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RESEARCH ARTICLE

Studies of threespine stickleback developmental evolution: progress and promise

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Abstract A promising route for understanding the origin and diversification of organismal form is through studies at the intersection of evolution and development (evo-devo). While much has been learned over the last two decades concerning macroevolutionary patterns of developmental change, a fundamental gap in the evo-devo synthesis is the integration of mathematical population and quantitative genetics with studies of how genetic variation in natural populations affects developmental processes. This micro-evo-devo synthesis requires model organisms with which to ask empirical questions. Threespine stickleback fish (*Gasterosteus aculeatus*), long a model for studying behavior, ecology and evolution, is emerging as a prominent model micro-evo-devo system. Research on stickleback over the last decade has begun to address the genetic basis of morphological variation and sex determination, and much of this work has important implications for understanding the genetics of speciation. In this paper we review recent threespine stickleback micro-evo-devo results, and outline the resources that have been developed to make this synthesis possible. The prospects for stickleback research to speed the micro-(and macro-) evo-devo syntheses are great, and this workhorse model system is well situated to continue contributing to our understanding of the generation of diversity in organismal form for many more decades.

Keywords Adaptive radiation · Stickleback · *Gasterosteus* · Development · Population divergence · Micro-evo-devo · Speciation

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Introduction

The biological disciplines of evolution and development were divorced at the beginning of the 20th century (Raff 2000; Hall 2003). Despite different approaches, great strides have been made independently in both fields toward defining the organismal and population-level processes that generate diverse phenotypes. A re-synthesis of these fields has recently occurred, with initial studies of the evolution of development (*evo-devo*) largely involving comparisons across large phylogenetic distances (Raff 2000; Gilbert 2003; Rudel and Sommer 2003). However, one of the great advances in biology has been the codification of evolutionary process in terms of mathematical population and quantitative genetic theory (Fisher 1930), and a true evo-devo synthesis will require integrating this theory with developmental reality (Wagner et al. 1997; Bolker 2000; Arnold et al. 2001; Johnson and Porter 2001; Jones et al. 2003; Wagner 2003). This *micro-evo-devo* synthesis promises great advances in our understanding of such things as the genetic basis of adaptation, how evolutionary change can be channeled or constrained by previously evolved developmental

programs, and how population divergence leads to speciation (Wagner 2000, 2001; Porter and Johnson 2002).

Similar to the need for *model developmental genetic systems*, such as *C. elegans* and *M. musculus*, the micro-evo-devo synthesis requires model systems in order to test predictions of emerging theory (Arnold et al. 2001; Johnson and Porter 2001; Jones et al. 2003, 2004; Force et al. 2004, 2005). Some desirable characteristics of *model micro-evo-devo systems* are the following:

(1) well-characterized evolutionarily relevant *population-level variation* in the wild
(2) the ability to perform *evolutionary analysis* at the phenotypic and genetic levels
(3) *developmental genetic tools* to decipher the genetic basis of phenotypes

Finding organisms that fulfill all these requirements is no small task. Here we show how threespine stickleback (*Gasterosteus aculeatus*), long a model system for studies of evolution and speciation, fulfill all of these criteria, and we describe work over the last decade to transform stickleback into a model for micro-evo-devo studies. Reviews of threespine stickleback as an evolutionary model system have been presented elsewhere (Bell and Foster 1994; Schluter 1996a, 2000; Rundle et al. 2000; McKinnon and Rundle 2002). In this review we briefly touch on the sea of work on phenotypic variation, population and quantitative genetics only as it pertains to very recent work to uncover the developmental genetic basis of stickleback phenotypic variation. A complete micro-evo-devo synthesis using stickleback will require integrating quantitative genetic studies of development with the identification of the genetic basis of trait variation, which is one of the major conceptual challenges facing evolutionary geneticists at the beginning of the 21st century.

A micro-evo-devo system allows research on *proximate* and *ultimate* mechanisms. Proximate mechanisms are those involved in the formation of the phenotype within the life of a single organism, such as allelic and environmental effects on single characters, or the effects of pleiotropic interactions of alleles at multiple loci on the genetic variance–covariance matrix (G) of a suite of traits. Proximate mechanisms also comprise, for example, the morphogenetic processes that make neural crest cells migrate or osteocytes to begin excreting an ossifying matrix. Ultimate mechanisms are the population-level processes (such as *mutation*, *population structure*, *genetic drift* and *selection*) that drive the evolution of proximate mechanisms. Thus, micro-evo-devo questions involve the ways that ultimate evolutionary mechanisms cause the evolution of proximate mechanisms, and the manner in which these proximate mechanisms bias subsequent evolution (Gibson and Wagner 2000; Mezey et al. 2000; Wagner 2000, 2001).

The most satisfying micro-evo-devo system would allow us to seamlessly connect genetic variation to changes in what cells do to differences in phenotypes that matter for the fitness of organisms in the wild. All of the tools for studying proximate and ultimate mechanisms could be developed for a completely novel system, which is a daunting task. Alternatively, microevolution of an existing developmental model organism could be studied. Unfortunately, little is known about natural phenotypic variation, and its fitness consequences, in most developmental model organisms. A promising approach is to develop micro-evo-devo models that are well studied evolutionarily, but which are also closely related to a model developmental organism. Developmental genetic tools in these wild *semi-model systems* can be developed quickly, and studies of these species are well informed by their laboratory relatives for things such as candidate genes and developmental processes. Sister species of *Drosophila melanogaster*, such as *D. mojavensis* (Etges and Jackson 2001), fill this role, as do wild relatives of *Arabidopsis thaliana* such as *A. lyrata* and *A. petraea* (Clauss and Mitchell-Olds 2003) and relatives of mice such as the rock pocket mouse *C. intermedius* (Hoekstra and Nachman 2003; Nachman et al. 2003).

The maturation of the latest vertebrate developmental model organisms, many of them bony fish, has provided a boost to evo-devo studies. Understanding of development and genome evolution has been greatly advanced by work on zebrafish, (*Danio rerio*; Amores et al. 1998; Postlethwait et al. 1998, 2000, 2004; Furutani-Seiki and Wittbrodt 2004), pufferfish (*Takifugu rubripes* Taylor et al. 2003; Van de Peer et al. 2003; Amores et al. 2004), and medaka, (*Oryzia latipes*; Wittbrodt et al. 2002; Furutani-Seiki and Wittbrodt 2004; Naruse et al. 2004). The use of these teleost model systems has opened the door to making many classic subjects for evolutionary studies, such as cichlids, salmon, charr, sunfish, whitefish and guppies, into micro-evo-devo systems (Schliewen et al. 1994; Robinson and Wilson 1994, 1996; Bernatchez et al. 1999; Danley et al. 2000; Hendry et al. 2000; Jonsson and Skulason 2000; Albertson et al. 2003; Foote et al. 2004; Kocher 2004). At the forefront of this parade is the threespine stickleback, *Gasterosteus aculeatus*, because of the long history of studies on phenotypic diversification, population and quantitative genetics, and speciation that has been well documented in a literature of

over 2000 papers spanning nearly a century (Bell and Foster 1994). Additionally, stickleback can also be crossed and efficiently reared in large numbers through numerous generations in the laboratory, have a fairly short generation time (4–6 months), and are amenable to experimental manipulation. Recently, significant progress has been made in developing genomic and developmental tools for stickleback (Hosemann et al. 2004; Kingsley et al. 2004), including the initiation of a stickleback genome project (Broad Institute, Massachusetts, USA). These characteristics make stickleback an excellent model system to significantly contribute to the micro-evo-devo synthesis.

Phenotypic variation in natural threespine stickleback populations

Threespine stickleback fish are restricted primarily to marine, brackish and coastal freshwater habitats of the northern hemisphere (Wootton 1976; Bell and Foster 1994). The morphology of the oceanic form of this small fish (<10 cm) is similar throughout its global range and has changed little in the last 10 million years (Bell and Foster 1994), allowing the use of extant oceanic populations as ancestor-surrogates for those in fresh water (Taylor and McPhail 1999, 2000; Hendry et al. 2002). Freshwater stickleback populations have undergone a rapid (often <20,000 years) endemic radiation in recently deglaciated regions, giving rise not only to a diverse array of phenotypes (Table 1), but also to new species (McPhail 1984; Schluter and McPhail 1992; Rundle et al. 2000; McKinnon and Rundle 2002; McKinnon et al. 2004). Fresh waters differ from oceanic in many biotic and abiotic factors, including predator, prey and competitor composition, temperature, osmolarity and availability of essential minerals. These differences can impose selection resulting in extensive parallel phenotypic evolution (Fig. 1; Schluter 1993, 1995, 2000; Walker 1997; Nagel and Schluter 1998; Walker and Bell 2000). Across a variety of geographic scales (continents, regions, drainages, populations within drainages, and even within populations), independently colonized freshwater habitats have given rise to similar derived stickleback phenotypes that are correlated with environmental conditions (Table 1), providing evidence of the evolution of these traits through natural selection (Endler 1986).

