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Ancestral Plasticity and Allometry in Threespine Stickleback Fish Reveal Phenotypes Associated with Derived, Freshwater Ecotypes

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Abstract

For over a century, evolutionary biologists have debated whether and how phenotypic plasticity impacts the processes of adaptation and diversification. The empirical tests required to resolve these issues have proven elusive, mainly because it requires documentation of ancestral reaction norms, a difficult prospect where many ancestors are either extinct or have evolved. The threespine stickleback radiation is not limited in this regard, making it an ideal system in which to address general questions regarding the role of plasticity in adaptive evolution. As retreating ice sheets have exposed new habitats, oceanic stickleback founded innumerable freshwater populations, many of which have evolved parallel adaptations to their new environments. Because the founding oceanic population is extant, we can directly evaluate whether specific patterns of ancestral phenotypic expression in the context of novel environments (plasticity), or over ontogeny, predisposed the repeated evolution of “benthic” and “limnetic” ecotypes in shallow and deep lakes, respectively. Consistent with this hypothesis, we found that oceanic stickleback raised in a complex habitat and fed a macroinvertebrate diet expressed traits resembling derived, benthic fish. Alternatively, when reared in a simple environment on a diet of zooplankton, oceanic stickleback developed phenotypes resembling derived, limnetic fish. As fish in both treatments grew, their body depths increased allometrically, as did the size of their mouths, while their eyes became relatively smaller. Allometric trajectories were subtly but significantly impacted by rearing environment. Thus, both environmental and allometric influences on development, along with their interactive effects, produced variation in phenotypes consistent with derived benthic and limnetic fish, which may have predisposed the repeated genetic accommodation of this specific suite of traits. We also found significant shape differences between marine and anadromous stickleback, which has implications for evaluating the ancestral state of stickleback traits.

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Introduction

Although considered intermittently for over a century (Baldwin, 1902; Morgan, 1896; Osborn, 1896; Simpson, 1953; Waddington, 1953), the recent emergence of evolutionary developmental biology has stimulated a renewed interest in how environmental influences on phenotypic development (phenotypic plasticity) might impact evolutionary processes (Ghalambor et al., 2007; Pigliucci, 2007; Price et al., 2003; Robinson & Dukas, 1999; Schlichting, 2004; West-Eberhard, 2003; Wimberger, 1994). Because the environment mediates how genetic variation translates into heritable, phenotypic variation, the environment directly affects the distribution of phenotypes upon which natural and sexual selection act. As such, phenotypic plasticity should substantially influence the processes of adaptation, speciation and diversification, a theory that is gaining empirical support (reviewed in Pfennig et al., 2010).

The goal of this study is to understand whether phenotypic plasticity can in part cause an adaptive radiation to exhibit parallel ecotypic variation: the repeated evolution of specific suites of traits in similar environments (reviewed in Schluter, 2000). One explanation for such “replicate radiations” is that similar selective pressures lead to similar adaptive outcomes in newly isolated lineages (Schluter & Nagel, 1995), which seems particularly likely if a source population provides the same standing genetic variation to multiple colonizing populations (Barrett & Schluter, 2008; Colosimo et al., 2005). However, a new environment not only imposes new selection pressures, it also determines how standing genetic variation translates into the phenotypic variation upon which those selection pressures act (Gibson & Dworkin, 2004; Le Rouzic & Carlborg, 2008; McGuigan et al., 2010a; Schlichting, 2008). When an ancestral stem group repeatedly encounters similar environments, the same phenotypes are expressed via plasticity and then exposed to similar selection pressures, leading to the evolution of parallel ecotypic variation (the “flexible stem hypothesis,” West-Eberhard, 2003). A key prediction of this hypothesis is that the phenotypes associated with derived ecotypes will resemble those that initially arose via plasticity in the ancestral colonists.

Testing the flexible stem hypothesis requires observing ancestral development in the context of derived environments—a challenge since ancestors are typically extinct or now adapted to derived conditions. Several recent studies have circumvented this problem either by inferring ancestral patterns of plasticity from the traits of derived taxa (e.g., Aubret et al., 2007; Gomez-Mestre & Buchholz, 2006; Ledon-Rettig et al., 2008; Losos et al., 2000), or by identifying systems in which both ancestral and derived populations are extant, permitting direct comparisons between ancestral plasticity and derived, adaptive variation (e.g., Scoville & Pfrender, 2010; Wund et al., 2008).

The threespine stickleback fish (*Gasterosteus aculeatus*) offers an outstanding opportunity to compare ancestral reaction norms to derived ecotypes. This small, holarctic fish has experienced an expansive freshwater radiation, much of which has taken place since the last glacial recession, which began 15 – 20,000 years ago. The stickleback radiation is characterized by parallel ecotypic variation along a number of phenotypic axes, including reproductive behavior (Foster et al., 2008; Shaw et al., 2007), defensive armor (Bell & Ortí, 1994; Moodie & Reimchen, 1976; Reimchen, 1994); foraging behavior (Hart & Gill, 1994), and morphology (Hart & Gill, 1994; Walker, 1997; Walker & Bell, 2000). Moreover, the oceanic population that produced the freshwater radiation is still widespread, allowing us to determine how ancestral, oceanic phenotypes developed in the context of novel, freshwater environments. Morphological and genetic evidence indicate that modern oceanic stickleback have changed little since giving rise to the freshwater radiation (Cresko, 2000; Deagle et al., 1996; Haglund et al., 1992; Mäkinen et al., 2006; Orti et al., 1994; Taylor & McPhail, 1999;

Walker & Bell, 2000; Withler & McPhail, 1985); thus, modern oceanic stickleback can be used to infer ancestral reaction norms.

