

When Phenotypes Do Not Match Genotypes—Unexpected Phenotypic Diversity and Potential Environmental Constraints in Icelandic Stickleback

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Divergent lateral plate phenotypes in stickleback represent one of only a few cases known, where a single gene underlies the phenotype under divergent selection between different habitats. However, the selection pressures leading to the repeated loss of lateral plates in freshwater are still not well understood. By genotyping 838 individuals from 9 independently colonized lakes and 1 marine population in Iceland, we found 1) that only in some lakes are phenotypes associated with the expected genotype and 2) that the independent repeated occurrence of a rarely described plate phenotype is expressed in the absence of an allele that is usually associated with this phenotype. This suggests that either other genes such as modifiers might be under divergent selection between lakes or that lateral plate expression in these populations is restricted due to environmental constraints.

Key words: *Eda*, *Stn382*, three-spine stickleback

Introduction

Identifying the genes underlying divergent phenotypes is central to the understanding of mechanisms involved in evolutionary diversification (Storz 2005). Examples of repeated parallel evolution of similar phenotypes provide particularly good opportunities to study such mechanisms. In addition, studying phenotypic expression of the same genotype in different environments allows for the investigation of changes and constraints on gene expression imposed by the environment. Only a few cases are known where one gene regulates a major phenotypic change as a response to divergent natural selection in different environments (Dalziel et al. 2009). One example comes from the three-spine stickleback *Gasterosteus aculeatus*, which has repeatedly evolved

parallel divergent ecotypes between similarly contrasting environments and has therefore become a model system in evolutionary biology (McKinnon and Rundle 2002). The ancestrally marine species invaded freshwater habitats across the northern hemisphere, many of which only became accessible after the last glacial retreat. While colonizing freshwater bodies, stickleback have repeatedly reduced and sometimes even completely lost their predator defense traits, consisting of bony lateral plates, a pelvic girdle, and dorsal and pelvic spines (reviewed in Barrett 2010).

The ecological mechanisms and evolutionary processes responsible for the repeated loss of lateral plates are not yet fully understood (Barrett 2010). However, several hypotheses have been postulated for the ecological mechanisms, relating to both biotic and abiotic factors. Biotic mechanisms include adaptations to different predation regimes (Reimchen 1994; Marchinko 2009), food availability (Bjaerke et al. 2010), and buoyancy (Myhre and Klepaker 2009), while abiotic mechanisms include adaptations to water chemistry such as the concentration of calcium and other ions (Bell et al. 1993; Marchinko and Schluter 2007).

In contrast, the genetic basis of the lateral plate phenotype in stickleback is relatively well understood with an almost Mendelian genetic basis (Le Rouzic et al. 2011). A single gene, *Ectodysplasin* (*Eda*), has been demonstrated in quantitative trait loci (QTL) analyses to explain ~75% of the phenotypic variation in the number of lateral plates (bony structures on the flanks) between marine and freshwater stickleback (Colosimo et al. 2004). Generally, marine sticklebacks have a full set of lateral plates from the head to the tail, which includes plates on the caudal keel. Freshwater fish, on the other hand, often have only a few bony plates close to the head. There are 2 classes of *Eda* alleles, further referred to as the *Eda^L* (low) and the *Eda^C* (complete) allele. Most marine stickleback populations have mainly *Eda^C* alleles, while most

freshwater stickleback populations are fixed or nearly fixed for *Edd^L* alleles (Cano et al. 2006; Schluter and Conte 2009). However, *Edd^L* alleles occur at low frequencies in marine stickleback (Colosimo et al. 2005; Schluter and Conte 2009). They may have arrived and be maintained there through low levels of introgression from freshwater populations into the marine population. As a consequence, *Edd^L* alleles may now be present as rare standing genetic variation in the sea, which can become selectively advantageous once marine stickleback colonize new freshwater habitats (Colosimo et al. 2005; Le Rouzic et al. 2011).