One of the best-studied suites of morphological characters in threespine stickleback are the body shape and trophic traits that distinguish *limnetic* and *benthic ecotypes* and *species* (Fig. 1; Larson 1976; Schluter 1993, 1995; Robinson and Wilson 1994; Walker 1997; Walker and Bell 2000). Limnetic ecotypes are specialized for feeding on plankton by having narrow mouths, long snout, large eyes, and long, closely spaced gill rakers (Lavin and McPhail 1985, 1986, 1987; Walker and Bell 2000). The benthic ecotype, in contrast, is specialized for feeding on bottom-dwelling invertebrates in the littoral zone (McPhail 1984; Walker and Bell 2000), and typically has character states opposite to those in the limnetic. Benthic and limnetic ecotypes exist in replicate at three distinct hierarchical levels (Foster et al. 1998): closely related sister species (McPhail 1984; Schluter and McPhail 1992; McKinnon and Rundle 2002), allopatric populations (Walker 1997; Walker and Bell 2000), and a polymorphism within a population (Cresko and Baker 1996; Robinson 2000; Baker et al. 2005; Bolnick 2004).

Threespine stickleback populations also vary extensively in *bony traits* (Reimchen 1980, 1995, 2000; Bell 1985, 1987, 2001; Bell et al. 1985, 1993, 2004; Bell and Orti 1994; Reimchen and Nosil 2002; Bergstrom and Reimchen 2003; Vamosi and Schluter 2004), with the oceanic form highly protected by a robust *set of spines*, a *pelvic girdle*, and numerous *lateral bony plates* (Fig. 1; Cresko et al. 2004). In freshwater populations, the level of armor development varies greatly, but repeatedly and independently, populations have evolved few or no plates (Hagen 1973; Hagen and Gilbertson 1973a, b; Baumgartner and Bell 1984; Banbura 1994; Banbura and Bakker 1995; Bell 2001), a reduction of spine size, and a diminution of the pelvis (Bell 1985, 1987; Bell et al. 1985, 1993; Bell and Orti 1994; Vamosi and Schluter 2004).

Work on benthic–limnetic species pairs of threespine stickleback (McPhail 1984) has provided extensive information on the processes involved in the evolution of ecotypes, and rekindled an appreciation for the role of ecological interactions in the formation of species (Rundle et al. 2000, McKinnon and Rundle 2002; McKinnon et al. 2004). However, the threespine stickleback system includes other replicated sets of diverging populations involving numerous different characters involved in mate recognition and breeding systems, such as nuptial coloration and body size (Foster 1994a; Candolin 1999, 2000; Boughman 2001; Scott 2004). Behavior also varies across populations including female preferences, diversionary displays, and levels of cannibalism (Foster 1988, 1994a, b). Life history traits, such as age and size at reproduction, longevity, and clutch and egg size also vary (Baker et al. 1995, 2005). Lastly, several studies have shown phenotypic variation

Table 1 A partial list of traits that vary across stickleback populations, including available genetic estimates and example developmental processes that may have evolved

Type trait	Specific trait	Genetic analysis		Candidate developmental process
Behavior	Aggressiveness		–	Many
	Cannibalism		–	Many
	Diversionary display		–	Many
	Female preference	QGen	H^2 = 0.43 (1)	Many
	Foraging		–	Many
	Male courtship		–	Many
	Male parental care		–	Many
	Nest site selection		–	Many
	Nuptial coloration (red)	Qgen	h^2 = 0.23 (1)	NC
	Nuptial coloration (black)	Map	Single Mendelian locus and polygenic modifiers (2)	NC
	Predator inspection		–	Many
Morphology	Anal fin ray number	QGen	h^2 = 0.04–0.54 (3)	NC, Os, Ch
	Abominal vertebrae number	QGen	h^2 = 0.02–0.47 (3)	NC, Os, Ch
	Body shape	QGen	h^2 = –0.28 to 0.78 for 33 different lengths (4)	Many
	Caudal vertebrae number	QGen	h^2 = 0.07–0.55 (3)	NC, Os, Ch
	Dorsal fin ray number	QGen	h^2 = 0.34–0.68 (3)	NC, Os, Ch
	Dorsal spine length	Map	2 QTLs (5)	NC, FO
	Fin size/shape		–	Many
	Gill raker length	Gene#	5–10 loci (6)	NC, Os, Ch
	Gill raker # total	Gene#	17–50 loci (6)	NC, Os, Ch
		Map	2 QTLs (5)	
		QGen	h^2 = 0.58 (7); 0.51 (*8*)	
	Gill raker number upper	QGen	h^2 = 0.02–0.33 (3)	NC, Os, Ch
	Gill raker number lower	QGen	h^2 = 0.34–0.42 (3)	NC, Os, Ch
	Jaw size/shape		–	Many
	Lateral plate morph	Map	Single Mendelian or major QTL and polygenic modifiers (8–16)	NC, Os
	Lateral plate number (in low morph)	Gene#	4–6 genes (6)	NC, Os
		Map	2 QTLs (*5*)	
		QGen	h^2 = 0.34–0.49 (3); 0.5–0.84 (7); 0.88 (8)	
	Muscle size/shape		–	Many
	Opercle size/shape	Gene#	3–5 genes (17)	NC Os, Ch
		Map	1 major QTL (17)	
	Pelvic spine length	Gene#	1–2 genes (6);	FO
		QGen	1 QTL (5, 18)	
	Pelvic morph	Map	Single Mendelian or major QTL and polygenic modifiers (10,18)	FO, FP, NC Os, Ch
	Pelvic lateral fork number	QGen	H^2 = –0.02 to 0.32(*3*)	Os, Ch
	Tooth number/size/shape		–	Many
Life History	Clutch size		–	Many
	Egg size		–	Many
	Anadromy		–	Many
	Life span		–	Many
	Age at reproduction		–	Many
	Adult size		–	Many

Table 1 continued

Type trait	Specific trait	Genetic analysis	Candidate developmental process
Physiology	Halotolerance	–	Many
	Thermotolerance	–	Many
	Swimming performance	–	Many

Gene # is an estimate of number of contributing loci, *Map* refers to results from standard experimental or QTL genetic mapping study, and *QGen* indicates heritability estimates from a quantitative genetic study. References are numbered in parentheses after each estimate and listed at the bottom of the table. Full references can be obtained from literature cited. This table is incomplete, and numerous additional traits vary within and among stickleback populations. *NC*: neural crest development, *Os*: osteogenesis, *Ch*: chondrogenesis, *FO*: fin outgrowth, *FP*: fin positioning

1. Bakker (1993), 2. Hagen and Moodie (1979), 3. Hermida et al. (2002), 4. Baumgartner (1994), 5. Peichel et al. (2001), 6. Hatfield (1997), 7. Hagen (1973b), 8. Aguirre et al. (2004), 9. Ziugnov (1983), 10. Cresko et al. (2004), 11. Avise (1976), 12. Colosimo et al. (2004), 13. Ikeda (1934), 14. Hagen and Gilbertson (1973b), 15. Munzig (1959), 16. Colosimo et al. (2005), 17. Kimmel et al. (2005), and 18. Shapiro et al. (2004)

related to parasite defense and physiological adaptation (Bakker and Mundwiler 1999; Barber et al. 2000, 2001; Overli et al. 2001; Heins et al. 2002; Barber and Svensson 2003; Heins and Baker 2003). The hundreds of thousands of freshwater threespine stickleback populations throughout the northern hemisphere, and the phenotypic diversity among them, ensure that the domain of stickleback micro-evo-devo studies is large (Table 1).