We are specifically interested in whether the repeated evolution of “benthic” and “limnetic” foraging ecotypes resulted in part from recurring patterns of developmental plasticity in their ancestors. In numerous lakes throughout the freshwater radiation, stickleback morphology has diverged in relation to the amount of shallow, littoral habitat present in a lake (Aguirre, 2009; Hart & Gill, 1994; Spoljaric & Reimchen, 2007; Walker, 1997; Willacker et al., 2010). Benthic stickleback inhabit shallow, relatively eutrophic lakes with much littoral habitat. They forage on zooplankton until they reach about 1 cm in length and then switch to feeding on benthic macroinvertebrates (Hart & Gill, 1994). Their adult morphology reflects this lifestyle, with deep, maneuverable bodies, deep heads with short snouts (useful for sucking invertebrates from the substrate, Carroll et al., 2004; Webb, 1984), large mouths and short, widely spaced gill rakers (Hart & Gill, 1994; Walker, 1997; Willacker *et al.*, 2010). Limnetic stickleback live in deep, relatively oligotrophic lakes and eat primarily zooplankton for their entire lives (Hart & Gill, 1994). They have a number of morphological adaptations associated with cruising through the water column, searching for and handling tiny prey. These include a long, streamlined head and body, relatively large eyes (although not exclusively, see Willacker *et al.*, 2010), and long, closely-spaced gill rakers that help them retain small prey in their buccal cavities as water exits their gills (Hart & Gill, 1994; Walker, 1997). This ecotypic variation is likely adaptive, given that it has been linked to foraging performance in shallow and deep water environments (Robinson, 2000); moreover stickleback of intermediate phenotype are outperformed by the more extreme forms in their respective environments (Hatfield & Schluter, 1999; Schluter, 1993).

If the flexible stem hypothesis is correct, oceanic stickleback should develop phenotypes resembling those of derived, benthic and limnetic ecotypes when reared in environments that mimic shallow and deep lakes, respectively. Previously, we performed a partial test of this prediction by rearing marine stickleback on a diet of either chironomid larvae, reflecting a benthic diet, or zooplankton, reflecting a limnetic diet (Wund et al., 2008). While plasticity of some traits did support the flexible stem hypothesis (e.g., head shape), other traits were either not plastic (e.g., body shape), or exhibited plastic variation contrary to predictions (e.g., eye size, gill raker length). Our interpretations of these outcomes might have been limited by the relatively simplistic nature of our experiment: we only considered a single environmental variable (diet) and we examined plasticity in only small fish of roughly uniform size. In order to come to a complete and accurate understanding of phenotypic evolution, it is important to more thoroughly consider the mechanisms of phenotypic development, which include the interacting inputs of environmental variation (plasticity), ontogeny and allometric relationships among traits (the “developmental norm of reaction,” Schlichting & Pigliucci, 1998).

In the present study, our aim is to more thoroughly characterize morphological plasticity in ancestral threespine stickleback in order to test the flexible stem hypothesis. This experiment diverges from our previous work in three important ways. First, to more completely represent natural environmental variation, alternative treatments differ not only in diet, but also in habitat structure. Second, by sampling individuals at regular intervals during the study we evaluate both the independent and interactive effects of the environment and ontogenetic allometry on ancestral trait development. We predict that the benthic treatment, with a diet of macroinvertebrates and a complex habitat structure, would lead to deep heads and bodies, relatively small eyes, and short, widely-spaced gill rakers. Conversely, when reared on a diet of zooplankton in a structurally simple environment, oceanic stickleback would develop narrow heads and bodies, relatively large eyes and long, closely-spaced gill rakers. Body shape in wild-caught, marine stickleback varies with size (Spoljaric &

Reimchen, 2011), and thus we characterized allometric changes in shape and asked whether the relationship between size and shape differed across the two rearing environments. Heterochronic shifts in body shape allometry may also have played a role in the evolution of benthic and limnetic stickleback (West-Eberhard, 2003) so it is important to establish ancestral allometric relationships to determine whether benthic- and limnetic-like phenotypes emerge over the course of individual ontogeny. Finally, oceanic stickleback exhibit two distinct life-history strategies, with some individuals being fully marine, and others living in the ocean but breeding in freshwater ("anadromy;" Baker, 1994). The marine form breeds in shallow (~ 0.25 – 0.5m depth) tide pools, whereas anadromous stickleback move into freshwater streams to breed. While habitat differences exist early in life (e.g., salinity, flow, prey), it is unknown as to whether the ecology of the two forms differ once they move out to the ocean after 1–2 months of age. Relative to the freshwater radiation, there is little morphological variation among oceanic stickleback (Walker & Bell, 2000); however, given that our work depends so strongly on our choice of a representative ancestor, one goal of this study was to characterize any differences in morphology (or morphological plasticity) between these two forms that could influence the choice of representative ancestors in future studies.

