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REVIEW PAPER

Adaptive evolution of lateral plates in three-spined stickleback *Gasterosteus aculeatus*: a case study in functional analysis of natural variation

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The three-spined stickleback *Gasterosteus aculeatus* is a model species for studying questions in ecology and evolution. The rapid diversification of *G. aculeatus* in post-glacial freshwater environments, combined with recently developed molecular tools, provides a unique opportunity to study the functional basis of fitness variation in natural populations. In derived freshwater populations, a number of morphological traits have diverged in parallel from the marine ancestral state, including the number of lateral armour plates. Evolution of reduced armour in freshwater populations is due to positive selection from both abiotic and biotic mechanisms. The major effect gene (ectodysplasin-A or *Eda*), along with several minor effect genetic regions, has recently been shown to control lateral plate variation. Field experiments have further determined the fitness consequences of allelic variation at the major effect locus. This work helps elucidate the mechanisms connecting genetic variation with phenotypic variation and fitness in the wild, a synthesis that should be applicable to many other phenotypic traits and species of fishes.

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INTRODUCTION

With close to 30 000 species covering a bewildering array of body forms and ecological roles, fishes represent a spectacular example of global biodiversity. Understanding the mechanisms responsible for the creation and maintenance of this diversity is a central goal of biological research, and one that requires the synthesis of knowledge from evolution, ecology and molecular biology. Precise fit between form and ecological function suggests the influence of adaptive evolution, but actually testing hypotheses about the role of natural selection in phenotypic differences have proven difficult. Historically, two parallel approaches have been used to study adaptive evolution, with population biologists focusing on how individuals differ in phenotypic traits across environments, and population geneticists investigating spatial

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and temporal changes in allele frequencies. These approaches are complementary, because although selection acts on phenotypes regardless of their genetic basis, the evolutionary response to selection is determined by the underlying genetic architecture of these traits. With the emergence of powerful genomic techniques, it is possible to combine these approaches by linking molecular changes to the phenotypes responsible for adaptive differences between populations (*i.e.* finding the genes that matter). While making the connection between genotype and phenotype is a laudable accomplishment, however, it cannot in itself identify the functional mechanisms or ecological context by which selection favours one allele over another and thereby creates phenotypic differences between populations. A powerful approach to solving this problem is to conduct field experiments with selected genotypes to evaluate directly the fitness consequences arising from the phenotypic effects of specific alleles (Bradshaw & Schemske, 2003; Lexer *et al.*, 2003; Baack *et al.*, 2008; Barrett *et al.*, 2008; Wegner *et al.*, 2008). This approach would represent a step towards the synthesis of genomics and population biology that should provide estimates of the timing and strength of selection, enhance understanding of the genetics of adaptation and yield insights into the mechanisms driving changes in gene frequency.

Progress towards the functional analysis of genetic variation in fishes is now being driven by research using model species with excellent genomic resources (Cossins & Crawford, 2005). For instance, comprehensive genomic tools have been developed in several actinopterygian groups: stickleback *Gasterosteus* sp., medaka *Oryzias latipes* (Temminck & Schlegel), fugu *Takifugu rubripes* (Temminck & Schlegel), zebrafish *Danio rerio* (Hamilton) and cichlids. These include full genome sequences, genetic maps, gene expression techniques, transgenic techniques, reverse genetic tools, forward genetic screens, express sequence tag (EST) databases and bacterial artificial chromosome (BAC) libraries (Abzhanov *et al.*, 2008). Furthermore, genomic resources for functional analyses are rapidly being developed and applied in several other groups of fishes, such as killifish *Fundulus heteroclitis* (L.) (Oleksiak *et al.*, 2005; Fanguie *et al.*, 2006) and salmonids [*e.g.*, lake whitefish *Coregonus clupeaformis* (Mitchill) and Atlantic salmon *Salmo salar* L.] (Waples & Naish, 2008; Bernatchez *et al.*, 2010). Natural characteristics of these groups, such as the presence of readily identifiable ecotypes and the viability of crosses from divergent populations, add to their usefulness for investigating adaptive genetic differences in the wild. In the case of *Gasterosteus aculeatus* L. and cichlids, decades of ecological and evolutionary study have preceded their more recent role as molecular model systems and provide a valuable real-world context within which to interpret genomic data.

Of all the taxa mentioned above, *G. aculeatus* may provide the best combination of diverse ecological variation, interesting evolutionary history and well-developed genomic resources. Three-spined *G. aculeatus* is a small (<7 cm L_S) holarctic fish with marine, anadromous and freshwater populations (Bell & Foster, 1994). The species can be distinguished by bony armour and spines, bright male nuptial colouration and elaborate courtship behaviour. Relative to marine *G. aculeatus*, the *G. aculeatus* inhabiting post-glacial freshwater habitats have evolved a wide diversity of armour, colour, diet, trophic morphology, body forms, behaviour and life-history traits. Some of this variation, such as presence or absence of a pelvis, rival interspecies differences in other groups (Bell, 1987; Cresko *et al.*, 2004). Furthermore, the evolutionary history of these fish provides an exceptional opportunity to study the genetic architecture of adaptive divergence. Genetic evidence shows that