Evolutionary genetic analyses of threespine stickleback diversification

An important factor that distinguishes micro- from macro-evo-devo studies is extensive intra- and inter-population genetic variation, shifting the focus from analysis and interpretation of gene sequence and functional conservation to analysis and interpretation of the functional effects of nucleotide polymorphisms (Stern 2000; Wray 2003; Phillips 2005). A major goal of micro-evo-devo is therefore to identify the nucleotide changes that form the quantitative genetic basis of phenotypic evolution (Stern 2000; Baguna and Garcia-Fernandez 2003; Gilbert 2003). Micro-evo-devo research involves not only the identification of loci and nucleotide variation that is causally associated with phenotypes, but also determining how environmental and population genetic conditions affect the nature of these associations.

The majority of genetic polymorphism in natural populations of stickleback is likely irrelevant to phenotypic divergence, making the hunt for causative nucleotide variation very difficult (Stern 2000). In addition to developmental genetic studies using experimental crosses, identifying the genetic basis of adaptation requires characterization of loci within the statistical frameworks of population and quantitative genetics. Population genetics provides models to help distinguish loci that have been under selection as compared to those evolving by random drift (Luikart et al. 2003), thus providing one link of genetic to phenotypic evolution. Central to quantitative genetics is the matrix of additive genetic variances and covariances of a suite of traits, termed *G*. As a statistical summary of complex genetic interactions it is necessarily incomplete (Schlichting and Pigliucci 1998), but this matrix is still a conceptual nexus that summarizes information about the molecular genetic basis of phenotypes, allowing predictions of phenotypic responses to evolutionary processes (Arnold et al. 2001; Jones et al. 2003, 2004; Phillips and McGuigan 2006).

Population genetic analyses of threespine stickleback

Existing population and quantitative genetic studies of threespine stickleback point to the difficulty and promise for identifying segregating genetic variants that underlay evolving phenotypes, and their findings are important for use of stickleback as a micro-evo-devo model system. Analyses of allozyme, mtDNA sequences, RFLP haplotypes and microsatellite alleles have revealed abundant genetic variation within and among stickleback populations (Avise 1976; Withler and McPhail 1985; Haglund et al. 1990, 1992; O'Reilly et al. 1993; Orti et al. 1994; Deagle et al. 1996; Higuchi and Goto 1996; Thompson et al. 1997; Taylor 1998; Taylor and McPhail 1999, 2000; Hendry et al. 2002; Reusch et al. 2001; Yamada et al. 2001; Watanabe et al. 2003; McKinnon et al. 2004; Raeymaekers et al. 2005). Although post-glacial freshwater threespine stickleback populations comprise only subsets of the genetic variation present in the oceanic fish, they are still highly polymorphic, and the distribution of alleles in freshwater populations supports their recent derivation from local oceanic threespine stickleback (Taylor and McPhail 1999, 2000; Reusch et al. 2001; Raeymaekers et al. 2005). Some of the genetic variation is partitioned among phenotypically or ecologically distinct freshwater populations (Reusch et al. 2001; Hendry et al. 2002; Watanabe et al. 2003), but the distribution of alleles supports the inference that the demographic processes (Fig. 2; founding events, genetic drift and selection) occurred independently in populations and species with similar phenotypes (Thompson et al. 1997; Taylor and McPhail 1999, 2000; Hendry et al. 2002, 2002; Watanabe et al. 2003; McKinnon et al. 2004).

Quantitative genetic analyses of threespine stickleback phenotypic variation

Phenotypic evolution, through neutral or adaptive processes, results from changes in allele frequencies at loci that contribute to the phenotype. The univariate heritability (h^2) is the proportion of phenotypic variance due to additive genetic variance. Univariate response to selection can be represented as the effect of selection filtered through the proportion of phenotypic variation with a genetic basis, commonly known as the breeder's equation ($R = h^2S$; Falconer and Mackay 1996; Lynch and Walsh 1998). In threespine stickleback, univariate heritabilities have been estimated (Table 1) for several morphological (Lindsey 1962a, b; Hagen 1973; Hagen and Gilbertson 1973b;

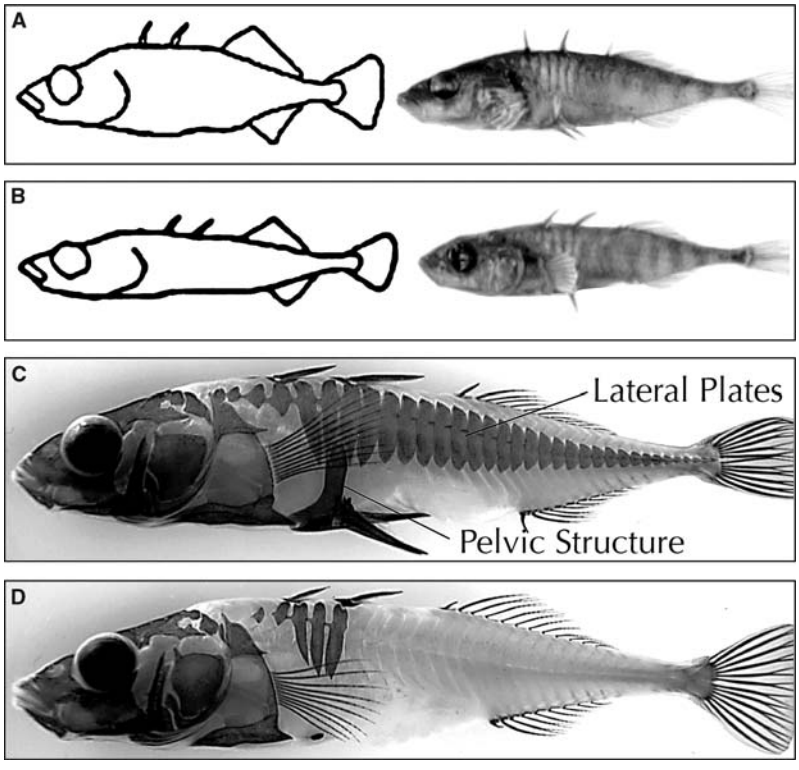

Fig. 1 Diagram and images of benthic (A) and limnetic (B) stickleback morphs, and the presence (C) and absence (D) of stickleback lateral plate and pelvic structure armor. Benthic and limnetic diagrams are modeled after Walker and Bell (2000). Images are of benthic and limnetic morphs in Benka Lake, Alaska (A, B), or are of fish from a genetic mapping cross between Alaskan freshwater and oceanic stickleback (C, D)