Methods

Field Collection, Generation of Laboratory-reared Families (Crosses) and Fish Maintenance

Adult stickleback were collected in June of 2008 using unbaited minnow traps. Two representatives of the ancestral stickleback were sampled: a fully marine population (Mud Bay), which breeds in tide pools near Homer, AK (59°38'16.5"N, 151°29'56.3"W) and an anadromous population that breeds in a freshwater creek, Rabbit Slough, near Wasilla, AK (61°32'9.87"N, 149°15'13.78"W). Five full-sib families were generated from each population. Crosses were made by extruding eggs from gravid females, who were then euthanized with an overdose of MS-222. After males were euthanized in MS-222, their testes were removed, macerated and sperm was spread over the eggs to induce fertilization. Fertilized embryos were washed each day with clean embryo medium (distilled water, 0.5 ppt Instant Ocean® sea salt). Within 2–3 days post-fertilization, clutches were disinfected in a 1% iodine solution and before day 5, were shipped overnight to Clark University in Worcester, MA. Fry were kept in Petri dishes until they absorbed their yolk sacs, after which they were fed "Platinum" ® brine shrimp nauplii (*Artemia* spp., Argent Chemical Co., Redmond, WA). As they grew, fry were moved to 0.45 L and then 0.9 L jars. After several weeks, they were transferred to 37 L aquaria in a recirculating aquarium system, and maintained on an 8 hour: 16 hour light:dark cycle at a temperature of 17°C. The short day length ensured that the fish would not become reproductive during the course of the study (nor any of the other fish in our facility), while the water temperature was maintained in a range to promote growth. Fish were fed brine shrimp nauplii until they became large enough to eat blended bloodworms (chironomid larvae). At this point, fish were placed in their respective experimental aquaria and the experiment began (fish ~1 cm standard length, and 2.5 months old).

Experimental Setup

The experiment was conducted in 37 L aquaria in the recirculating systems with two treatments: benthic and limnetic. Twenty-four individuals from each of the ten families were divided into two adjacent tanks, each representing one of the two treatments. Each tank contained 12 full siblings, and all families were represented in both treatments (Fig. 1.). To simulate the structural complexity of a benthic environment, benthic tanks contained gravel bottoms, two inverted, plastic "strawberry" baskets and nine small, plastic plants, 8 of which

were anchored in the tops of the plastic baskets with the 9th being rooted in the gravel substrate. The arrangement of these structures was identical for all benthic tanks. Fish in this treatment were fed bloodworms twice a day, with the food being distributed within the gravel, plants and baskets, thus requiring fish to maneuver through a complex habitat and search for food. Bloodworms were briefly blended for the first 50 days of the experiment so they would be small enough for the fish to ingest. Subsequently, the fish were large enough to eat full sized, unblended worms. Three times a week, benthic fish were fed nutrient rich "Cyclop-eze" ® flakes (Argent Chemical Co., Redmond, WA).

For the limnetic treatment, aquaria were kept completely bare to simulate an open, freshwater environment. Fish in this treatment were also fed twice daily, and continued their diet of "Platinum" brine shrimp nauplii for the duration of the experiment. This treatment was also supplemented three times a week with Cyclop-eeze, but in this case, as whole freeze-dried crustaceans, a neutral-bouyancy, planktonic food. Thus, both treatments received the same type of dietary supplement, but which was size-appropriate to each treatment. This strategy was designed to minimize morphological differences due to diet quality (Wimberger, 1993). Every 6 weeks for 24 weeks, two fish were haphazardly chosen from each tank, euthanized with MS-222 and preserved in 10% formaldehyde. Fish were then stained with Alizarin red S and stored in 60% isopropanol in individual vials. Three Rabbit Slough families suffered some mortality due to a microsporidial infection in some individuals, so out of the 160 possible individuals included in the experiment, 151 survived (Mud Bay, Benthic: N = 40; Mud Bay, Limnetic: N = 40; Rabbit Slough, Benthic: N = 37; Rabbit Slough, Limnetic: N = 34).

Morphometric analyses

The 151 specimens were photographed with a 10 Megapixel Canon Digital Rebel XTi under identical lighting and exposure conditions. Slightly bent specimens were pinned in place to ensure that all photos were taken from an identical lateral view. Geometric morphometrics (reviewed in Zelditch et al., 2004) was used to characterize shape variation among subjects. We used the same set of 16 landmarks as in our previous work (Wund et al., 2008; Fig. 2), which were selected to capture variation in both cranial and postcranial external morphology. Landmarks were identified on each specimen using TPSdig v. 2.10 (Rohlf, 2004). A single observer (SV) placed all landmarks, and was blind to treatment and population of origin. To determine the location of landmark 3, a straight line was created between landmarks 1 (the tip of the snout) and 2 (the center of the eye), and landmark 3 was placed at the intersection between this line and the edge of the eye.

Generalized Procrustes Analysis superimposition was used to obtain shape variables that are independent of size, rotation and translation (Rohlf, 1990; Walker, 1997; Zelditch et al., 2004). A consensus configuration of all specimens, specimen centroid sizes (a measure of size independent of shape), as well as principal and partial warps from a thin plate spline bending energy matrix were generated using IMP geometric morphometric software (Sheets, 2003a; Sheets, 2003b). The partial warps ("shape variables") of the bending energy matrix can be analyzed with conventional multivariate statistical analysis (Zelditch et al., 2004). Multivariate analysis of covariance (MANCOVA, Sokal & Rohlf, 1995) was performed on partial warp scores and uniform components to determine if shape varied between populations, treatments and among families (nested within population), with centroid size as a covariate. Centroid size and preservation stage were highly correlated ($r = 0.934$; $P < 0.001$), so stage was not included in the MANCOVA models to avoid multicollinearity. It is also worth noting that we chose to preserve fish at six week intervals to achieve a final range of sizes to use as a continuous covariate, not because they represented some inherent ontogenetic stages. We also included population x treatment, population x size, treatment x size and population x treatment x size interactions in our initial models to determine whether

populations and/or treatments exhibited different allometric changes in shape, or if populations exhibited different patterns of plasticity across treatments. Backwards elimination was used to remove interaction terms that did not explain a significant amount of variation in shape. A one-way ANCOVA was performed on standard lengths and centroid sizes to determine whether fish from different populations and/or treatments differed in size.