marine *G. aculeatus* are ancestral to many freshwater populations, which have repeatedly colonized lakes and streams created after the retreat of the Pleistocene glaciers (Hagen & McPhail, 1970; Rafinski *et al.*, 1989; McPhail, 1994; Taylor & McPhail, 1999; Reusch *et al.*, 2001; Raeymaekers *et al.*, 2005). Thus, the profound morphological, behavioural and physiological differences between marine and freshwater populations in recently deglaciated regions have evolved in a remarkably short period of time, presumably in response to local ecological conditions (McKinnon & Rundle, 2002). In this short review, several decades of research investigating the adaptive divergence of the lateral plate armour phenotype in three-spined *G. aculeatus* are outlined. This topic has been reviewed previously (Reimchen, 1994; Bell, 2001), and so a focus is placed on recent efforts to experimentally measure selection on the genes that underlie this trait. This work will hopefully serve as an example of how connecting genotype to phenotype to fitness allows a more comprehensive understanding of the mechanisms that create and maintain biological variation in fishes.

PHENOTYPIC VARIATION IN LATERAL-PLATE NUMBER

One of the most characteristic morphological differences between marine and freshwater *G. aculeatus* is the reduced number of bony lateral plates in many freshwater populations (Bertin, 1925; Heuts, 1947). Lateral plates are superficial dermal bones that form a single row numbering between 0 and 36 along each side of the body (Scott & Crossman, 1973). There is quantitative variation both within and among populations. Marine *Gasterosteus* species typically have a continuous row of over 30 plates spanning from head to tail (complete morph) (Fig. 1). In contrast, freshwater *Gasterosteus* species typically possess <10 plates, usually at the anterior end of the body (low morph). A small proportion of both marine and freshwater fish have intermediate numbers of plates, most often with plates absent from the middle of the body (partial morph). The sequence of plate development proceeds with anterior plates developing first, followed by posterior plates and finally middle plates (Bell, 2001).

Consistent changes in the frequency of lateral-plate morphs have been observed during contemporary evolution following colonization of fresh water by marine *G. aculeatus*. Significant reductions in lateral-plate number have been documented in recently founded freshwater populations in Norway, Iceland and Alaska (Klepaker, 1993; Kristjansson *et al.*, 2002; Bell *et al.*, 2004; Kristjansson, 2005). These changes have occurred progressively over several generations during time periods of ≤ 40 years, consistent with evidence that lateral-plate phenotype is heritable and subject to selection (Bell, 2001). A number of hypotheses have been proposed as potential mechanisms driving the evolution of reduced armour in freshwater environments. These hypotheses can loosely be grouped into those involving either biotic or abiotic mechanisms. Below, the work that has tested each is outlined.

BIOTIC MECHANISMS

It has been frequently suggested that lateral-plate morphology is under divergent selection across environments because of differing predation risks (Hagen & Gilbertson, 1973; Moodie *et al.*, 1973; Bell & Haglund, 1978; Reimchen, 1983, 1991, 1992,



FIG. 1. Lateral-plate morphs in marine *Gasterosteus aculeatus*. Complete morph (top), partial morph (middle) and low morph (bottom). Fish were stained with Alazarin red to highlight bone (Barrett *et al.*, 2008).

2000; Bell & Foster, 1994; Bell, 2001; Vamosi, 2002; Kitano *et al.*, 2008; Marchinko, 2009). Marine and lake habitats with a high abundance of large fish usually have *Gasterosteus* populations composed of the complete morph (Reimchen, 1994). Increased piscivore predation intensity has been associated with positive selection on lateral plate number during contemporary evolution in Lake Washington, U.S.A. (Kitano *et al.*, 2008). Lateral plates serve as a defensive role against predatory fish. Anterior plates connect dorsal and pelvic spines, mechanically bracing the structures against each other. This allows a *G. aculeatus* to maintain erect spines, which creates the mechanical integrity required to pierce the mouth of piscivorous vertebrates and also provides a greater effective diameter, which increases the difficulty of ingestion by gape-limited predators (Reimchen, 1983). More obviously, plates can act as a protective barrier against puncture injury from toothed predators. Experimental evidence shows that *G. aculeatus* with greater numbers of anterior plates are more probable to survive after capture by predatory fish (Reimchen, 1992), helping contribute to the high (90%) failure rate of these attacks (Reimchen, 1991).

Greater overall numbers of plates can also obstruct swallowing by piscivorous fishes, most likely by hindering pharyngeal jaw retraction. The increased difficulty of swallowing leads to greater handling time and more escape opportunities (Reimchen, 2000). Cumulatively, these various biomechanical advantages of lateral plates are thought to result in positive selection on lateral plate number in environments where *G. aculeatus* are regularly captured by predatory fish.

