

# Should I stay or should I go? The *Ectodysplasin* locus is associated with behavioural differences in threespine stickleback

Rowan D. H. Barrett<sup>\*,†</sup>, Tim H. Vines<sup>†</sup>, Jason S. Bystriansky and Patricia M. Schulte

Department of Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, British Columbia, Canada V6T 1Z4

\*Author for correspondence (rbarrett@zoology.ubc.ca).

<sup>†</sup>These authors contributed equally to this work.

**Adaptive divergence may be facilitated if morphological and behavioural traits associated with local adaptation share the same genetic basis. It is therefore important to determine whether genes underlying adaptive morphological traits are associated with variation in behaviour in natural populations. Positive selection on low-armour alleles at the *Ectodysplasin* (*Eda*) locus in threespine stickleback has led to the repeated evolution of reduced armour, following freshwater colonization by fully armoured marine sticklebacks. This adaptive divergence in armour between marine and freshwater populations would be facilitated if the low allele conferred a behavioural preference for freshwater environments. We experimentally tested whether the low allele is associated with preference for freshwater by measuring the preference of each *Eda* genotype for freshwater versus saltwater after acclimation to either salinity. We found no association between the *Eda* low allele and preference for freshwater. Instead, the low allele was significantly associated with a reduced preference for the acclimation environment. This behaviour may facilitate the colonization of freshwater habitats from the sea, but could also hinder local adaptation by promoting migration of low alleles between marine and freshwater environments.**

**Keywords:** behaviour; stickleback; adaptation; evolution; gene; pleiotropy

## 1. INTRODUCTION

Adaptive divergence is hampered when gene flow from the ancestral range introduces locally deleterious alleles and thus impedes local adaptation (Kawecki & Ebert 2004). This effect may be mitigated if alleles are more likely to move to environments where they have high fitness (Jaenike & Holt 1991). This ‘matching habitat choice’ (Edelaar *et al.* 2008) can arise when locally adaptive loci have pleiotropic effects on habitat preference, or when they are tightly linked to loci that confer preference. There is evidence that pleiotropic effects are common for candidate genes affecting behaviour, but examples are still limited to just a few systems (Sokolowski 2001; Fitzpatrick *et al.* 2005).

The gene *Ectodysplasin* (*Eda*) is largely responsible for variation in defensive armour (bony lateral plates) in threespine stickleback *Gasterosteus aculeatus* populations (Colosimo *et al.* 2005). Fish homozygous for ‘complete’ alleles are common in the ocean and typically possess a row of 30 to 36 plates (complete morph), whereas homozygotes for ‘low’ alleles are common in freshwater and typically possess 0 to 9 plates (low morph) (Hagen & Gilbertson 1972; Bell & Foster 1994). Heterozygotes are rare in both environments and possess an intermediate number of plates (partial morph) (Hagen & Gilbertson 1972; Bell & Foster 1994). Lateral plates play a defensive role in stickleback, increasing the difficulty of ingestion by predatory vertebrates (Reimchen 1983) and also the probability of escape and survival after capture (Reimchen 1992, 2000). The complete allele is probably favoured in oceanic habitats, because sticklebacks are often far from cover and experience intense vertebrate predation pressure (Reimchen 2000; Bell 2001; Colosimo *et al.* 2005; Marchinko 2009). By contrast, the low allele is favoured in freshwater owing to beneficial effects on growth rate that lead to higher overwinter survival (Marchinko & Schluter 2007; Barrett *et al.* 2008). The low allele originated more than two million years ago (Colosimo *et al.* 2005), but the postglacial lakes commonly inhabited by low-armour freshwater populations have only existed for approximately 10 000 years, implying that the evolution of the low-armour phenotype has occurred by recurrent local selection on an ancient allele brought repeatedly into freshwater environments by marine founders.

Here, we use a laboratory experiment with the  $F_1$  progeny of wild marine sticklebacks heterozygous at the *Eda* locus to test whether there are significant differences in behaviour between genotypes. Specifically, we test whether the genetic variation at *Eda* is associated with preference for freshwater versus saltwater after acclimation to each salinity. Adaptive divergence in stickleback armour morphology would be facilitated if the low allele conferred a consistent preference for freshwater environments.

## 2. MATERIAL AND METHODS

### (a) 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 to 32 ppt, in which phenotypically partially plated fish occur at an approximate frequency of 0.01. This population breeds in saltwater, and the rare sticklebacks with reduced plate number are marine in all other phenotypic traits (shape, size, colour, spine length; Foster 1994; Saimoto 1995; Barrett *et al.* 2008). Fish with reduced armour predominantly (96% of *Eda* heterozygotes; Barrett *et al.* 2008) carry alleles consistent with marine residency at a single nucleotide polymorphism within an  $\alpha 1$  subunit of Na<sup>+</sup>-K<sup>+</sup>-ATPase (Jones *et al.* 2006). We sampled approximately 10 000 fish using unbaited minnow traps. We genotyped partially plated fish to confirm that they were *Eda* heterozygotes (discussed subsequently). Using artificial fertilization, we generated families from these heterozygotes and raised offspring in 1021 freshwater (0 ppt) aquaria on a diet of brine shrimp during juvenile growth and bloodworms during adult growth. Fish were kept at 17°C and 16 L:8 D regime and were an average of 44.0 mm ( $\pm 5.0$  s.d.) in length and 18 months old at the start of the experiment.