As a complement to our MANCOVA approach, we also employed discriminant function analysis (Kleinbaum et al., 1988) to identify axes of shape variation that best distinguished populations and treatments. Because MANCOVA indicated a significant treatment x size interaction (see below), we performed a DFA to look for treatment differences using size-adjusted residual Procrustes coordinates. A regression of Procrustes coordinates on size was performed separately for each treatment, and then DFA was performed on the combined residuals from these two analyses. Thin plate spline was used to generate deformation grids that permit a visual interpretation of shape variation along the discriminant functions. DFA and thin plate spline analyses were performed using MorphoJ v. 1.02h (Klingenberg, 2011).

Gill raker measurement

In addition to examining external morphology, we also examined variation in the gill rakers, an important internal trophic character that helps retain food in the buccal cavity as water exits through the gills. We dissected out the first gill arch on the left side of all fish and placed them in glycerin on a microscope slide. Once mounted and viewed under a dissecting microscope, gill arches were photographed via MIAS2006 and measured in ImageJ version 1.43r (http://rsbweb.nih.gov/ij/). ImageJ's sequential line tool was used to measure the lengths of the 2^{nd}, 3^{rd}, and 4^{th} rakers on the descending (long) branch of the first arch, which are typically the longest gill rakers. These three lengths were then averaged for each fish. We also measured the linear distance along the gill arch spanned by these three gill rakers. All fish were scored blind by a single observer (SW) with respect to treatment and population of origin. We were able to dissect out and measure gill rakers from 147 of the 151 specimens used in the morphometric analyses.

Nested ANCOVAs were used to determine if population, family within population, treatment or head centroid size (covariate) could explain variation in average gill raker length or spacing. Both of these characters were more highly correlated with head centroid size (r = 0.97 and 0.96, respectively) than with standard length (r = 0.91 and 0.89, respectively). Size-adjusted gill raker lengths and spacing were generally normally distributed within population x treatment groups (Shapiro-Wilks test for normality: all $P > 0.1$, except benthic treatment/Mud Bay population, in which $P = 0.046$). Population and treatment were treated as fixed effects, and family nested within population as a random effect. As with the MANCOVAs described above, we also included population x size, population x treatment, treatment x size and population x treatment x size interactions in initial MANCOVA models, and used backwards elimination to remove non-significant interaction terms from subsequent analyses.

Results

The 151 fish included in the study ranged in size from a standard length of 17.0 mm to 46.3 mm (mean ± sd = 31.65 ± 8.5 mm), spanning all four preservation stages. The two populations did not differ in size, whether measured by standard length ($F_{1, 149} = 1.096$; $P = 0.297$), or morphometric centroid sizes ($F_{1, 147} = 1.111$; $P = 0.293$). Fish from the two treatments also did not differ in size ($F_{1, 147} = 2.285$; $P = 0.126$).

Shape

Shape differed between the two populations, among families within populations and between the two treatments, and these differences were associated with moderate to large effect sizes (Table 1). Fish also changed shape as they grew, and this allometric affect differed between the two environmental treatments (as indicated by a significant treatment x centroid size interaction; Table 1). No other interactions were significant. With respect to population differences, Mud Bay fish exhibited a more streamlined shape than Rabbit Slough fish ($T^2 = 159.0$; $P < 0.001$; Mahalanobis D = 2.06; Fig. 3). With respect to plasticity, fish in the limnetic treatment had shallower bodies and more elongate heads than fish in the benthic treatment ($T^2 = 122.5$; $P < 0.001$; Mahalanobis D = 1.802; Fig. 4). Despite a significant treatment x size interaction in the MANCOVA (Table 1), fish in both treatments exhibited similar changes in shape as they grew, with larger fish exhibiting relatively deeper bodies, smaller eyes, larger mouths, and a more dorsal positioning of the more posterior portion of the skull (Figs. 5a and 5b).

Gill rakers

When adjusted for head centroid size, gill raker length and spacing differed between treatments, but not between populations, although the effect size of treatment on gill raker spacing was quite low (Tables 2 and 3). For a given body size, fish in the limnetic treatment had longer, more closely-spaced gill rakers than those in the benthic treatment (Figure 6). Gill raker length also differed significantly among families (Table 2). Interactions among treatment, population and head size did not affect either gill raker length or spacing (all $P > 0.05$). Considering the partial effect sizes of these traits, the environment had a much larger effect on gill raker length than spacing.