In contrast to the open-water habitat of many marine environments, freshwater environments possess greater proportions of littoral habitat where *G. aculeatus* can presumably be closer to cover (Reimchen, 1994). Under these conditions, *Gasterosteus* species may be more likely to successfully evade attacks and the importance of evasion may supersede the importance of escape and survival after capture (Reimchen, 2000; Bell, 2001). Greater numbers of plates are associated with reduced body flexure and velocity during fast-start escapes, suggesting that completely plated *G. aculeatus* may be easier to catch (Taylor & McPhail, 1986; Bergstrom, 2002). This may be especially relevant in cases of significant avian predation because diving birds, which only submerge for brief periods and hold *G. aculeatus* with compression rather than puncturing, decrease the protective advantage of plates compared with burst speeds required to escape (Reimchen, 1994). Furthermore, in some freshwater habitats, aquatic invertebrate predation may also select for reduced armour. Aquatic insects prey on juvenile *G. aculeatus* (Foster *et al.*, 1988), and it has been hypothesized that armour serves as a point of leverage for the predators to grasp during capture (Reimchen, 1980). Thus, a reduction in lateral-plate number may be advantageous in freshwater habitats because it improves the likelihood of avoiding predation entirely.

ABIOTIC MECHANISMS

Abiotic differences between marine and freshwater environments have long been considered possible mechanisms causing diversification in armour morphology (Heuts, 1947). Teleosts absorb calcium from ambient water for skeletal development (Peterson & Martin-Robichaud, 1986; Marshall, 2002; Evans *et al.*, 2005). Reduced concentrations of calcium, phosphate and other ions in many freshwater environments relative to the ocean are hypothesized to make it increasingly costly to mineralize the skeletal components required to build lateral plates (Giles, 1983). Under this hypothesis, possessing fewer plates would be advantageous in low ion concentration freshwater lakes and streams but not necessarily disadvantageous in high ion concentrations. Correlations between ion concentration and amount of skeletal armour across freshwater environments provide evidence that is consistent with ion limitation leading to reduced numbers of lateral plates (Giles, 1983; Bell *et al.*, 1993; Bourgeois *et al.*, 1994; Schluter, 1995). It should be noted that ion limitation may also have an indirect effect on lateral-plate number in fresh water by limiting the distribution of predatory fish, which cannot tolerate the low pH associated with calcium-limited lakes (Muniz, 1991). Ion limitation and reduced predation are therefore not mutually exclusive hypotheses for the evolution of reduced armour (Bell *et al.*, 1993).

An additional hypothesis involving abiotic differences between marine and freshwater environments is that there is a trade-off between salinity tolerance and lateral-plate number. An early experiment investigating this idea found that

complete-plate morphs survive longer and hatch more successfully in high salinities, whereas low-plate morphs survive longer and hatch more successfully in low salinities (Heuts, 1947). While suggestive, the plate morphs used in this study were collected from different environments, and thus, there may be other local adaptations contributing to the performance of the morphs in addition to lateral plates. A more recent study avoided this problem by testing the salinity tolerance of plate morphs collected from the same populations. The results supported earlier findings of reduced growth rate of complete plate morphs in freshwater environments but did not find evidence of a difference in growth in salt water or differences in hatching success in either ionic strength (Marchinko & Schluter, 2007). Together, this work suggests that positive selection for low-plate morphology may be the result of a correlated response to selection for increased growth rate under low salinity conditions. If the predominance of the low-plate morph in fresh water is due to differences in salinity tolerance between morphs or the difficulty of building plates under ion limitation, a key point is that the mechanism driving evolution of plate morphology could differ between environments (Marchinko & Schluter, 2007). In freshwater habitats, functional constraints imposed by abiotic properties of the environment may result in the evolution of reduced armour, whereas in the ocean, complete armour may persist due to selection from high-intensity fish predation.

GENETICS OF LATERAL-PLATE PHENOTYPE

The genetics of lateral plates in *Gasterosteus* species have been intensively studied for decades (Banbura & Bakker, 1995; Bell, 2001). Here, the recent studies that have greatly advanced understanding of this topic through the use of genome-wide linkage mapping approaches have been focused to identify the specific molecular changes responsible for lateral-plate differences. Despite the diversity of life-history, morphological and behavioural traits in *Gasterosteus* sp., viable crosses can be produced from almost any two populations from around the world, greatly facilitating the use of genetic mapping (Peichel, 2005). This approach obviates the need for a more traditional strategy using candidate genes. By definition, a candidate gene approach is limited to looking for variation in already known genes that have been conserved across widely divergent taxa. These genes have typically been chosen based on mechanistic knowledge about the links between a phenotypic trait and a candidate locus (Hoffmann & Willi, 2008). In contrast, genetic mapping makes no prior assumptions about the genes involved in a phenotypic trait and is therefore capable of finding previously unknown loci.

To identify genes responsible for variation in lateral plates, genotype–phenotype associations were investigated in the F₂ offspring from a divergent cross. A complete-plate morph from the Japanese Sea was crossed with a low-plate morph from Paxton Lake in British Columbia [Fig. 2(a)]. The resulting F₁ progeny were then crossed to produce an F₂ generation that segregated variation in lateral plate number and a number of other traits that distinguish the two grandparents. These F₂ individuals were genotyped with 428 microsatellite markers spanning the *G. aculeatus* genome (Colosimo *et al.*, 2005) [Fig. 2(b)]. The markers identified six loci that interact semi-additively to determine lateral-plate number. One of these loci, located on linkage group 4, was associated with a quantitative trait locus (QTL) controlling 80% of