Furthermore, it has been suggested that the different alleles of the *Eda* gene might have pleiotropic effects, which could explain the repeated fixation of different *Eda* alleles and their respective plate phenotypes. Barrett et al. (2008) found that individuals carrying the *Edd^L* allele had a growth advantage and a better winter survival in freshwater ponds, suggesting hitherto unknown costs for the development of lateral plates. The enhanced growth rates for low-plated (LP) morphs in freshwater suggests the existence of a fitness trade-off between defense and growth rate.

Although the *Edd^L* allele has repeatedly been found to explain phenotypic shifts in freshwater populations across the Northern Hemisphere, an increasing number of cases are known where the *Eda* genotypes do not match the lateral plate phenotypes (Colosimo et al. 2005; Cano et al. 2006; Le Rouzic et al. 2011). Cano et al. (2006) found that the presence of at least one *Eda^C* allele was necessary to express a keeled phenotype, suggesting a dosage effect. It should also be noted that both fully plated (FP) and partial plated phenotypes are generally described to have a keel, although phenotypically LP fish with a keel (LPK) are known from several systems (Bañbura and Bakker 1995; Kristjánsson et al. 2002; Cano et al. 2006; Lucek et al. 2010). However, our understanding of the underlying genetics of the expression of keel plates is poor (Cano et al. 2006) and only a few populations have yet been investigated for both *Eda* and the presence of keel plates. In order to test for an association of *Eda* with lateral plates and especially the keeled phenotype, we investigated 9 independently colonized freshwater systems in Iceland in relatively close geographic proximity and compare them with a putatively ancestral marine population.

Materials and Methods

Three-spine stickleback were sampled between August and September 2010 in 9 lakes and 1 marine site in Iceland (Table 1), using minnow traps and by hand netting. Previous studies on 2 of these lakes (Frostastadavatn and Myvatn) indicated the presence of different plate phenotypes (Kristjánsson et al. 2002). All fish were euthanized with an overdose of clove oil and stored in 70% ethanol. A fin clip was taken and preserved in absolute ethanol for genetic analyses. Sample sizes per lake ranged from 30 to 198 (mean: 84 ± 59 standard deviation, Table 1). A total of 838 individuals were included in our analysis. Lateral plates were counted on the left side under a dissection microscope; anterior and keel plates were counted separately.

Table 1 Sampling sites used in this study, with sample size and location, Snt382 C allele frequency, and lateral plate numbers (mean and range)

Lake	N	Latitude	Longitude	C allele frequency	Mean and range of lateral plates	Ca ⁺⁺ [mg/l]	N _{keeled} Ind.	LP			LPK			PPNK			PPK			FP		
								0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
Apavatn	41	64°11.7'	20°37.9'	0	3.0 (0–5)	5.37 ^a	2	39	0	0	2	0	0	0	0	0	0	0	0	0	0	0
Flodid	30	65°29.5'	20°21.6'	0	5.3 (3–7)	—	0	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Frostastadavatn	137	64°01.4'	19°02.5'	0	7.4 (5–17)	4.35 ^a	14	119	0	0	12	0	0	4	0	0	2	0	0	0	0	0
Galtaból	57	65°15.7'	19°44.5'	0	5.2 (4–6)	3.76 ^a	0	57	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hraunfjörðr	79	64°55.9'	23°01.7'	0.228	8.9 (3–34)	—	25	45	7	1	1	11	0	0	1	0	0	11	1	0	0	1
Mjóavatn	61	65°15.7'	19°48.0'	0	6.0 (4–8)	5.70 ^a	1	60	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Momelar	34	65°25.3'	20°39.9'	0	5.1 (4–7)	—	0	34	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Myvatn	198	65°38.0'	16°37.4'	0.247	6.0 (3–12)	9.70 ^b	52	109	36	1	4	36	12	0	0	0	0	0	0	0	0	0
Thingvallavatn	156	64°09.0'	21°02.8'	0	4.7 (2–8)	3.7 ^b	1	155	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Marine	45	65°02.7'	22°27.5'	0.989	28.8 (14–35)	412.1 ^c	45	0	0	0	0	1	1	0	0	0	0	0	20	0	0	23

Reported calcium concentrations are given where available. Where present, the number of individuals per plate phenotype is given with their respective number of *Eda* C alleles (LP = low plated without keel; LPK = LP with keel; PPNK = partially plated without keel; PPK = partially plated with keel; FP = fully plated).