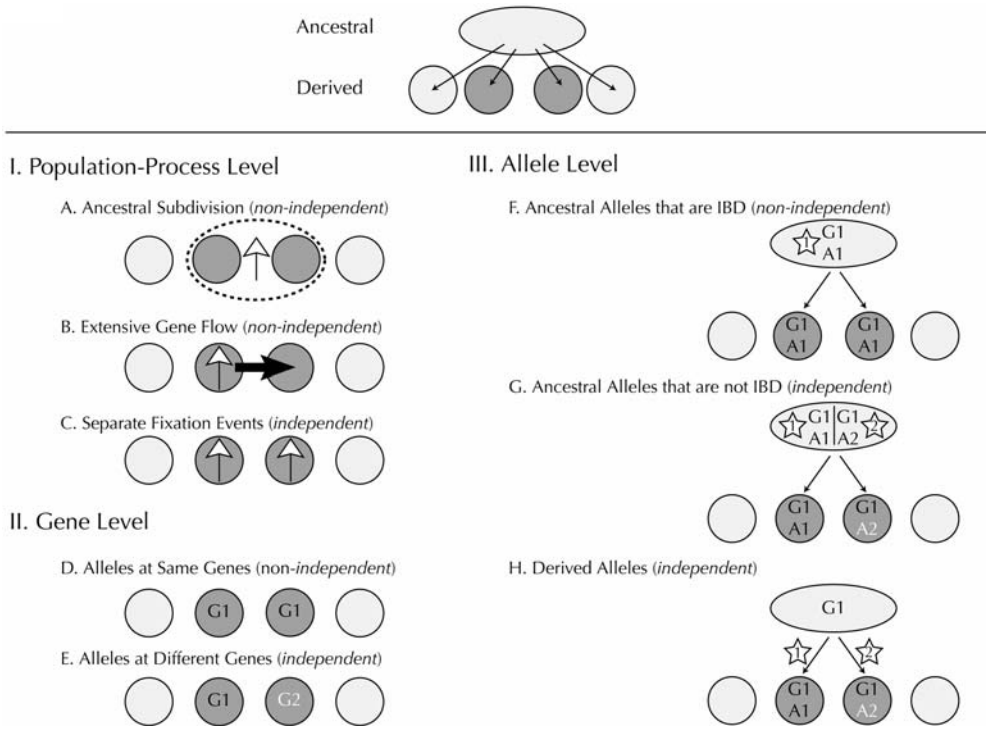

Fig. 2 Hierarchical levels of independent evolution at the population, gene, and allelic levels. Darkly shaded circles represent the parallel evolution of a similar phenotypic state. Section 1 represents population-level processes (drift, selection, and gene flow) as they affect the parallel evolution of similar phenotypes in two populations. Open arrows represent increases in frequency of a phenotype (increasing darkness of shading) either through selection or drift. The closed horizontal arrow represents high levels of gene flow from one population to another, causing the similarity of phenotypes, even if maladaptive in the population receiving the migrants. The dashed line in IA indicates the boundary of the original population where fixation occurred only once before it was divided into two populations. Only IC shows population-level independence, or parallel phenotypic evolution, which provides support for the action of natural selection when it is correlated with a common environmental condition. Section ''Phenotypic variation in natural threespine stickleback populations'' represents independence at the level of the gene. Both IID and IIE show independent population-level processes of fixation (IC), but only IIE indicates additional independence at the genetic level. In this case, two different genes (G1 and G2) have fixed alleles that lead to a common phenotype. Section ''Evolutionary genetic analyses of threespine stickleback diversification'' represents independent evolution at the allele level when independent evolution has occurred at the population level (IC) that is non-independent at the gene level (IID). Ancestral alleles that are Identical by Descent (IBD) due to a single mutational event (star) may be ancestral and used in replicate in the derived populations (IIIF). Additionally, ancestral alleles with equivalent phenotypes could be produced by two mutational events at the same locus that are present in the ancestor and driven to fixation in the different populations (IIIG). Lastly, the mutational events could occur independently after colonization of freshwater (IIIH). Both IIIG and IIIH show independent evolution at the allele level even though non-independence at the genetic level may have occurred. Thus, all three levels must be taken into account when addressing questions of parallel evolution in stickleback. In particular, discovering how often IID occurs in combination with IIIG or IIIH will provide insight into genetic biases that might exist in the evolvability of developmental genetic pathways that underlie rapid phenotypic evolution. Of particular importance is deciding whether the occurrence of common developmental genetic biases changes our inference of the importance of natural selection even if we find that the population-level processes are independent (i.e. independence at population and allelic levels, but not at the genetic level)

Schluter 1996b) and physiological traits (Garenc et al. 1998), and all have demonstrated heritable variation. In a particularly thorough study, Hermida et al. (2002) calculated heritabilities for eight meristic traits using several approaches in the wild and lab.

However, exclusive focus on single trait heritability can hamper attempts to understand phenotypic evolution because of the importance of genetic covariance among traits that result from linkage and pleiotropy (Falconer and Mackay 1996; Phillips and McGuigan 2005). The direction and rate of multivariate evolution (Δz—the vector of change in mean trait values) depends on both the vector of selection gradients (β) and on G, which is the multivariate matrix of additive genetic variances and covariances, which summarize the total allelic contribution to phenotype.

($\Delta z = G\beta$; Lande 1979; Lynch and Walsh 1998; Steppan et al. 2002). Because of genetic correlations, unselected traits can evolve if they share a genetic basis with selected traits. Similarly, traits under directional selection might not respond due to antagonistic selection on a correlated trait (Lande 1979).

The overall impression from the stickleback literature is that sufficient univariate additive genetic variance exists for a response to directional selection (Schluter et al. 2004). However, little to no genetic (co)variance might exist for particular multivariate *trait combinations* (Blows 2004), limiting the multivariate response to selection. Therefore, determining the structure of genetic covariances in ancestral and derived threespine stickleback populations is essential to understanding the replicate phenotypic evolution of freshwater populations. The only multivariate estimate of additive genetic (co)variance in threespine stickleback was by Schluter (1996b), who observed negative and positive genetic covariation among several meristic and morphological traits. He demonstrated that the major axis of evolution among 10 threespine stickleback populations was in the direction of maximum genetic variance, which corresponded to the distribution of phenotypes along the benthic–limnetic axis. Schluter's result suggests that less genetic variation exists for other combinations of benthic and limnetic characters. The observation that these traits both co-vary through evolutionary time, and are genetically correlated within contemporary populations, implies that they share a genetic basis through pleiotropic gene action that could bias evolution (Falconer and Mackay 1996; Lynch and Walsh 1998).

Schluter's results point to the useful role of G as an organizational tool for micro-evo-devo studies of the genetic basis of evolving traits. These results are from a single experiment using one population under constant environmental conditions, making extension of inferences to other populations difficult (Falconer and Mackay 1996; Lynch and Walsh 1998). The phenotypic effect of each allele at contributing loci depends both on the environmental (Garcia-Dorado and Marin 1998; Weining et al. 2003a, b, Weining and Schmitt 2004) and genetic contexts (Agrawal 2001; Lenski et al. 2003; Wilke 2003), thus affecting both univariate heritabilities (h^2), and multivariate genetic correlations (G). Particularly for organisms evolving after invasion of new habitats, such as freshwater stickleback, inferring the response to selection necessitates studies in multiple populations of the direct effect of the environment on the structure of genetic (co)variances of traits.

Direct role of environment for rapid evolution

In addition to studies of how standing genetic variation affects trait evolution, the direct role of the environment must be incorporated into our understanding of rapid post-glacial evolution in stickleback. Many nucleotide substitutions will have no detectable effect on the phenotype in one particular environmental condition, either because they are true neutral mutations, resulting in functionally equivalent alleles, or because variation arising from the mutation is suppressed epistatically through the action of alleles at other loci. Altering environmental conditions during development, such as invasion of novel habitats, can change the functional neutrality or epistatic interactions of alleles, both of which may expose genetic variation for phenotypes (Gibson and Dworkin 2004).

Phenotypic plasticity, defined here in general terms as the consistent production of a distribution of phenotypes by the same genotype in different environmental contexts, has a genetic basis and may have an important role in rapid adaptive evolution (Brakefield et al. 1996; Via and Conner 1995; Robinson and Parsons 2002; Price et al. 2003; West-Eberhard 2003; Weining and Schmitt 2004). This consistent relationship between environmental conditions and phenotypic variation can be described as a *developmental reaction norm* (DRN; Schlichting and Pigliucci 1998; West-Eberhard 2003). DRNs are traits that have evolved in response to environmental conditions in order to differentially suppress and expose the phenotypic effects of genetic variation. Because DRNs have a genetic basis themselves, populations can harbor variation in DRNs that can change in frequency through drift or selection. Still unclear is the evolutionary relationship between genetic variation for a particular character, and variation for developmental systems that differentially suppress or expose character variation.

Under novel environmental conditions, extremes of DRNs can be exposed to selection. The hypothesis that the phenotypic effects can be suppressed (*canalization*), and that this suppression could be released and the exposed variation genetically fixed within the population (*genetic assimilation*), was first explored in the middle part of the 20th century (Waddington 1942, 1952, 1953; Schlichting and Pigliucci 1998). The importance of canalization and assimilation for rapid evolution from the standing genetic variation is presently being reappraised (West-Eberhard 2003). Under constant environmental conditions, larger populations should harbor more phenotypically neutral genetic variation than smaller populations. Both the lack of biogeographic boundaries, and molecular population

genetic data, indicate that oceanic stickleback populations might contain large amounts of variation that is phenotypically neutral in oceanic environments, and the change in developmental environment from ocean to freshwater might alter the distribution of genetic variation in threespine stickleback traits available to selection.