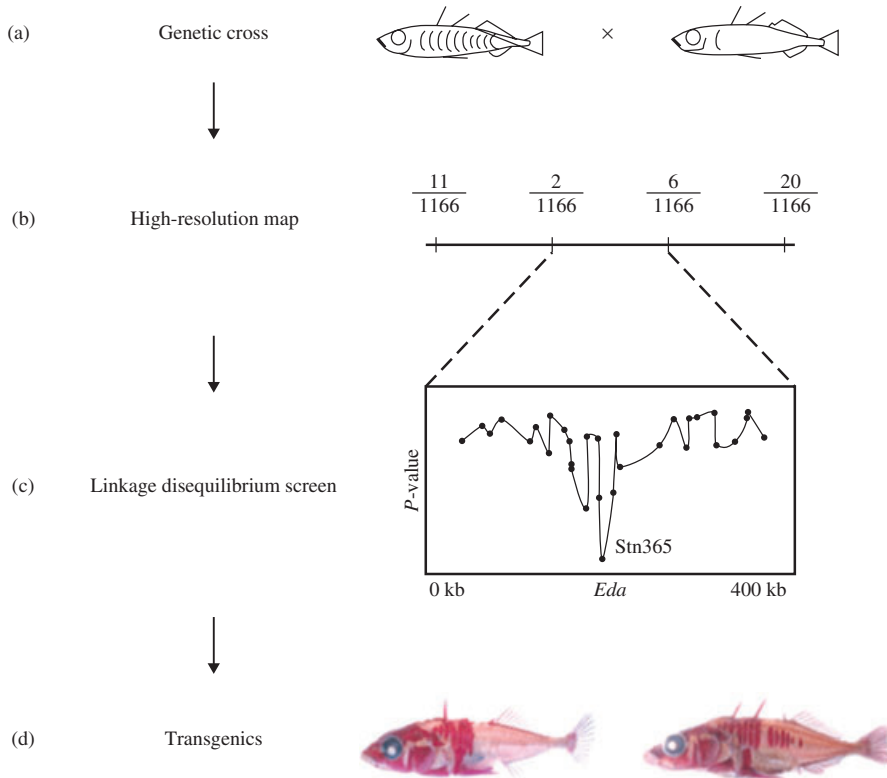


FIG. 2. Identifying the genetic basis of lateral-plate phenotype. (a) A complete-plate morph from the Sea of Japan was crossed with a low-plate morph from Paxton Lake in British Columbia. The resulting F1 progeny were then crossed to produce an F2 generation that segregated variation in lateral-plate morph. (b) High-resolution genetic mapping identified microsatellite markers that rarely recombine with the plate morph locus (number of recombinants in 1166 chromosomes shown). (c) Linkage disequilibrium screening demonstrated that marker Stn365, located within the *Eda* locus, showed large differences in allele frequency in completely and low-plated fish from Friant, CA, U.S.A. (d) Introduction of an *Eda* cDNA construct stimulates lateral-plate formation. Fish on the left is a control low-plated *Gasterosteus aculeatus*, and fish on the right is a sibling from the same clutch after introduction of the transgene. Note the six extra lateral plates that have formed in the transgenic *G. aculeatus* [modified from Colosimo, P. F., Hosemann, K. E., Balabhadra, S., Villarreal, G., Dickson, M., Grimwood, J., Schmutz, J., Myers, R. M., Schluter, D. & Kingsley, D. M. (2005). Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. *Science* **307**, 1928–1933. Reproduced with permission from AAAS.] kb, kilobase.

the variation in plate number (Colosimo *et al.*, 2004). To identify the specific gene underlying this QTL, genetic mapping was used to discriminate a 0.68 cM interval and bacterial artificial chromosome (BAC) clones covering this interval were produced (Colosimo *et al.*, 2005). Two BACs that covered the majority [400 kilobases (kb)] of the interval were then sequenced, which helped identify a number of genes in this region. The interval was further narrowed with linkage disequilibrium mapping, leaving a 16 kb region containing a few genes likely to be responsible for plate variation [Fig. 2(c)]. Among these genes is the secreted signalling molecule ectodysplasin-A (*Eda*), a member of the tumour necrosis family. *Eda* was originally identified by its role in hypohidrotic ectodermal dysplasia (HED) in humans,

which results in defects in the development of multiple ectoderm-derived structures, including hair, teeth and sweat glands (Mikkola & Thesleff, 2003). Hypohidrotic ectodermal dysplasia (HED) patients also show characteristic changes in flat ectodermal bones of the skull, showing that *Eda* is required for normal development of some skeletal structures. In addition, mutations affecting the *Eda* receptor (*Edar*) impede formation of scales in *D. rerio* (Harris *et al.*, 2008) and *O. latipes* (Kondo *et al.*, 2001), which share a developmental origin with the dermal bone used in *G. aculeatus* lateral plates. The importance of the ectodysplasin-signalling pathway for development of dermal bone and scales makes *Eda* a good candidate for controlling lateral plate number, but to conclusively show that the gene is causally responsible, transgenic techniques were used to test whether plate development could be altered by changing levels of *Eda* signalling (Colosimo *et al.*, 2005). Embryos from low-plated *G. aculeatus* parents were injected with an *Eda* cDNA construct that is known to restore development of teeth, hair and sweat glands when introduced into mutant mice carrying a null mutation at the *Eda* locus. Introduction of this construct resulted in higher levels of *Eda* expression and in the development of extra plates in a small number of fish, suggesting that *Eda* transgenes are sufficient to trigger development of lateral plates [Fig. 2(d)]. Despite identification of a strong association between *Eda* and lateral plates, however, the causative regulatory mechanism still remains unknown (Knecht *et al.*, 2007).