^a Karst-Riddoch et al. (2009).

^b Olafsson (1979).

^c Libes (2009).

All individuals were genotyped for the marker *Stn382*, a microsatellite flanking a 60 bp indel in intron 1 of the *Eda* gene. It yields either a 158 bp allele (further referred to as “L” allele), associated with the *Eda^L* allele and the LP phenotype or a 218 bp allele (“C” allele), associated with the *Eda^C* allele and the completely plated phenotype (Colosimo et al. 2005). DNA was extracted using a 10% chelex solution, following the manufacturer’s protocol (Biorad, Switzerland). Polymerase chain reaction (PCR) protocols followed Peichel et al. (2004). PCR products were analyzed on a 1.5% agarose gel, stained with ethidium bromide. All bands were scored by eye.

Following previous studies, individuals were assigned to different plate phenotype categories (Hagen and Gilberts 1973; Ziuganov 1983; Barrett 2010; but see, Bañbura and Bakker 1995) explicitly incorporating the presence or the absence of a keel (Bañbura and Bakker 1995; Cano et al. 2006; Lucek et al. 2010) (Figure 1): FP fish have lateral plates contiguously along the whole body (total 31–35 plates) from the head to the caudal peduncle, where the plates form a keel. LP fish have only 0–10 anterior plates and lack the keel. LPK have only 0–10 anterior body plates and, in addition, plates on the caudal peduncle. Partially plated fish have more than 10 anterior plates, and may or may not have a keel (PPK and

PPNK, respectively). In most populations hitherto studied, the 3 phenotype classes (FP, LP, and PP) normally correspond very well to being homozygous for *Eda^C*, homozygous for *Eda^L*, and heterozygous, respectively (Colosimo et al. 2005; Lucek et al. 2010). All collected data were deposited on the Dryad Digital Repository (doi:10.5061/dryad.7dp3126p).

Results

The marine population is almost fixed for the *Stn382* C allele (98.9%), yet PP individuals with missing plates are common and even LPK individuals are present, albeit at low frequency (Figure 2, Table 1). Marine individuals have a keel with a higher number of plates than freshwater fish with a keel ($t = 22.2$, $df = 132.0$, $P < 0.001$). In the freshwater populations, we found only 2 lakes where the C allele was present (Myvatn and Hraunsfjodr). Hraunsfjodr had a relatively high proportion (19.0%) of marine-like FP phenotypes. Individuals of all *Eda* genotypes seem to be able to express a keel in these 2 lakes, including individuals with only few anterior body plates—a phenotype not observed in the marine population (Figure 1). Nonetheless, individuals with a C allele are likely to have a keel in both lakes (logistic regression of presence of keel against number of C alleles: Myvatn: $\chi^2_1 = 86.18$, $P < 0.001$, Hraunsfjodr: $\chi^2_1 = 43.9$, $P < 0.001$). In these 2 lakes, *Eda* is still significantly correlated with lateral plate phenotypes (Myvatn: $R^2 = 0.415$, $P < 0.001$, Hraunsfjodr: $R^2 = 0.514$, $P < 0.001$), but explains less variation than has previously been described (41.5% and 51.4%, respectively, compared with ~75% in Colosimo et al. 2005). Furthermore, in both Myvatn and Hraunsfjodr, we find individuals belonging to the LP phenotype class even in the presence of 1 or 2 C alleles, often without a keel (Figure 2).