### (b) Genotyping

We genotyped fish at diagnostic sites within the *Eda* gene that distinguish 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 used a diagnostic indel locus, Stn381, to identify low and complete *Eda* alleles (Colosimo *et al.* 2005). *Eda* alleles were amplified by PCR using a DNA Engine Peltier Thermal Cycler (MJ Research, Inc.) in 10  $\mu$ l reactions containing 5–15 ng of genomic DNA, 1  $\mu$ M of each forward and reverse primer, 10  $\times$  PCR buffer, 0.25 mM of each dNTP, 1.5 mM MgCl<sub>2</sub> and 0.25 U of AmpliTaq Gold polymerase (Applied Biosystems). 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, five 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).

### (c) Preference experiment

We conducted the preference experiment in 21 l (40  $\times$  20  $\times$  15 cm) aquaria, each containing a Plexiglas sheet 3 cm shorter than the height of the aquaria. We randomly selected one side of each aquarium to fill with artificial saltwater (30 ppt, 17°C; Instant Ocean synthetic sea salt, Aquarium Systems, Inc., Mentor, OH, USA) up to the level of the dividing sheet. We then filled the other side of the aquarium with freshwater (0 ppt, 17°C) up to the top of the aquarium, creating a 2 cm freshwater bridge between the two sides. We placed size-matched non-experimental fish in jars with a mesh top on either side to promote normal schooling behaviour (Barber & Ruxton 2000) (figure 1). To initiate the preference experiment, we introduced a freshwater-acclimated test fish into the saltwater side of the aquarium. After a 5 h settling period, we recorded fish location every 15 min for 4 h using a QuickCam Pro 9000 by Logitech (Fremont, CA, USA) webcam and EVOCAM software (<http://www.evological.com>). Data from fish that did not visit both sides prior to the data collection period ( $n = 2$ ) were discarded. We tested all fish within 24 days. We then acclimated fish to saltwater for between 35 and 50 days under the same laboratory conditions. Stickleback plasma osmolarity, which is a strong indicator of osmotic condition, takes 7 days to stabilize, following transfer from freshwater to saltwater, so this acclimation period is more than sufficient to allow osmotic acclimation to saltwater (Schaarschmidt *et al.* 1999). We then repeated the preference experiment using the protocol mentioned earlier, except that fish were first introduced to the freshwater side of the aquaria. All fish were tested in the second trial within 28 days. We introduced fish to the unacclimated salinity in each trial, so that they would initially experience osmotically stressful conditions and be encouraged to sample both environments. We tested 81 fish in the freshwater acclimation trial (30 homozygous complete, 28 heterozygotes and 23 homozygous low) and 75 in the saltwater trial (29 homozygous complete, 25 heterozygotes and 21 homozygous low).

We determined preference for freshwater as the proportion of time spent by a fish in freshwater during a trial. We calculated this by scoring location as 1 if the fish was observed in freshwater and 0 if observed in saltwater and then averaging over all observation periods. Similarly, we determined preference for acclimation environment by scoring location as 1 if observed in the acclimation environment and 0 if observed in the alternate environment and then averaging over all observation periods. To determine the influence of *Eda* genotype on preference, we employed a linear mixed effects model in R 2.7.0 (R Development Team 2008 Foundation for Statistical Computing, Vienna, Austria: <http://www.r-project.org>) to test for an association between the number of low alleles possessed by an individual (0 for homozygous complete, 1 for heterozygote and 2 for homozygous low) and preference score. Genotype and environment (saltwater versus freshwater or acclimated versus unacclimated) were treated as fixed effects and individuals were treated as random effects. To account for the possibility that differences in preference may be influenced by differences in activity level between genotypes, we also scored the number of times a fish had moved between environments and included this term as a fixed effects covariate in our model.