A recent review of fish in post-glacial lakes in North America suggested phenotypic plasticity might play an important role during initial divergence in the new environments (Robinson and Parsons 2002). The authors found that patterns of plastic relationships among traits generally matched, in kind but not necessarily in magnitude, patterns of multivariate evolutionary response of fish populations and species. Although receiving scant attention in stickleback, when phenotypic plasticity has been directly examined it has been found. Both salinity and temperature (Heuts 1947; Lindsey 1962a,b) were found to have a direct (although mild) effect on the number of lateral plates. More recently, Day et al. (1994) recorded a plastic response to diet for several body shape and trophic characters when studying a benthic–limnetic species pair (Day et al. 1994), and environmental influences on life history characters of a stream population of stickleback have been described (Baker and Foster 2002). Notably, the variation in environmentally produced stickleback phenotypes is similar to that produced by segregating alleles (Day et al. 1994; Schluter 1996b). Still unknown, however, is the general importance of direct environmental effects during the initial stages of rapid evolution in threespine stickleback (see Bell et al. 2004), and should be a prominent focus of stickleback research. Part of the promise of this work is unraveling the molecular genetic basis of phenotypic plasticity by determining the relationship between genetic variation for characters, and variation in developmental systems that differentially expose this character variation (West-Eberhard 2003).

Developmental genetic analyses of threespine stickleback traits

What is the molecular genetic basis of evolving traits in stickleback? Just a decade ago performing the research necessary to address this question would have been impossible. With the development of model fish species such as zebrafish, medaka and pufferfish (Furutani-Seiki and Wittbrodt 2004), and the decreasing cost of performing molecular genetic studies, this work has begun in earnest in stickleback (Foster and Baker 2004). Initial studies have involved examination of candidate gene expression patterns, construction of cDNA and genomic libraries, development of a large number of genetic markers, production of the first stickleback genetic linkage map (Peichel et al. 2001), initiation of a stickleback genome project, and formation of mapping crosses to identify the genetic basis of trophic morphology, armor variation, and sex determination (for review see Kingsley et al. 2004). In addition, techniques have been developed to reduce gene function using morpholinos, and to make transgenic stickleback to test gene function and perform cell lineage studies (Hosemann et al. 2004). Below we discuss each of these approaches and results, but first provide a brief description of some physiological and developmental work that the recent molecular work uses as a base. Although each type of research is presented individually, we would like to emphasize that integrated work at all levels is necessary to fully address the evolutionary developmental genetic basis of evolving stickleback traits (Fig. 3).

Studies of threespine stickleback physiology and development

Although small compared to the extent of work on behavior, ecology and evolution, research on stickleback physiology and development is fairly broad and has focused primarily on morphological, histological and hormonal aspects of the phenotype. A normal development table for threespine stickleback was developed by Swarup (1958), who made use of the fact that, like zebrafish and medaka (Kimmel et al. 1995; Furutani-Seiki and Wittbrodt. 2004), the chorions of developing stickleback are optically clear, allowing morphogenetic processes to be observed. However, significant differences exist among populations and environments (Wright et al. 2004), and standard growth tables need to be made for reference stickleback populations (oceanic and freshwater) using modern rearing conditions and microscopy techniques. Four areas of focus have predominated in work on threespine stickleback development: the formation of lateral plates (Igarashi 1964, 1970), asymmetric development of the pelvic structure (Bell 1985; Bergstrom and Reimchen 2000, 2002, 2003), effects of hormones on sex differentiation and the development of secondary sexual characteristics (Borg et al. 1982, 1987, 1992, 2004; Andersson et al. 1992; Borg and Mayer 1995; Mayer et al. 1997, 2004; Bornestaf and Borg 2000; Hellqvist et al. 2001; Katsiadaki et al 2002; Olsson et al. 2003), and the development of stickleback visual and olfactory sensory systems (Ekstreöm et al. 1983, 1985, 1992; Hokanen and Ekstreöm 1992; van Veen 1980, 1984).

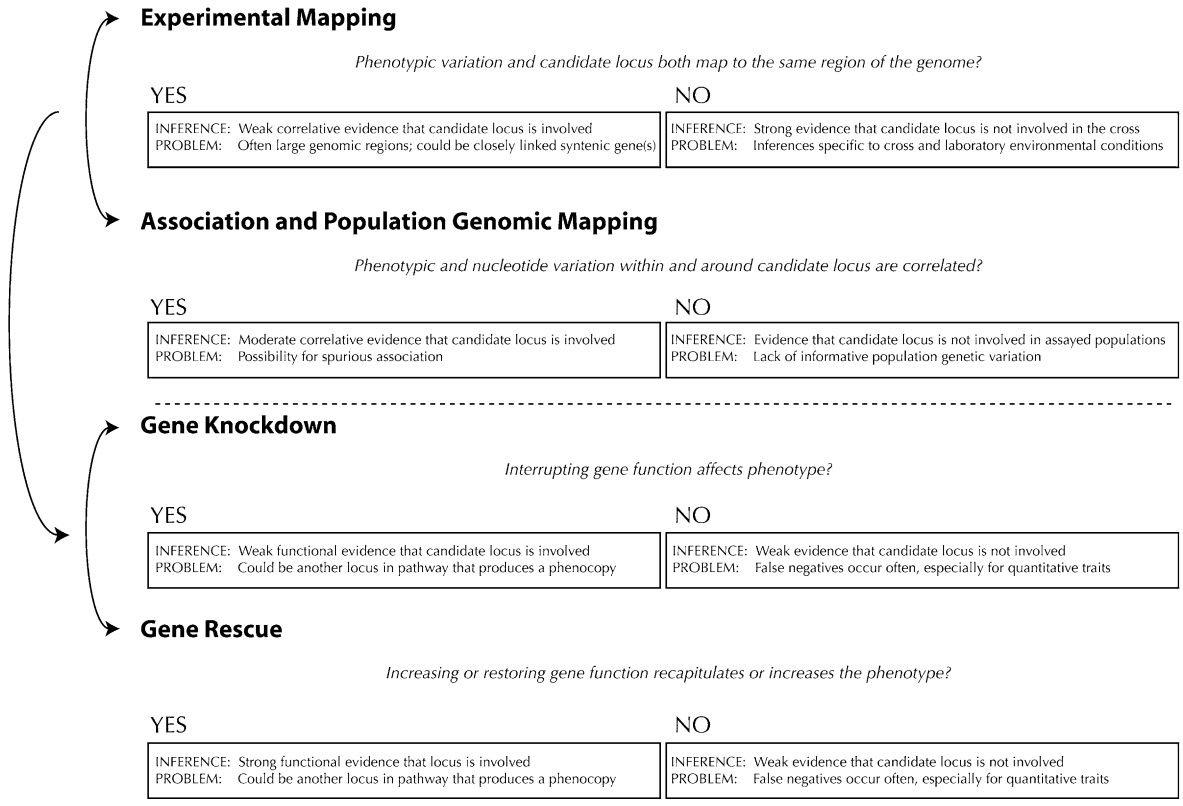

Fig. 3 Diagram illustrating decision chart for determining causation regarding the genetic basis of evolving traits. This diagram presents questions a researcher might address with a technique, with possible inferences to be drawn from answers, including potential problems with those inferences. Experimental and association or population genomic mapping (above dashed line) are correlative approaches to reducing the range of candidate genetic factors to a smaller region of the genome. Both approaches can be used as the initiation point for localizing a gene, and data from one can be used to inform the other (bi-directional arrows). Once mapping has reduced the complexity of the problem, functional studies through knockdown and/or rescue of gene function can be used to test candidates in a region of the genome. If strong evidence exists from other organisms that points to a candidate gene, testing of this locus through genetic manipulation can proceed in the absence of mapping data. All of these techniques have their benefits and deficits, and are best used together as an integrated toolkit for uncovering the genetic basis of evolving traits

Studies of gene expression

Patterns of gene expression can be examined by in situ hybridization using RNA probes made from a single gene. This gene is often a *candidate* for phenotypic variation, as indicated by previous work on the focal organism, or studies of homologous developmental processes in other species. Frequently the candidate gene will be a *transcription factor* that regulates other genes, often in complicated ways (Davidson 2001). Empirically understanding the logic of regulation is extremely difficult even in model organisms (Davidson et al. 2002). However, significant progress has been made already in examining expression patterns of candidate genes in stickleback by adopting and modifying in situ techniques that were developed for zebrafish (Jowett and Yan 1996). The first threespine stickleback *in situ* studies examined expression of *Hox* (*homeobox-containing*) *genes* during early embryogenesis (Ahn and Gibson 1999a, b, c). *Hox* genes are fundamental developmental regulators in all animals (Carroll et al. 2001), and the work of Ahn and Gibson was an attempt to correlate threespine stickleback axial variation (e.g. number of vertebrae) with expression of *Hox* genes. The results of these studies were somewhat equivocal, which can partially be explained by the fact that stickleback, like other teleosts, have approximately twice the number of *Hox* genes as tetrapods (Amores et al. in preparation; Tanaka et al. 2005), a fact not known for stickleback at the time of Ahn and Gibson's work.