In 4 of 7 lakes where the C allele is absent (Apavatn, Frostastadavatn, Mjoavatn, and Thingvallavatn), phenotypes did not match predictions based on their respective *Eda* genotype. In these lakes, even LP individuals homozygous for the L allele may express a keel (Table 1). These keeled individuals had usually only one or few plates on the caudal peduncle compared with the populations where the C allele is present. We even observe a few PPK individuals in Frostastadavatn with up to 15 anterior plates, which are otherwise only found in the marine population and Hraunsfjodr. Hence, there is no clear association between genotype and phenotype class in these 4 lakes, as only L alleles seem to be present, and yet there are LP individuals expressing plates on the caudal peduncle. In 3 other lakes where the C allele is absent (Flodid, Galtabol, and Momelar), all fish show the predicted LP phenotype.

Discussion

Most Icelandic freshwater stickleback populations are low plated, whereas the marine population has almost exclusively highly plated PP and FP phenotypes. Marine fish also

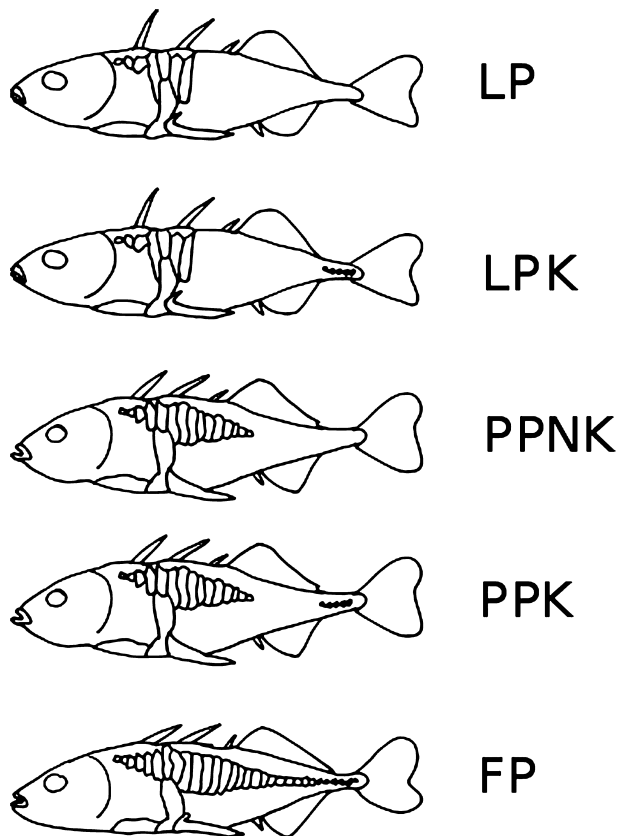


Figure 1. Lateral plate morph categories, from top to bottom: LP, low plated (0–10 plates); LPK, LP with keel; PP, partially plated with intermediate plate counts either having a keel (PPK) or not (PPNK); FP, fully plated (31–35 plates).

