, Volume 2nd, (Hillsdale, NJ: Lawrence Erlbaum Associates).

S2. Fox, J., and Weisberg, S. (2011). An R companion to applied regression, (Los Angeles, CA: Sage).

S3. Hagen, D.W., and McPhail, J.D. (1970). The species problem within *Gasterosteus aculeatus* on the Pacific coast of North America. J. Fish. Res. Board Can. *27*, 147-155.

S4. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods *9*, 671-675.

S5. Fry, B. (2006). Stable isotope ecology, (New York, NY: Springer).

S6. Post, D. (2002). Using stable isotopes to estimate trophic position: Models, methods, and assumptions. Ecology *83*, 703-718.

S7. Gray, R.H., and Dauble, D.D. (2001). Some life history characteristics of cyprinids in the Hanford Reach, mid-Columbia River. Northwest Sci. *75*, 122-136.

S8. Groot, C., and Margolis, L. (1991). Pacific salmon: life histories, (Vancouver, BC: UBC Press).

S9. Kiljunen, M., Grey, J., Sinisalo, T., Harrod, C., Immonen, H., and Jones, R.I. (2006). A revised model for lipid-normalizing δ13C values from aquatic organisms, with implications for isotope mixing models. J. Appl. Ecol. *43*, 1213-1222.

S10. Ingram, T., Matthews, B., Harrod, C., Stephens, T., Grey, J., Markel, R., and Mazumder, A. (2007). Lipid extraction has little effect on the δ15N of aquatic consumers. Limnol. Oceanogr. Methods *5*, 338-343

S11. Agresti, A. (2010). Analysis of ordinal categorical data, (Hoboken, NJ: Wiley).

S12. Rousset, F. (2008). GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol. Ecol. Resour. *8*, 103-106.

S13. Peichel, C.L., Nereng, K.S., Ohgi, K.A., Cole, B.L.E., Colosimo, P.F., Buerkle, C.A., Schluter, D., and Kingsley, D.M. (2001). The genetic architecture of divergence between threespine stickleback species. Nature *414*, 901-905.

S14. Albert, A.Y.K., Sawaya, S., Vines, T.H., Knecht, A.K., Miller, C.T., Summers, B.R., Balabhadra, S., Kingsley, D.M., and Schluter, D. (2008). The genetics of adaptive shape shift in stickleback: pleiotropy and effect size. Evolution *62*, 76-85.

S15. Rogers, S.M., Tamkee, P., Summers, B., Balabahadra, S., Marks, M., Kingsley, D.M., and Schluter, D. (2012). Genetic signature of adaptive peak shift in threespine stickleback. Evolution *66*, 2439-2450.

S16. Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological), 289-300.

S17. Castric, V., Bernatchez, L., Belkhir, K., and Bonhomme, F. (2002). Heterozygote deficiencies in small lacustrine populations of brook charr *Salvelinus fontinalis* Mitchill (Pisces, Salmonidae): a test of alternative hypotheses. Heredity *89*, 27-35.

S18. Luikart, G., and Cornuet, J.-M. (1999). Estimating the effective number of breeders from heterozygote excess in progeny. Genetics *151*, 1211-1216.