The only three subsequent studies of gene expression have concentrated on transcription factor genes involved in the formation of bone and cartilage (*sox9a* and *sox9b*; Cresko et al. 2003), and development of the pelvic structure (Cole et al. 2003; Shapiro et al. 2004).

The former paper provided evidence that stickleback share a whole-genome duplication with most teleosts, and indicated that functions of genes may be partitioned independently in different fish lineages. The Cole et al. (2003) paper provided an important advance in our understanding the development and evolution of the threespine stickleback pelvic armor by examining genes known to function in the formation of vertebrate limbs, specifically *Pitx1*, *Pitx2*, *Tbx4* and *Tbx5*. The pelvic fins of fishes are highly evolutionarily labile, and the ventral spines of threespine stickleback have long been thought to be highly derived pelvic fins. Cole et al. (2003) demonstrated that these genes are in fact expressed during development of the pelvic structure of threespine stickleback, and confirmed this inference of homology. Furthermore, these authors showed that spineless threespine stickleback in a Scottish population have changes in expression patterns of these genes that are consistent with changes in regulation of *Pitx1* or some upstream factor that regulates *Pitx1*. They also proposed that a previously observed left pelvic asymmetry in threespine stickleback (Bell 1985; Bell et al. 1993; Bergstrom and Reimchen 2000, 2002, 2003) may be due to changes in *Pitx1* expression uncovering the effects of the asymmetrically expressed *Pitx2* gene, with which *Pitx1* is functionally redundant. Shapiro et al. (2004) subsequently confirmed the Cole et al. (2003) in situ results in a genetic mapping study that used fish from Japan and British Columbia, and their data pointed to *Pitx1* as segregating the causative variation (see below).

A significant weakness of studying expression of one or a few genes is that these candidates are only a small subset of all genes potentially segregating variation in the wild. The difficulties become more acute when the trait of interest has no obvious homologous developmental pathway from which to draw candidates. Assessing global changes in gene expression, through the use of *microarray technologies* (Gibson 2002; Oleksiak et al. 2002), can potentially mitigate each of these problems. For example, RNA from the developing structure in divergent stickleback populations can be competitively hybridized to a cDNA microarray, potentially revealing a large number of candidate genes and pathways, even if these have not yet been studied in developmental model organisms (Gibson 2002). Furthermore, the effects of varying environmental conditions on gene expression patterns can be ascertained using microarray techniques. No microarray studies have been performed to date on stickleback, but initial genomic microarrays have been developed at the University of Oregon and additional work is underway.

All analyses of gene expression, whether individual or global, can be ambiguous with respect to the genetic basis of phenotypic variation. Differences in expression of many genes may have nothing to do directly with the genetic basis of trait variation, but may simply be correlated or coincidental. Additionally, failure to detect differences in expression does not rule out the importance of a gene, as changes might be so subtle as to escape detection, and/or the stage at which to look for critical changes in gene expression may be unknown. Studying variation in gene expression, either focused or globally, is a tool best used with genetic mapping approaches. Strong inferences about causative genetic variants can be drawn when both types of techniques, used in populations that have independently evolved the same phenotypes, point to the same genes (Fig. 1).

Genetic mapping using experimental crosses

Experimental crosses can be used to associate regions of a genome with heritable phenotypes. This approach requires the ability to cross and rear organisms through several generations, a possibility in threespine stickleback. Experimental mapping also necessitates the development of molecular markers spread throughout the genome that can be associated with either the presence/absence of Mendelian traits (*standard genetic mapping*), or a statistical association of a region of the genome with a proportion of the variance of a quantitative trait (*QTL mapping*). There are two major benefits of experimental crosses over other approaches (see below) for initial identification of a region of a genome that contains a causative genetic variant. First, because only two individuals are generally chosen as the parents for a cross, the genetic complexity of the population is reduced to the alleles carried by these individuals. Secondly, because the extent of linkage disequilibrium is constrained by the number of meioses in a cross, which is usually on the order of hundreds or thousands, the requisite number of markers needed to find an association is reduced because only one is needed for each non-recombining block of the genome (Phillips 2005).

Stickleback linkage map and benthic–limnetic QTLs

A significant advance for the mapping of phenotypes in experimental stickleback crosses was the formation of the first stickleback genetic map by Peichel et al. (2001). The stickleback genome is approximately 750 million base pairs (750 mBases) over *21 chromosomes*. The published map coalesced to *26 linkage*

groups (LGs) and covered a sex-averaged genetic distance of approximately *1000 centiMorgans* (cM), with every one cM of genetic distance equal to approximately 400 kBases of physical distance (Peichel et al. 2001). The map was constructed by following the segregation of a large number of microsatellite loci (Rico et al. 1993, Taylor 1998, Largiader et al. 1999; Peichel et al 2001) in a cross between benthic and limnetic threespine stickleback (Fig. 1) from Priest Lake, British Columbia. Body shape, trophic characters, length of pelvic spines and number of anterior lateral plates segregated in this cross in a quantitative manner, and for several of these traits QTLs were localized. Because the genetic markers are non-anonymous, the linkage map provides an excellent opportunity to compare mapping results of studies using different threespine stickleback populations.

Stickleback sex determination

Previous work has shown that threespine stickleback have a *genetic sex determination system* that is linked to the Idh locus (Avise 1976; Withler and McPhail 1985, Withler et al. 1986). These findings allowed the development of a sex-specific DNA marker (Griffiths et al. 2000). Peichel et al. (2001) mapped the sex determination locus to the distal end of LG XIX. They subsequently found that, although threespine stickleback chromosomes are not cytologically heteromorphic, this region of the genome is apparently a proto-sex chromosome with reduced recombination and extensive divergence between the presumptive 'X' and 'Y' chromosomes (Peichel et al. 2004). Comparisons across divergent *G. aculeatus* populations, and with the sister species, *G. wheatlandi* (which, unlike *G. aculeatus* has heteromorphic sex chromosomes), indicates that the threespine master sex determination switch evolved between 2 and 10 million years ago (Peichel et al. 2004). The molecular basis of this sex determination gene is not yet known.

Lateral plate armor reduction

Other experimental mapping work has examined in more detail variation in the extent of lateral plate and pelvic armor formation between ancestral and descendant threespine stickleback populations. Crosses between complete and low-plated populations (Fig. 1) revealed that a single major QTL or Mendelian factor governs the number of all but the most anterior lateral plates, and that the completely plated phenotype is specified by a dominant allele (Colosimo et al. 2004; Cresko et al. 2004). *Genetic complementation* of major loci did not occur in crosses among three low-plated Alaskan populations (Cresko et al. 2004), indicating alleles at the same major gene, or between low- armor British Columbia and California populations (Colosimo et al. 2004). Furthermore, analysis showed that the major lateral plate loci mapped to the same region of the genome (LG IV) in the three Alaskan populations, and the populations in British Columbia and California (Colosimo et al. 2004; Cresko et al 2004). Although the Alaskan major lateral plate locus was originally reported to map to LG XVIII (Cresko et al. 2004), it was subsequently discovered that this LG XVIII actually coalesces into LG IV near the region where the British Columbia major lateral plate QTL mapped (Colosimo et al. 2004).

The significance of this similarity in mapping position between the two studies became clear in subsequent work by Colosimo et al. (2005). These authors performed fine scale mapping that restricted the plate locus in their crosses between Japanese and British Columbia fish to a region covering less than 1 million base pairs. In this region are several candidate loci, one of which showed a pattern of linkage disequilibrium in a phenotypically polymorphic population in California (Friant Lake; Avise 1976) indicating that the gene *Ectodysplasin* (*Eda*) is segregating alleles that are associated with reduced numbers of lateral plates. The authors were unable to detect expression of *Eda* when and where plates are developing, and further tested the hypothesis of *Eda* being causative by injecting a genetic construct that expressed the mouse *Eda* transcript into low-plated stickleback. The authors found that 3 of 14 transgene-positive individuals produced a small number of ectopic plates. Changes in the coding region of the gene do not appear responsible for armor loss, and the authors therefore concluded that changes in regulation of *Eda* must be responsible.