Discussion

The environment directly impacts how genetic variation translates into the expressed phenotypic variation upon which selection pressures act, so patterns of plasticity that emerge following a change in selection pressures should influence the nature of adaptive evolution. The results of this study support a key prediction of this hypothesis, that the phenotypes revealed by plasticity in a colonizing population will resemble the type of adaptive phenotypes that ultimately evolve. When reared in aquaria with no structure and only planktonic prey, oceanic stickleback exhibited features similar to what is observed in the derived, limnetic ecotype: long heads, narrow bodies, and long, narrowly spaced gill rakers. When reared in an environment simulating a shallow lake, oceanic stickleback exhibited benthic-like features: a deep head and body, and short, widely-spaced gill rakers. Thus, given that plasticity consistently revealed these patterns of variation, it is not surprising that in lake after lake, selection would produce the same general outcomes. Both body depth and head depth also increased disproportionately with overall size, so selection on allometric trajectories should further reinforce evolution along these phenotypic trajectories. Indeed, it has been proposed that the evolution of the limnetic ecotype represents a heterochronic shift in allometric growth, with adult limnetics retaining a juvenile-like, narrow body shape (West-Eberhard, 2003).

Considering the effect sizes of in our MANCOVAs and ANCOVAs, it is clear that allometric effects on shape were quite substantial. However, available evidence suggests that Rabbit Slough stickleback harbor little to no additive genetic variance in the slope of static allometry (the relationship between size and shape among individuals of the same age; McGuigan et al., 2010b), so additive variance in the slopes of ontogenetic allometry (allometries of individuals of different ages/sizes) is likely not present, since the latter leads to the former (Shingleton et al., 2007). Allometry, and the expression of genetic variance

thereof, might be environmentally contingent. A key strategy in our experimental design was to consider the interactive effects between the environment and allometry, espousing Schlichting and Pigliucci's "developmental reaction norm perspective" ("DRN;" 1998). Doing so demonstrated that allometric effects on body shape are subtly, but significantly contingent upon the developmental environment. Experiments currently underway, examining developmental plasticity within freshwater benthic and limnetic stickleback, are exploring whether the ancestral DRN established in the present study was altered via genetic accommodation in the course of adaptation to freshwater environments.

The observed plasticity in head shape was generally consistent with our previous work (Wund et al., 2008), however, there are a number of notable differences between the outcomes of the two experiments, which we attribute to our expanded experimental approach in the present study. In the present study, plasticity of body shape and gill raker morphology were consistent with the flexible stem model, and eyes no longer appeared larger in the benthic treatment as in our previous experiment. These results are also broadly consistent with those of Day and colleagues (Day & McPhail, 1996; Day et al., 1994), who examined diet-induced plasticity of head morphology in sympatric, freshwater benthic and limnetic stickleback populations in British Columbia. However, our results contrast with those of another study that examined plasticity in response to habitat structure alone, which found that wild-caught, riverine stickleback held in spatially complex environments had shallower bodies than those held in spatially simple environments (Garduño-paz et al., 2010). The discrepancy between these two sets of results possibly reflects differences in experimental design, the use of laboratory-bred versus wild-caught fish, and/or the use of riverine versus marine populations. These discrepant outcomes highlight the care that must be applied to designing and interpreting developmental plasticity experiments. By design, the outcomes are contingent upon the environments chosen, a decision that must be made based upon the biology of the organisms and the objectives of the study. Our first experiment laid the groundwork for testing the flexible stem hypothesis, but led to perplexing outcomes in some instances. Moving forward, the developmental reaction norm approach detailed by Schlichting and Pigliucci (Schlichting & Pigliucci, 1998) has led to a more thorough characterization of ancestral patterns of development in relevant ecological settings.

The two populations we examined, one marine (Mud Bay) and one anadromous (Rabbit Slough), differed in body shape, with Rabbit Slough fish having generally deeper bodies than Mud Bay fish. The population effect size was nearly twice as large as that due to plasticity (although the effects of plasticity were manifest both directly, and via an interaction with allometry). Previous work on wild-caught fish has documented relatively little morphological variation among oceanic sampling sites, even between stickleback from the Atlantic and Pacific basins (Walker & Bell, 2000). The fish in this study were reared in freshwater conditions, which could have revealed intrinsic differences between marine and anadromous fish that remain cryptic in the wild. In a recent experiment using Rabbit Slough stickleback, the same anadromous population used in the present study, additive genetic variance was significantly higher when were reared in freshwater versus saltwater (McGuigan *et al.*, 2010a). Perhaps marine stickleback respond differently than anadromous stickleback when exposed to freshwater rearing conditions. It is not currently known why some oceanic stickleback breed in freshwater while others breed in saline tide pools and little is known about their habits in the open ocean or how they choose breeding sites as adults. Our results indicate that marine and anadromous stickleback cannot be considered equivalent with respect to ancestral character state determination; given the importance of stickleback as a model system for studying evolutionary processes, it is important to understand why any such differences exist, and they affected patterns of freshwater diversification.

Although mainly considered on theoretical grounds for the better part of a century, recent empirical evidence from a variety of systems indicates that phenotypic plasticity can indeed impact patterns of diversification (reviewed in Pfennig et al., 2010). Plasticity can facilitate a population's persistence under novel circumstances, and can allow individuals to explore new ecological niches, bringing newly-expressed phenotypes under novel selective regimes (e.g., Aubret *et al.*, 2007; Badyaev, 2009; Tebbich et al., 2010; Yeh & Price, 2004). The adaptations that result from this process will necessarily reflect the expressed phenotypic variation in the ancestral populations—variation that was induced by a change in the developmental environment (West-Eberhard, 2003). A number of recent studies have found that patterns of plasticity within taxa parallel evolved differences between taxa (e.g., Gomez-Mestre & Buchholz, 2006; Kolbe & Losos, 2005; Losos *et al.*, 2000; Magalhaes et al., 2009; Stauffer & van Snick Gray, 2004), suggesting a role for plasticity in diversification. Other studies have inferred ancestral reaction norms from derived taxa in order to evaluate plasticity's role in adaptation (e.g., Ledon-Rettig et al., 2008). The strength of the stickleback system, and others like it (e.g., Badyaev, 2009; Parsons & Robinson, 2006; Scoville & Pfrender, 2010), is that ancestral reaction norms can be directly observed in extant taxa, and then linked to patterns of divergence in their descendants (McCairns & Bernatchez, 2010; Shaw et al., 2007; Wund et al., 2008; the present study).