An exciting new development for high-throughput fine-scale genetic mapping of the lateral-plate phenotype is the use of restriction-site-associated DNA (RAD) tags (Baird *et al.*, 2008). These markers are short fragments of DNA adjacent to each instance of a particular restriction enzyme recognition site. When RAD tag libraries are combined with next-generation sequencers, it is possible to rapidly discover and map thousands of SNPs at low cost. RAD marker density is an order of magnitude greater than existing microsatellite marker density, which facilitates both fine mapping of previously known genetic regions associated with a particular phenotype and also the discovery of additional unknown regions. This approach has recently been used to discover regions associated with lateral plate phenotype that share the same linkage group but are 7–12 megabases (Mb) physically distinct from *Eda* (Miller *et al.*, 2007; Baird *et al.*, 2008). Importantly, when reanalysed without taking advantage of any available reference genome information, tags linked to lateral-plate phenotype were again found in the same three regions, suggesting that sequencing of RAD tags would provide useful markers even in fish that do not have a reference genome.

SELECTION ON *EDA* AND THE MECHANISMS RESPONSIBLE

Identifying differences in the ecological performance of phenotypic variants and the genes that underlie them are the first steps in the functional synthesis of evolutionary biology (Arnold, 1983; Kingsolver & Huey, 2003; Dalziel *et al.*, 2009). The final element is to quantify natural selection acting on the genes to shed light on the functional mechanisms and evolutionary forces that have shaped patterns of variation. Although they cannot provide an estimate of the strength of selection, instances of parallel evolution give strong evidence of past selection. Natural selection has almost certainly played a role whenever the same genetic changes and

phenotypic traits evolve repeatedly and consistently in association with the environment (Simpson, 1953; Endler, 1986; Schluter & Nagel, 1995; Schluter *et al.*, 2004). Parallel evolution could also occur through genetic drift, but it is highly unlikely that transitions would be consistently associated with the environment. Genetic mapping and complementation crosses show that *Eda* is responsible for the repeated evolution of the low-plate morph in fresh water throughout the world (Awise, 1976; Colosimo *et al.*, 2004; Cresko *et al.*, 2004; Schluter *et al.*, 2004; Colosimo *et al.*, 2005). Almost all low-plate freshwater populations examined share a number of nucleotide changes within and near the *Eda* gene, while a different set of related haplotypes is shared by completely plated marine populations. Phylogenetic analysis of these sequences grouped almost all populations according to their plate morph. This pattern, and the discrepancy with the topology of the tree obtained with 25 random neutral markers, which grouped populations by geography rather than plate phenotype, strongly suggests that the low-plate haplotype is favoured in fresh water because genetic drift is unlikely to create such a strong correlation between genotype and environment.

A few recent studies have explicitly tested whether freshwater populations have experienced selection at the *Eda* locus by analysing patterns of sequence polymorphism at and around the gene in comparison to neutral markers in other regions of the genome. Separating the effects of selection from demography can complicate detection of this signal because population expansions or bottlenecks can result in similar patterns to the sweep of a beneficial allele (Slatkin & Wiehe, 1998; Nielsen, 2001; Kim & Stephan, 2002; Santiago & Caballero, 2005). The prediction, however, is that if *Eda* mutations resulting in the low-plate morph are under strong positive selection in freshwater, there should be a reduction in polymorphism that is restricted to the neighbouring region, whereas demographic effects would leave a genome-wide signal. This is because the low-plate alleles will occur with only a sub-set of neutral variants at linked sites, creating linkage disequilibrium between them. Although mutation and recombination can obscure this signature, the fixation of low-plate alleles is expected to be recent enough (10–15 000 years) to often leave a distinguishing signature of selection in the surrounding genomic region. In agreement with this prediction, a genome scan of 103 microsatellite and two indel markers in four freshwater and three marine Fennoscandian populations found that the strongest signal of directional selection emerged from markers within the intronic regions of *Eda* (Makinen *et al.*, 2008). Similarly, F_{ST} values at markers within the *Eda* gene were significantly lower than F_{ST} values at neutral markers in an analysis of six Belgian freshwater populations (Raeymaekers *et al.*, 2007). In contrast, when a marker more distantly linked to *Eda* was analysed in marine and freshwater populations, no allele frequency differentiation was found, despite greater differentiation in plate number than that expected by genetic drift ($Q_{ST} > F_{ST}$; Cano *et al.*, 2006). These studies demonstrate that the major locus controlling variation in lateral plates does experience positive selection upon colonization of fresh water, and therefore provide indirect evidence supporting an adaptive role for reduced armour in this environment.