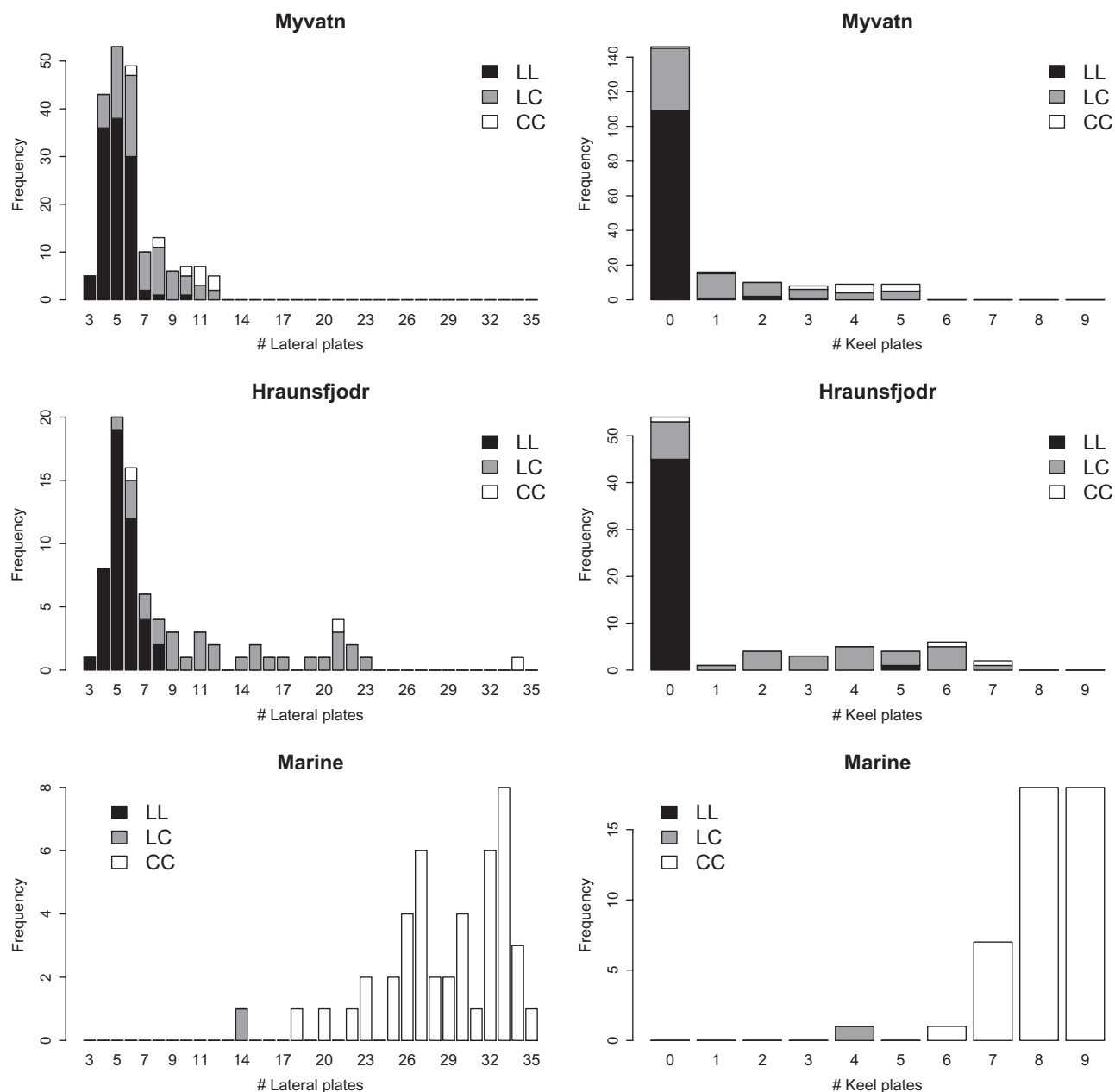


Figure 2. Frequency distributions of lateral plate counts for the 3 *Stn382* genotypes (black, LL; gray, LC; and white, CC) in Myvatn (top), Hraunsfjodr (middle), and the marine population (bottom). The total number of plates are shown in the left panels, and the number of keel plates are shown in the right panels.

have more keel plates than freshwater fish. This is consistent with the widely observed pattern of lateral plate loss in freshwater stickleback. However, there are several anomalies in these freshwater populations. 1) In many lakes, there is a reduced association between the *Eda* genotype and plate phenotype in the wild compared with a previous QTL study (Colosimo et al. 2005). 2) Despite the absence of the *C* allele in some lakes, we find repeated occurrence of the LPK and even the PPK and PPNK phenotypes, which is inconsistent with the proposed dosage effect of *Eda^C* alleles (Cano et al.

2006). Thus, the observed phenotypic variation can only partially be explained by the underlying *Eda* genotype.