Here, we have strengthened support for the hypothesis that ancestral plasticity predisposed the evolution of benthic and limnetic stickleback ecotypes. Inter-population and interspecific divergence in body shape and trophic morphology characterize many lacustrine fishes, and plasticity has been implicated, either directly or indirectly, in many of these instances (e.g., Adams & Huntingford, 2004; Collyer et al., 2007; Magalhaes *et al.*, 2009; Parsons & Robinson, 2006; Stauffer & van Snick Gray, 2004; Wimberger, 1994, for review). These plastic responses can manifest in complex ways, impacting the functional integration among traits and their development over ontogeny, so understanding phenotypic evolution requires consideration of the complex interactions among traits, across environments and through time (Schlichting & Pigliucci, 1998). When comparing ancestral and derived traits to further elucidate mechanisms of adaptation, it is important to consider how the ancestral traits were expressed not in the ancestral environment, but rather in the context of the derived selective environment. When a population's adaptive landscape changes, plasticity could lead to an immediate, within generation shift in the distribution of heritable phenotypic variation—it is this altered distribution of traits upon which novel selection pressures will act, thus influencing the subsequent adaptive process. The stickleback radiation is an outstanding model for addressing how ancestral development in novel environments impacts subsequent adaptive radiation (Foster & Wund, 2011; McGuigan *et al.*, 2010a; Shaw *et al.*, 2007; Wund *et al.*, 2008).

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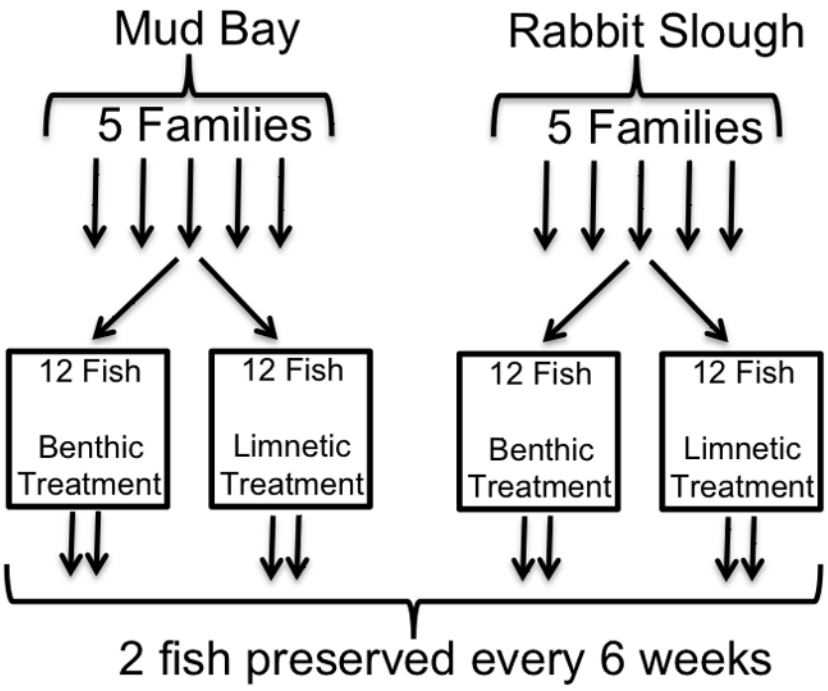

Figure 1.
Experimental design. Two fish from each aquarium were preserved every 6 weeks, for a total of 24 weeks (8 fish per aquarium).

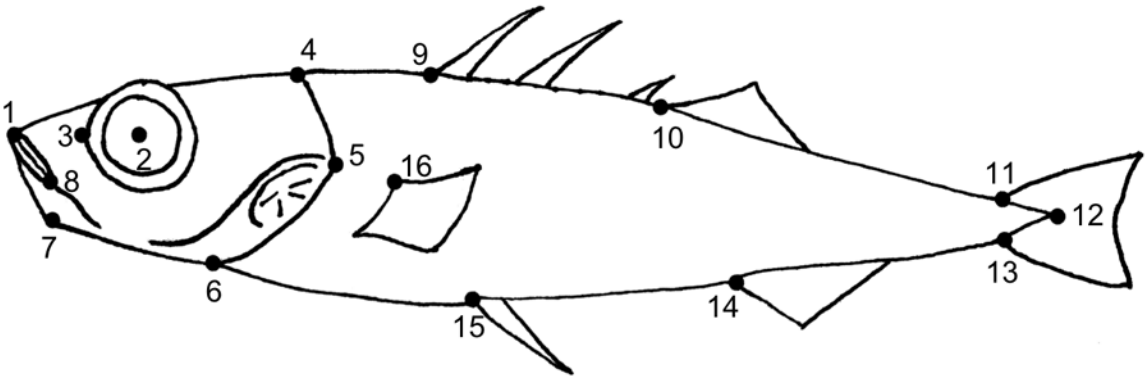

Figure 2.