Although identifying a molecular signature of selection can provide supporting evidence that a gene contributes to production of an adaptive phenotype, it cannot reveal the functional mechanism by which selection is acting on the gene. To address this, studies are now beginning to incorporate the growing knowledge of the genes underlying putatively adaptive traits with experiments to directly test hypotheses about the fitness consequences of functional differences between genotypes under

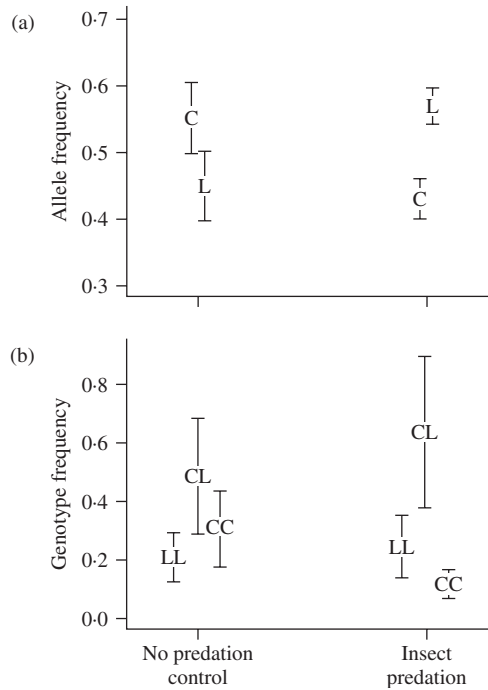


FIG. 3. Insect predators selection against the *Eda* complete allele. Allele frequency (a) and genotype frequency (b) at the ectodysplasin locus in F2 juveniles from control and predation treatments. Uppercase letters indicate among family mean (± 1 S.E.). In the top panel, L corresponds to the low allele and C represents the complete allele. In the bottom panel, LL, CL and CC represent the low allele homozygote, the heterozygote and complete allele *Eda* genotypes, respectively. Data are from the six F2 families generated from crosses made from fish collected from Paxton Lake (specifically the benthic population) and Oyster Lagoon, British Columbia [modified from Marchinko, K. (2009). Predation's role in repeated phenotypic and genetic divergence of armor in threespine stickleback. *Evolution* **63**, 127–138. Reproduced with permission from John Wiley & Sons.]

natural conditions. This approach has recently been used to clarify the mechanisms driving evolution of reduced armour in freshwater *G. aculeatus*. Marchinko (2009) tested whether insect predation would favour reduced armour by measuring selection in juvenile F2 families from crosses between freshwater (low-plate) and marine (complete-plate) populations. Families were split between control and insect predation treatments in experimental ponds. Predation was greatest on individuals carrying the complete allele [Fig. 3(a)], providing support for the hypothesis that selection from aquatic insect predation contributes to the evolution of reduced armour in freshwater *G. aculeatus*. In conjunction with the evidence for positive selection on armour when fish predation is high (Reimchen, 1992, 2000), these results indicate that divergent selection driven by differences in predation regime is one mechanism contributing to armour polymorphism across environments.

In two complementary studies, Barrett *et al.* (2008, 2009) tested the hypothesis that when marine *Gasterosteus* invade freshwater environments, natural selection favours the low allele because it confers an advantage in growth rate. Under this hypothesis, the evolution of low-plate populations in freshwater environments may be the result of a correlated response to positive selection for increased growth rate

rather than negative selection on armour (Marchinko & Schluter, 2007). Barrett *et al.* (2008) tracked adaptive evolution over a complete generation at the *Eda* locus in replicated transplants of marine *G. aculeatus* to freshwater environments. The experiment was designed to test whether the *Eda* low allele is positively selected in fresh water because it permits a reallocation of energy from lateral plates to growth rate. Increased growth rate can increase lipid stores and result in higher overwinter survival, a key component of fitness (Schultz *et al.*, 1991; Curry *et al.*, 2005). To control for the effects of background genetic variation, the study used only rare marine *Gasterosteus* that were heterozygous at *Eda*. These fish carry the same genetic variation that selection acts on in nature and because the variation at *Eda* is embedded within a marine genetic background, they can be used to help isolate the effects of selection on the locus from many of the other genetic differences between marine and freshwater environments. Over 35 000 fish were sampled from the ocean to obtain 182 heterozygotes, which were used to produce an F1 generation with all three *Eda* genotypes. In agreement with predictions, the low allele was associated with higher juvenile growth and improved overwinter survival, with a selection coefficient of *c.* 0.5 against the complete allele over the winter months (Fig. 4). Barrett *et al.* (2009a) used laboratory rearing experiments to test if *Eda*'s effects on growth rate are environmentally determined. If pleiotropy between armour and growth is only present in fresh water, this would facilitate divergent selection on *Eda* even with parallel selection on armour across environments. Similar to the field experiment by Barrett *et al.* (2008), this study used only F1 fish produced from crosses of rare marine *Eda* heterozygotes. When raised in fresh water, *Gasterosteus* carrying the low allele had increased growth rate relative to those carrying the complete allele. In salt water, this growth advantage was present during juvenile growth but lost during adult growth, suggesting that in this environment *G. aculeatus* are able to develop full armour plates without sacrificing overall growth rate (Fig. 5). These experiments show that, in freshwater *G. aculeatus*, the fish experience a trade-off between armour and growth, because fish carrying the complete allele have high armour but reduced growth rate. Given the importance of growth for overall fitness (Schultz *et al.*, 1991; Curry *et al.*, 2005; Barrett *et al.*, 2008), this result helps explain how the low allele could be favoured even in freshwater environments where fish predation is common.