Global genealogical analysis of nucleotide variation around the *Eda* locus showed that this region of the genome forms two monophyletic clades, with low morph populations coalescing in one, and complete morph populations in the other. This pattern leads to the inference that most of the repeated reduction in lateral plates is due to repeated fixation of only one allele from the standing genetic variation. Finding the low form of the allele in detectable frequencies in oceanic populations further supported this inference and a prediction by Lindsey (1962a) that rapid evolution of stickleback traits is largely due to fixation of standing genetic variation in ocean populations. Interestingly, a lateral-plate reduced population from Japan did not fit this genealogical pattern, despite

previous complementation results showing that the same locus was responsible (Colosimo et al. 2004). These data can be interpreted as indicating a separate origin for the low-armor *Eda* allele in Japan. However, because of the outbred nature of the stickleback populations being crossed, and variation in the dominance relationships of the genes underlying lateral plate variation, the possibility of *non-allelic non-complementation* (see Mackay 2001) leads to the alternative hypothesis of an additional locus responsible for the low lateral plate morph. This hypothesis needs to be tested through independent mapping analyses on this Japanese population. Taken together, however, results from several recent studies (Colosimo et al. 2004, 2005; Cresko et al. 2004; Schluter et al. 2004) lead to the general conclusion that much of the parallel evolution in lateral plate reduction that has occurred at the phenotypic level around the world is due the repeated fixation of the same *Eda* allele produced by a single mutational event that occurred millions of years ago (Fig 2). The Colosimo et al. (2005) data also support a *metapopulation* hypothesis, proposed by Klepaker (1993, 1995, 1996), for the long-term maintenance of this allele through repeated gene flow from low-armor populations back into oceanic populations through intermittent hybridization.

Pelvic armor reduction

A single Mendelian factor (Cresko et al. 2004) or major QTL (Shapiro et al. 2004) was also found to exert substantial influence over pelvic expression, with the full pelvic structure specified by a dominant allele. Similar to the results for lateral plate variation, both mapping and complementation crosses suggest that the same locus may be involved in pelvic reduction in geographically disparate populations in Alaska and British Columbia (Cresko et al. 2004; Shapiro et al. 2004). Both studies reported that the major pelvic-loss locus mapped to the distal end of LG VII, and both found significant contributions to pelvic variation from additional loci. Some of these loci were isolated as minor QTLs (Shapiro et al. 2004), and it was shown that different minor loci contribute to pelvic loss across independently derived freshwater populations (Cresko et al. 2004). Building upon the work of Cole et al. (2003; discussed above), Shapiro et al. (2004) further demonstrated that one of these genes, *Pitx1*, maps to the region where the major locus contributing to pelvic reduction resides. As there were no differences in *Pitx1 protein coding sequence* (*CDS*) between full or pelvic-reduced fish, Shapiro et al. (2004) concluded that *cis-regulatory* changes in *Pitx1* are responsible for the effects of this major locus on pelvic reduction.

Taken together, the lateral plate and pelvic data suggest that the independent population-level processes for armor loss utilize the same genes (Fig. 2), but this conclusion is tentative until causative loci are identified in each population. Similar Mendelian phenotypes in independently derived freshwater threespine stickleback populations could be the result of fixation of the same allele segregating at a low frequency in the ancestral marine population as is indicated by some of the lateral plate data. Alternatively, the marine population might be segregating several alleles at the same locus with equivalent phenotypic effects, with daughter populations fixing alternative alleles. Lastly, new mutations in the same gene could occur independently in each population subsequent to invasion of fresh water (Fig. 2). If the same loci are clearly identified in each stickleback population, determining allelic independence will require genealogical studies from numerous populations. However, if mutational biases exist for the allowable allelic states of a particular gene, independent mutations will produce a genealogical pattern that appears to be due to inheritance of a common ancestral allele. Testing this *mutational bias hypothesis* may require more than observation of pattern in natural populations, necessitating work on such things as mutation accumulation studies in laboratory populations of stickleback.

Stickleback head bone variation

Given the huge number of stickleback traits, populations and contributing loci, experimental mapping in stickleback promises a significant contribution to our knowledge of the genetic basis of vertebrate traits. Bony armor is only one type of the many characters that vary among stickleback populations, and there is little reason to expect that all variable stickleback phenotypes have a predominantly Mendelian genetic basis. Genetic analysis of another dermal bone, the *opercle*, revealed quantitative variation in shape differences between anadromous and freshwater stickleback populations, as well as among freshwater populations (Kimmel et al. 2005). Despite the quantitative nature of the variation, a major QTL that mapped to LG XIX accounted for a significant proportion of the shape variation in this bone. Thus, although other stickleback traits may not have an identifiable Mendelian locus, mapping may still reveal major loci that are open to developmental genetic analysis.

Limits to experimental mapping

Despite the initial success of mapping Mendelian and quantitative traits in threespine stickleback, the major benefits of experimental crosses—reduced genetic complexity and increased linkage disequilibrium—are double-edged swords for the identification of the molecular basis of evolving traits (Fig. 3; Phillips 2005). Stickleback crosses are resource expensive in both time and money, and the relatively few offspring in a stickleback cross (100–300 embryos per clutch) is a limitation for the precision with which experimental laboratory mapping of loci can be done. Pooling across families when each is segregating Mendelian or major QTLs that are likely to have the same effects in all backgrounds and environments can ameliorate this precision problem. However, pooling can be problematic for minor loci. Because stickleback are highly polymorphic, each cross (even half-sib designs) will likely segregate different minor alleles that will have variable effects. At least until inbred stickleback lines are produced, small family sizes will impose a significant constraint on the isolation of minor or moderate QTLs through experimental mapping.

Care should also be taken in extending inferences from a small number of crosses to multiple populations, or the stickleback system a whole. Only a small proportion of the genetic variation segregating in a single natural population, let alone across populations, will be present in any laboratory cross. Furthermore, the identification of contributing genetic variants may depend on the laboratory conditions in which studies are performed (Weining et al. 2003a, b; Weining and Schmitt 2004). Despite the precision and mapping problems, experimental crosses will continue to be crucial in identification of the genetic basis of evolving traits, but like expression studies, are best augmented by additional approaches (Fig. 3).

Genetic mapping using association and population genomics

Association and *population genomic* mapping in natural stickleback populations may provide a means by which to correlate phenotypic variation with smaller regions of the genome than can be achieved in experimental laboratory crosses (Fig. 3; Luikart et al. 2003; Streelman et al. 2003; Phillips 2005; Storz 2005). Both techniques rely on the extent of linkage disequilibrium in natural populations, which should be much smaller than that seen in laboratory crosses due to numerous generations of recombination (Luikart et al. 2003). *Association mapping* examines the pattern of sequence differentiation at candidate loci in natural populations. As such, it is a natural partner for experimental laboratory crosses. A problem is the need to identify all genes in a candidate region, which may involve extensive sequencing if the identified region is large. An alternative approach, and one that is particularly applicable to semi-model species such as threespine stickleback, is to use *conserved syntenic* (genes on the same chromosome) arrangements of genes in a developmental model organism (Postlethwait et al. 2004; Naruse et al. 2004). The homologous genomic region for a QTL in stickleback can be identified in a fully sequenced developmental model such pufferfish. Each gene in the pufferfish region becomes a candidate, and can be cloned, sequenced and mapped individually in stickleback without the need to sequence non-genic regions, which are the majority of a vertebrate genome. Unfortunately, this approach will miss genes that are novel or that do not share a syntenic arrangement. Ultimately, the complete sequence of the region, or better yet the entire genome sequence, might be necessary for successful association mapping. Fortunately, sequencing of the stickleback genome has begun (Broad Institute, Massachusetts, USA), a very exciting development for stickleback biologists and all researchers who use fish to study the genetic basis of adaptation.