Although we cannot rule out the possibility that the indel marker used in this study might have become dissociated from the respective *Eda* alleles by recombination or mutation, it must have had occurred independently in several lakes or is maintained at very low frequency in the marine population. This, plus its widespread association with the respective *Eda* alleles in the Northern Hemisphere, suggests that genetic factors other than the *Eda* gene may

contribute to the expression of the keel on the caudal peduncle and lateral plates in general. Indeed, Ziuganov (1983) already suggested the presence of an additional gene closely linked but separate from the plate morph locus. This has, however, been questioned by Bañbura (1994, but for discussion, see Bañbura and Bakker 1995). Another explanation could be a recurrent mutation in the same modifying gene, leading to different expression of phenotypes, which is what was found for pelvic spines in stickleback (Chan et al. 2010). This may suggest that modifier genes are also important to the expression of the keel and that they may affect the expression of plates as well.

In addition, our results may suggest that either the underlying genetic basis or the association between *Eda* and the lateral plate phenotype could differ in wild populations compared with a former QTL study (Colosimo et al. 2005). The relative high genotype–phenotype association (~75%) reported in F2 crosses therefore might represent a maximal association, which may differ in the wild. However, our observed pattern could also be driven by uneven allelic frequencies in the wild. Nevertheless, we find only limited support for a dosage effect of the *Eda^C* allele in the expression of a keel based on the linked Stn382 C allele as was proposed by Cano et al. (2006). In fact, we find in both Myvatn and Hraunsfjodr individuals of all *Eda* allele combinations expressing a keel. Moreover, in both lakes, a few individuals express an LP phenotype despite carrying 1 or 2 C alleles. In all other lakes, where the C allele is either absent or very rare if present, the keel cannot be explained by the dosage effect. Further support comes from the marine population, where individuals with missing plates are common despite having 2 C alleles.

Abiotic factors, such as the low calcium concentrations in Icelandic waters could be a possible explanation for the low numbers of lateral plates in Icelandic freshwater stickleback (Table 1). The calcium concentration of Icelandic lakes rarely exceeds 10 mg/l (Karst-Riddoch et al. 2009), which is below the proposed threshold of 12 mg/l, below which the expression of calcium structures might be limited (Bell et al. 1993). Hence, this may result in a high cost to express calcified armor structures. This could be the case in Lakes Myvatn and Hraunsfjodr, where many individuals have no keel or low plate counts despite the presence of the C allele resulting in a lower variation explained by *Eda*.

However, we cannot exclude biotic factors such as higher winter mortality and lower growth rates as a selective force against the C allele (Barrett et al. 2008). Another proposed source of selection are invertebrate predators, which promote an armor reduction, where less-armored individuals should have a higher escape rate (Reimchen 1980; Marchinko 2009). However, this is unlikely to play a major role in the Icelandic system, as only one small odonate species is described (Gislason and Olafsson 1989) and other known invertebrate predators are either absent (Notonectoids) or species poor (Dytiscids; Fauna Europaea 2010) and appear to be rare (personal observation K.L.). Given that invertebrate predators are virtually absent in Icelandic lakes but predators such as piscivorous arctic charr abound (Kristjánsson et al. 2002), one would expect that

Icelandic freshwater stickleback should express the fully plated phenotype and yet the large majority are low plated.

Despite the large number of studies aiming to identify the selective forces acting on different body armor phenotypes, the adaptive relevance of a caudal keel is not yet resolved. It has been argued that a keel may be beneficial for various swimming behaviors (Baumgartner 1992). Indeed, stream velocity gradients and plate phenotypes seem to be correlated (Bañbura and Bakker 1995). However, this could also reflect different predation pressures, which covary along such gradients (Bañbura and Bakker 1995). Such effects would play a minor role in our lake systems, as flow regimes are absent. Further experimental evidence is needed to test the adaptive significance of a keel and the effects of environmental calcium concentrations.

In conclusion, we find the repeated occurrence of genotype–phenotype mismatches in a phenotype whose genetic basis was thought to be well understood. This indicates the general need to integrate a broad range of the natural distribution of a species when genotype–phenotype associations are studied. Recognizing both the genetic and environmental mechanisms leading to variation of such associations in the wild will allow us to understand the adaptive landscape and its evolutionary consequences. In the Icelandic lake stickleback populations that we have studied, the *Eda* gene may not be the only determinant of the lateral plate phenotype involving the caudal keel.

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