The patterns linking the low *Eda* allele with increased growth rate and survival suggest that positive selection in fresh water can result from both direct and indirect effects of having fewer armour plates. Some results of these experiments, however, cannot be explained by selection on lateral plates. For instance, although *Eda* heterozygotes are characterized by an intermediate level of armour plating relative to the homozygotes, they did not show intermediate fitness relative to homozygotes in these studies. *Eda* heterozygotes showed lower mortality from insect predation and overwinter conditions than did *Eda* homozygotes (Barrett *et al.*, 2008; Marchinko, 2009) [Figs 3(b) and 4(c)]. In contrast, during early life-history stages before the development of lateral plates, heterozygotes had lower relative fitness than homozygotes (Barrett *et al.*, 2008) [Fig. 4(c)]. These anomalous patterns imply that either variation at the *Eda* gene has direct or epistatic effects on other phenotypic traits contributing to fitness or it is linked to other, unidentified loci affecting fitness. The evolution of plates will therefore be determined not solely by the selective consequences of armour but also by the other effects of the major gene underlying this trait (Barrett *et al.*, 2009b), and the effects of genes that are in linkage disequilibrium with it. It

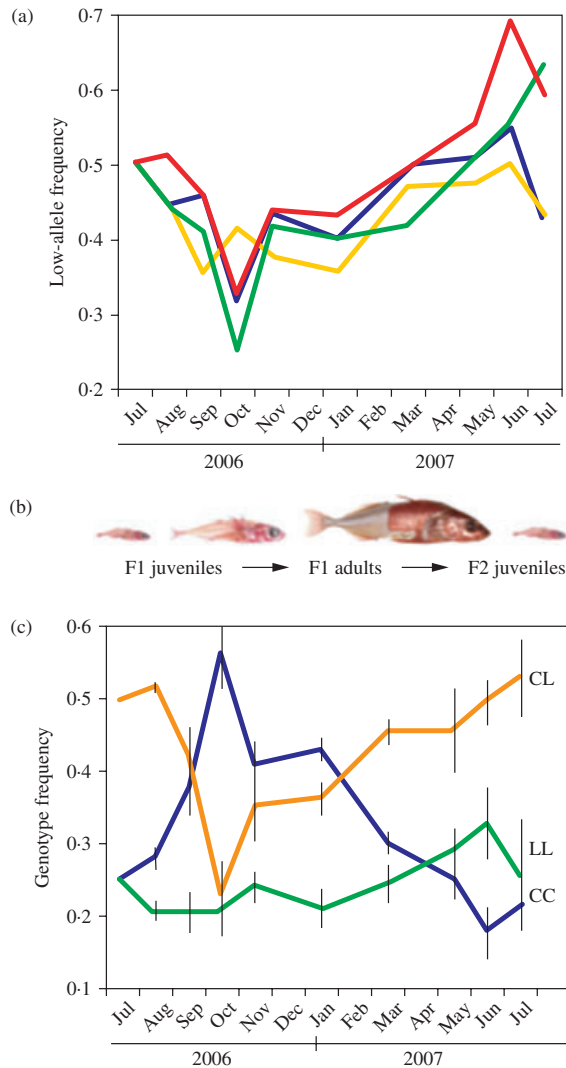


FIG. 4. Natural selection on *Eda* in natural populations of marine *Gasterosteus aculeatus* transplanted to freshwater ponds. (a) Frequency of the low allele in four replicate ponds (different coloured lines). All samples are from the first (F1) cohort of offspring except the June and July 2007 samples, which are from the second (F2) pond generation. (b) Approximate life-history stages through the course of the experiment. Fish stained with alazarin red to highlight plate morphology. (c) Genotype frequencies averaged across all four ponds. All samples as in (a). Purple, homozygous complete genotype (CC); orange, heterozygote genotype (CL); green, homozygous low genotype (LL). Vertical bars show S.E. on the basis of $n = 4$ ponds [modified from Barrett, R. D. H., Rogers, S. M. & Schluter, D. (2008). Natural selection on a major armor gene in threespine stickleback. *Science* **322**, 255–257. Reproduced with permission from AAAS.]

would not have been possible to discover the early selection against heterozygotes without knowing the major gene for the phenotypic trait, since selection occurred before it was possible to distinguish between lateral-plate morphs. These studies underscore the utility of field experiments to measure selection on genes, which can