With the increasing efficiency of genetic marker development and screening, using *population genomic* approaches to identify the genetic 'signatures of selection' of evolving traits has become feasible. Population genomics is the statistical detection of loci that have had a different history with respect to the rest of the genome, and uses population genetic statistics such as F_{st} *values* to identify different pattern of genetic variation between morphs in a population, or among populations (see Storz 2005 for review). Most of the distribution of genetic variation will be due to processes such as drift and population structure that affect the entire genome roughly equally. In contrast, locus-specific patterns will occur due to processes, such as selection, that only affect a subset of the genome. These statistical *outlier genomic regions* can indicate processes such as diversifying or stabilizing selection (Luikart et al. 2003). Identification of the same outliers in populations that have independently evolved similar phenotypes adds further confidence to the inference that these loci contribute to phenotypic variation that is under selection. This approach has not been applied to stickleback, but the phylogenetic relationship of freshwater to oceanic stickleback makes population genomics very promising. A significant problem is the need for numerous, densely spaced markers spread

throughout the genome. Although it is presently difficult to perform such a study using only specific markers (such as *SSRs* or *SNPs*), the use of anonymous markers, such as Amplified Fragment Length Polymorphisms (AFLPs), should allow this approach to be successful, as has been shown for other fish (i.e. whitefish; Bernatchez et al. 1999; Campbell and Bernatchez 2004).

Confirming causation of genes

Experimental, association and population genomic mapping techniques are a necessary first step in identifying the genetic basis of evolving traits, but they each only provide associations. Further testing through manipulative studies is required to show a specific pattern of nucleotides causes the observed change (Fig. 3; Phillips 2005). These approaches are the same as for confirming the genetic basis of a mutant phenotype in laboratory model organisms: *allelic introgression, genetic interference* and *phenotype rescue* (Fig. 3). Introgression involves the incorporation of the smallest region of the genome of an organism with a divergent phenotype of interest into a different 'genetic background' that will still produce the phenotype. Ideally, but not absolutely necessary, the genetic background of the alternative population would be homozygous through inbreeding. For example, repeated back crosses of a complete lateral plate stickleback to a low-plate fish, with selection of offspring that show the high armor phenotype in each generation, will eventually produce fish that are 'low plate' everywhere in the genome except for the causative lateral plate region. Of course, these crosses would require a significant expenditure of time and money and have not been completed yet in stickleback.

A second approach is to ablate the effects of candidate loci through direct injection of molecular constructs into the developing embryo to reduce gene function. In fish, normal gene function can be interrupted by use of *morpholino* antisense nucleotides that bind to messages and do not allow for proper maturation of the *RNA transcript*, or blocks *translation into a protein* (Draper et al. 2001). Interruption in oceanic complete-armor stickleback of a candidate gene that is actually segregating the causative variation would then cause a phenotypic change similar to the evolved trait (*phenocopy*). Use of morpholinos in stickleback is in its infancy, but we have shown their usefulness in disrupting the proper formation of the hindbrain by interrupting expression of threespine stickleback *krox20* genes. Identifying specific versus non-specific effects of morpholino can be difficult, however, and interrupting a gene in the same pathway as the causative one would also lead to a phenocopy. A last, and very significant limitation of morpholino techniques is that their effects are confined to the earliest stages of development, up until approximately 4 days post-fertilization.

The gold standard of genetic causation in developmental genetics is the injection of the candidate region of a genome that produces or 'rescues' the phenotype. In threespine stickleback, a large BAC clone (approximately 200 kBases) that has been correlated with the phenotypic variation through mapping and expression could be injected into a fish that does not have a trait. The presence of the structure, or *rescue of the phenotype*, would confirm the BAC clone contains the causative genetic variant. This approach requires the ability to inject and incorporate the construct into the genome of the organism so that it can be functional. This procedure is quite difficult but significant progress has been made in this area in threespine stickleback already (Hosemann et al. 2004), and has been used to support the role of *Eda* in lateral plate variation (discussed previously; Colosimo et al. 2005). Rescue works best with Mendelian traits, where one needs only to assay for the presence of a trait. However, the principal holds for dominant QTLs, but is practically much more difficult because it requires ascertaining a change in the distribution of the trait between injected and control individuals. This problem becomes more acute as less of the variance is explained by a QTL of interest, or when more complex patterns of dominance or epistasis occur. Establishing causation of nucleotide changes in QTLs is a major technical and conceptual challenge for the next few decades (Stern 2000, Gibson and Mackay 2002), especially micro-evo-devo studies of natural populations. The solution to this problem, at the moment, is not at all clear (Gibson and Mackay 2002; Palsson and Gibson 2004).

Extending molecular genetic inferences to evolution in natural populations

Identifying the developmental genetic basis of evolving stickleback traits requires a plethora of molecular developmental tools and approaches. However, regardless of the difficulty and detail, these studies are only the first step in identifying the molecular history of the evolutionary process. Even seemingly obvious answers, such as the large size of the evolutionary step in armor loss, should be treated as tentative. The unit for the size of evolutionary steps is the fixation of mutations, and an allele of large effect may actually be the product of numerous mutations (Phillips 1999,

2005). The millions of years in which the low lateral plate *Eda* has been segregating at low frequencies in oceanic populations, and then repeatedly driven to fixation in fresh water (Colosimo et al. 2005), has made it an ancient mutational target. The present allele of major effect may actually be the product of numerous rounds of fixation of alleles of small effect at this locus (Phillips 2005). The same may be true for the genetic basis of other traits, and care should be taken in extending inferences too deeply in stickleback history.

In addition, proximate developmental genetic data must be interpreted in the context of population and quantitative genetics. The developmental genetic basis of traits is specific to the stickleback population under study, and may be affected by things such as the extent of time since colonization. Developmental genetic analysis of two populations of different age, both evolving in parallel along the same evolutionary genetic trajectory toward a common phenotype, might reveal different sets of contributing minor loci. In old populations the initial causative genetic changes may be obscured by compensatory changes elsewhere in the genome. What might appear to be different evolutionary genetic bases are due to sampling at different time points along the common trajectory (Nijhout 2002).

The temporal issue of population genetic context extends to a geographic concern when choosing populations for mapping crosses. When crossing ancestral and derived stickleback populations from the same locality, we can have some confidence that at least some of the variation that is observed recapitulates that seen during the evolutionary trajectory of the populations (Cresko et al. 2004). However, crosses from very divergent populations should be analyzed with care, especially considering the deep phylogeographic structure of stickleback populations from the Atlantic and Pacific basins (Orti et al. 1994). Even if major loci are the same, such as in the case of lateral plate reduction (Colosimo et al. 2005), the different population genetic contexts introduced into the cross may affect our understanding of additional minor loci involved, their contributions to the variance of the trait, and even the proportion of variance explained by the major locus itself (Nijhout 2002).

Summary

Integrating developmental and mathematical evolutionary genetics promises great advances in our understanding of the generation of organismal form. The threespine stickleback is well situated to provide empirical data for this micro-evo-devo synthesis. An obvious area of research is to continue the identification of the genetic basis of traits that vary within and among stickleback populations. Initial stickleback micro-evo-devo studies have revolved around variation in bony characters, but numerous other traits are potentially amenable to analysis (see Table 1). Importantly, because threespine stickleback populations straddle the species boundary, micro-evo-devo studies can be directed at traits that form the basis of variable stickleback mate recognition systems (pre-zygotic isolation), as well characters that are involved in the reduction in hybrid fitness (post-zygotic isolation). Despite the clear scientific advances to be made by identifying the standing genetic basis of stickleback trait variation, it is necessary to periodically step beyond the minutiae of proximate genetic mechanisms to ask specific questions about the ultimate processes behind the evolution of development. We propose concentrating research efforts both on the rearrangement of the standing genetic variation, and the input of mutational variance, thereby examining not just the genetic architecture of traits but the evolutionary dynamics or *evolvability* (*sensu* Wagner and Altenberg 1996) of these architectures (Schluter et al. 2004). While this synthesis might be achieved in many ways, we feel exploring the developmental genetic basis of trait (co)variance (G) across populations and environmental conditions to be one of the most promising. In particular, the following four questions are foremost in our minds:

- What is the functional genetic basis of G?
- What are the developmental and morphogenetic influences on G?
- What are the effects of different population genetic and external environments on G?
- How do these changes in G bias evolution via fixation of the standing variation or new mutations?

Doing so would provide an empirical and conceptual bridge between proximate and ultimate evo-devo mechanisms (*sensu* Arnold et al. 2001). The threespine stickleback system, a model organism for the study of behavior and evolution for most of the 20th century, is well situated to contribute to this micro-evo-devo synthesis for much of the 21st century.

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