The 16 digital landmarks used to describe fish shape in left lateral view: (1) anterior tip of upper lip; (2) center of eye; (3) eye radius as defined by line created by points 1 and 2; (4) supraoccipital notch lateral to dorsal midline; (5) farthest posterior point in skull; (6) junction of head to body on ventral midline; (7) posterior edge of angular; (8) posterior end of mouth; (9) anterior junction of first dorsal spine; (10) base of dorsal fin ray; (11, 12, 13) caudal peduncle; (14) base of first anal fin ray; (15) junction of pelvic spine on pelvic girdle; (16) upper edge of junction of pectoral fin to body.

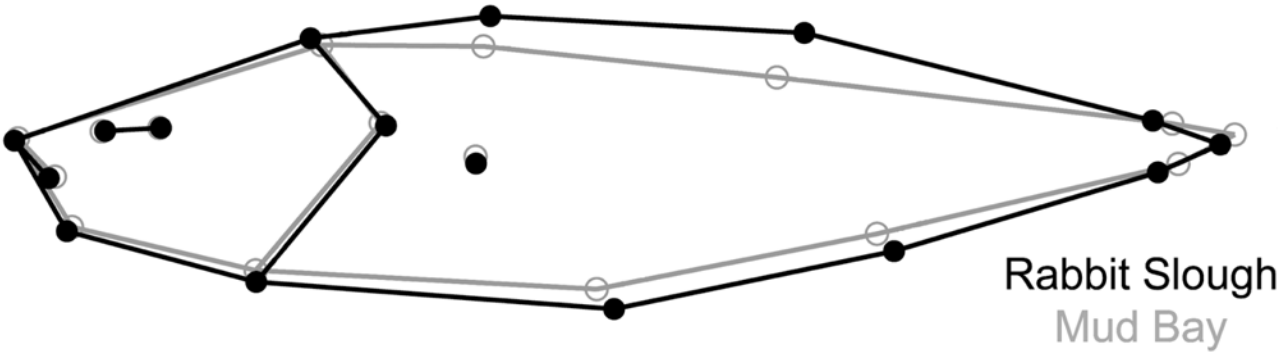

Figure 3.
Wireframe graphs depicting the average body shapes of Rabbit Slough fish (black) and Mud Bay fish (grey). Deformations are exaggerated 4× to illustrate differences.

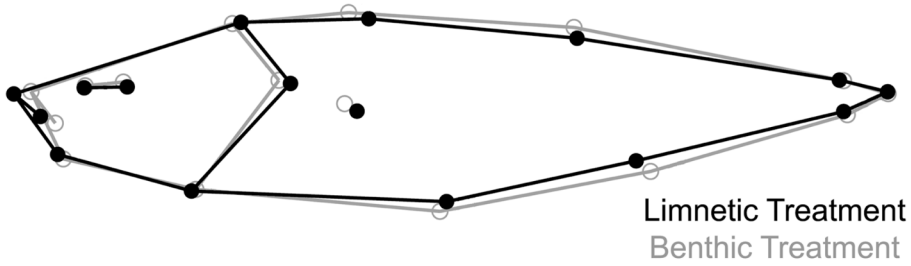

Figure 4.
Wireframe graphs depicting the average body shapes of limnetic treatment fish (black) and benthic treatment fish (grey). Deformations are exaggerated 4× to illustrate differences.

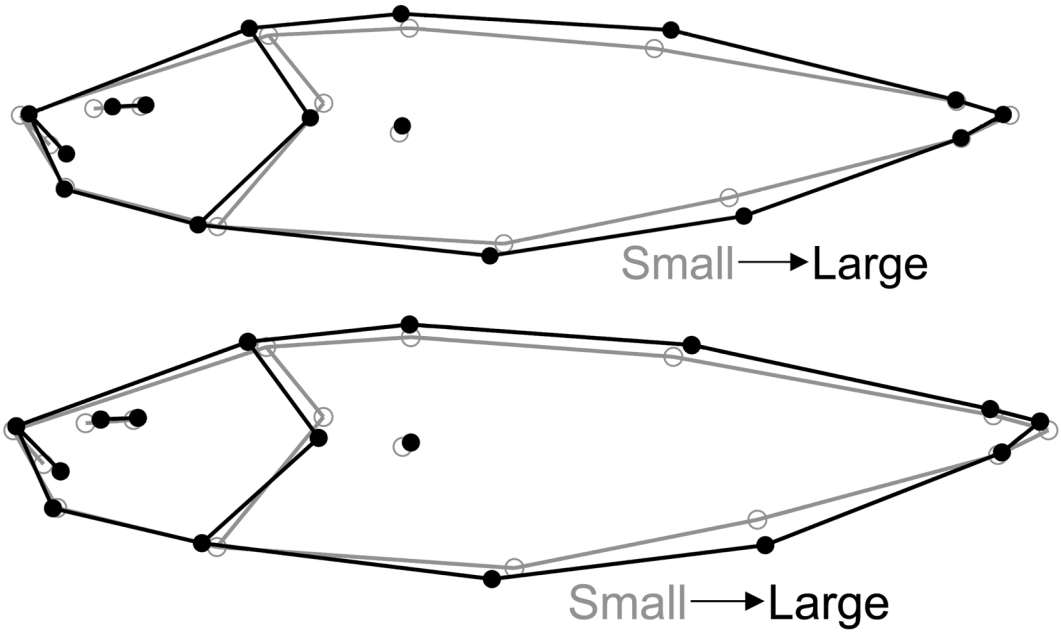

Figure 5.
Wireframe graphs depicting change in shape as fish grew (small fish = grey, large fish = black). Given the significant treatment x size interaction, allometric shape changes are depicted separately for the (A) limnetic and (B) benthic treatments. Deformations exaggerated 4× to illustrate differences.