## 3. RESULTS

We found no significant difference between genotypes in preference for freshwater, but we found a strong interaction between genotype and acclimation environment (linear mixed effects model on preference for freshwater,

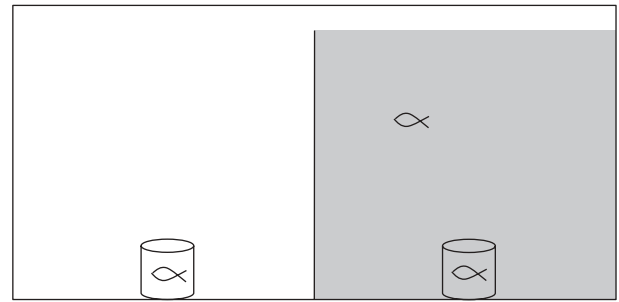


Figure 1. Schematic of the experimental design for preference trials. White represents freshwater (0 ppt) and grey represents saltwater (30 ppt); see §2.

genotype:  $F_{1,80} = 0.231$ ,  $p = 0.632$ ; genotype by acclimation environment:  $F_{1,66} = 8.104$ ,  $p = 0.006$ ). This interaction indicates that homozygous complete genotypes spend a greater proportion of time in the acclimation environment than homozygous low and heterozygous genotypes. If the data are re-analysed using a linear mixed effects model on preference for acclimation environment and genotype, we find strong support for an effect of *Eda* genotype (figure 2;  $F_{1,80} = 7.368$ ,  $p = 0.008$ ). We found no evidence for differences in movement rate between genotypes (genotype by movement:  $F_{1,66} = 0.407$ ,  $p = 0.526$ ).

## 4. DISCUSSION

We found, to our knowledge, the first evidence of a locus associated with behaviour in threespine stickleback. Our results provide no support for an association between the low allele and preference for freshwater. Instead, fish carrying a low allele at the *Eda* locus showed a preference for the alternative environment over the acclimation environment. The main behavioural difference between *Eda* genotypes is thus that fish carrying the complete allele prefer to stay in the salinity to which they have been acclimated, whereas fish carrying the low allele prefer to move to different salinities. The mechanistic basis for this observation is unknown, but could be due to either direct effects of the *Eda* locus or the effects of closely linked genes. Mutations in both *Eda* and the *Eda* receptor (*Edar*) have been shown to affect the number and structure of gill rakers in zebrafish (Harris *et al.* 2008), opening the possibility of effects on the functional properties of gills. Alternatively, *Eda* is tightly linked to vacuolar proton translocating ATPase subunit a isoform 3 and sodium/hydrogen exchanger 6, two proteins known to be important in ion uptake mechanisms of freshwater fish (Evans *et al.* 2005), and *Eda* could be acting as a marker for variation at these loci. Thus, it is possible that the low allele is associated with physiological changes that allow increased tolerance of altered ionic conditions. However, it is unclear why this would lead to a preference for these conditions over the acclimation conditions.

The differences in behaviour observed between *Eda* genotypes in this study may have implications for the long-term maintenance of variation at this locus in natural populations. Our results suggest that marine sticklebacks carrying the low allele may be more

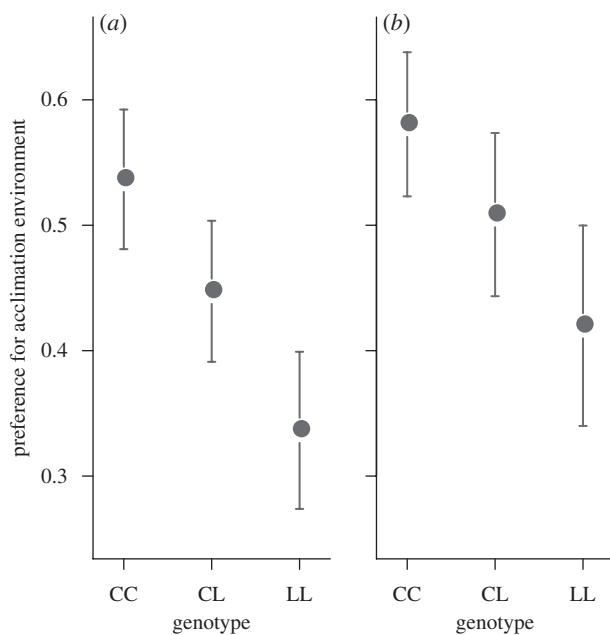


Figure 2. Preference for acclimation environment after acclimation to (a) freshwater and (b) saltwater. Preference represents the average preference score for all individuals of each *Eda* genotype. A preference of 1 indicates complete preference for the acclimation environment and 0 indicates complete preference for the alternate environment. Error bars represent  $\pm 1$  standard error. *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.

likely to colonize freshwater environments, where positive selection can then act to increase its frequency (Colosimo *et al.* 2005; Barrett *et al.* 2008). However, freshwater acclimated sticklebacks carrying the low allele will be more likely to leave freshwater for the ocean. This trait may impede local adaptation by promoting the migration of low alleles between environments and could help to explain the persistence of the allele at low frequencies in the ocean, despite presumably negative selection in this environment (Colosimo *et al.* 2005). Experimental tests of the behavioural effects of genes under divergent selection allow for a more comprehensive understanding of the mechanisms that drive local adaptation and the maintenance of genetic variation.

The treatment of experimental animals was in accordance with the British Columbia Animal Care protocol no. A07-0293 (Department of Zoology, University of British Columbia).

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