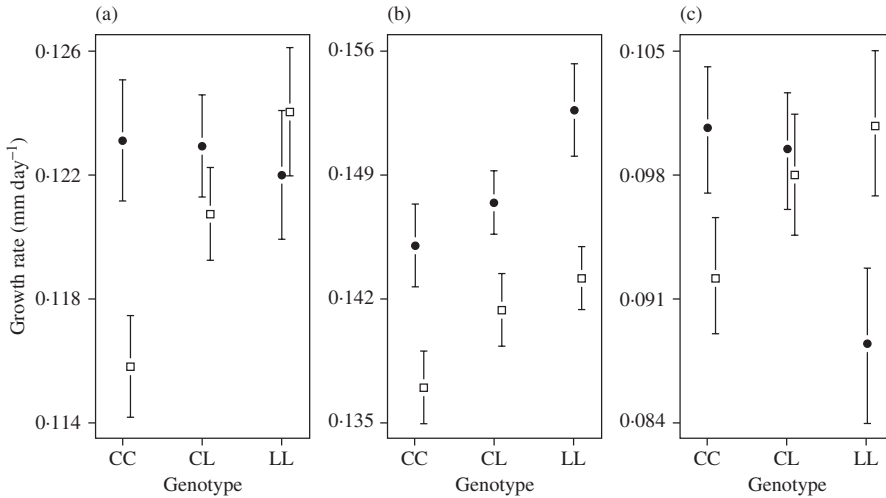


FIG. 5. Environment-specific effects of *Eda* on growth rate in salt (●) and fresh water (□). (a) Total growth rate by *Eda* genotype (b) Juvenile growth rate by genotype, calculated from birth to the end of adult plate number development at average standard length (L_S) of 27 mm. (c) Adult growth rate by genotype, calculated between the juvenile growth and the end of the experiment at average $L_S = 44$ mm. Error bars show ± 1 S.E. Note different scale on vertical axis in each panel [modified from Barrett, R. D. H., Rogers, S. M. & Schluter, D. (2009a). Environment specific pleiotropy facilitates divergence at the ectodysplasin locus in threespine stickleback. *Evolution* **63**, 2831–2837. Reproduced with permission from John Wiley & Sons.]

provide powerful tests of hypotheses for the mechanisms responsible for variation in fitness, and also lead to discovery of unanticipated fitness effects.

CONCLUSIONS

The availability of new genomic resources has recently made it possible to find the genes contributing to phenotypic variation in natural populations. Incorporating *a priori* knowledge about the functional properties of genes and their resulting effects on phenotypic traits, organismal performance and fitness will facilitate a more comprehensive understanding of the role that ecology can play in driving evolutionary patterns (Dalziel *et al.*, 2009). The functional analysis of adaptive variation in three-spined *G. aculeatus* lateral-plate number provides a useful case study showing the power of this approach. This research programme has helped address basic questions about the timing and strength of selection, the genetic architecture of adaptation, the mechanisms driving changes in allele frequency and the source of genetic variation used in adaptation to new environments. Significant investment was required to build a suite of new genomic tools for *G. aculeatus* (Peichel *et al.*, 2001; Colosimo *et al.*, 2004, 2005; Cresko *et al.*, 2004, 2007; Kingsley *et al.*, 2004; Peichel, 2005; Kingsley & Peichel, 2007; Baird *et al.*, 2008), yet these resources have allowed researchers to add a molecular component to a solid foundation of evolutionary and ecological research built over many decades (Bell & Foster, 1994). An exciting prospect for researchers studying other species of fish is that the increasing accessibility of high-throughput sequencing, together with improvements in bioinformatics

tools, will make it far less laborious and expensive to develop their own favourite species into a model species.

Future studies investigating the functional properties of candidate genes may be able to identify the specific mutations that cause ecological differences between genetic variants. There are very few cases in wild populations in which a particular molecular change has been functionally associated with an ecologically significant trait (Hoekstra *et al.*, 2006). Moreover, this level of resolution has only been successful for single major effect genes, whereas most trait differences are probably governed by complex interactions between mutations at different genes. Thus, the gap in the knowledge of the molecular interactions responsible for ecologically relevant traits makes it difficult to generate predictive models of adaptive evolution for most variation in nature. The functional analysis of natural variation will benefit greatly from a focus on dissecting oligogenic traits (*i.e.* traits controlled by several, but not hundreds, of genes with major and minor effects) to identify multiple mutations contributing to an adaptive phenotype. Even lateral-plate number in *G. aculeatus*, which is often cited as an example of a single locus trait because of the major effects of *Eda*, is determined by more complicated inheritance patterns than originally thought (Peichel *et al.*, 2001; Colosimo *et al.*, 2004; Cresko *et al.*, 2004; Miller *et al.*, 2007). Increased marker density provided by new genomic tools will greatly facilitate the discovery of minor effect loci and the epistatic interactions between them (Baird *et al.*, 2008). Fine-scale analysis of the genetic architecture underlying lateral plates and other putatively adaptive traits will make it possible to distinguish the effects of physical linkage and pleiotropy in driving the changes in gene frequency that have been observed in selection experiments (Barrett *et al.*, 2008, 2009a; Cresko, 2008; Marchinko, 2009).

The research programme described in this review demonstrates the utility of using a strategy that starts with a phenotype of interest, identifies genotype and finally evaluates the fitness consequences arising from the phenotypic effects of specific alleles under natural conditions. This approach can improve the chances that underlying phenotypes and genotypes are relevant for adaptation (Rogers & Bernatchez, 2007), thus improving the understanding of the ecological mechanisms responsible for evolutionary change in natural populations. With the current molecular revolution, these methods will soon be accessible to researchers studying species from across the broad range of fish diversity.

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