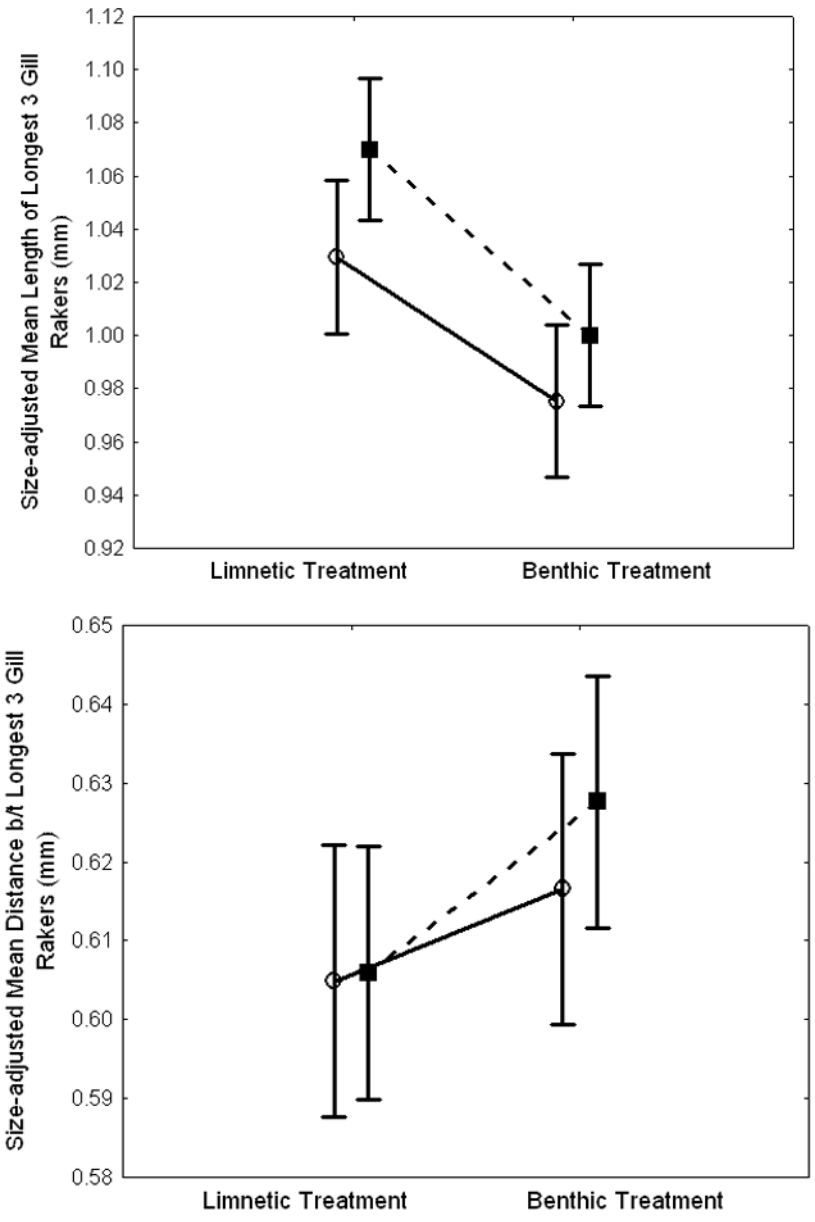

Figure 6.

2-D reaction norm graphs depicting differences in (A) the length of the three longest gill rakers on the 1st gill arch and (B) the mean distance spanned by these gill rakers. Dashed lines = Mud Bay, solid lines = Rabbit Slough. Values are LS means adjusted for head centroid size, ± 95% CI.

Table 1

MANCOVA results for shape

Effect	Wilks Lambda	df	F	P	Partial eta^2
Population	0.3950	28, 111	6.073	<0.001	0.605
Family(Population)	0.0222	224, 873	2.486	<0.001	0.390
Treatment	0.6664	28, 111	1.985	0.006	0.334
Centroid size	0.0925	28, 111	38.901	<0.001	0.908
Treatment x Centroid Size	0.7110	28, 111	1.611	0.043	0.289

Table 2

ANCOVA results for gill raker length

Effect	df	F	P	Partial eta^2
Population	1, 8.1	1.017	0.343	0.112
Family (Population)	8, 135	5.376	<0.001	0.242
Treatment	1, 135	19.979	<0.001	0.129
Centroid size	1, 135	2775.138	<0.001	0.954

Table 3

ANCOVA results for gill raker spacing along the gill arch

Effect	df	F	P	Partial eta^2
Population	1, 8.4	0.483	0.506	0.055
Family (Population)	8, 135	1.096	0.370	0.061
Treatment	1, 135	4.156	0.043	0.030
Centroid size	1, 135	1783.748	0.000	0.930