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ADAPTIVE DIVERGENCE BETWEEN FRESHWATER AND MARINE STICKLEBACKS: INSIGHTS INTO THE ROLE OF PHENOTYPIC PLASTICITY FROM AN INTEGRATED ANALYSIS OF CANDIDATE GENE EXPRESSION

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Debate surrounding the integration of phenotypic plasticity within the neo-Darwinian paradigm has recently intensified, but is largely dominated by conceptual abstractions. Advances in our capacities to identify candidate genes, and quantify their levels of expression, now facilitate the study of natural variation in inherently plastic traits, and may lead to a more concrete understanding of plasticity's role in adaptive evolution. We present data from parapatric threespine stickleback (*Gasterosteus aculeatus*) demes inhabiting geologically recent, freshwater and saltwater zones of a large estuary. Reaction norms for survival confirm adaptation to local salinity conditions. Analysis of osmoregulatory candidate gene expression within an ecological quantitative genetics framework suggests putative mechanisms underlying adaptive variation, and provides insights into the role of ancestral trait plasticity in this divergence. A sodium–potassium ATPase (ATP1A1) is identified as a candidate gene for freshwater adaptation. In addition to heritable variation for gene expression, we infer significant correlation between measures of expression and individual fitness. Overall results indicate a loss of plasticity in the freshwater deme. We discuss how this is consistent with adaptation facilitated by ancestral plasticity as a heuristic example that may prove useful for future, explicit tests of the genetic assimilation hypothesis.

KEY WORDS: **Adaptive plasticity, ATPase, cystic fibrosis transmembrane regulator (CFTR), evolutionary physiology, genetic assimilation, osmoregulation.**

The study of intraspecific, adaptive divergence has yielded many valuable insights into the rate and processes underlying evolutionary changes; however, most empirical work appears to be biased toward morphological divergence (Hoffmann et al. 1995; Sinervo and Svensson 2002; Russell and Bauer 2005). Yet it has been suggested that more inherently plastic traits, such as behavior or physiology, may be among the first to diverge and evolve during the process of adaptation to novel environments (Mayr 1963; Skúlason et al. 1993; Rogers et al. 2002). This is not to say that morphological traits cannot be plastic. Indeed, examples of morphological phenotypic variance in response to environmental effects abound (West-Eberhard 2003). Moreover, it has been demonstrated that selection acting directly on such trait plasticity can ultimately yield adaptive divergence, even distinct ecotypes (de Jong 2005). Nevertheless, a distinction is often drawn between inherently "labile" traits for which expression

can change throughout the lifetime of an individual, and traits whose expression may be modified only during a critical developmental period, beyond which they are fixed (Alpert and Simms 2002; Gabriel 2006; Crispo 2008). Regardless of whether it is labile or developmental in nature, there is an emerging view that phenotypic plasticity likely plays a significant role in evolution (West-Eberhard 2003, 2005; Pigliucci et al. 2006; Pigliucci 2007). This is in stark contrast, however, to Wright's (1931) notion that "individual adaptability" [*sic*; plasticity] is "a factor in evolution tending to dampen the effects of selection." Indeed, modern interpretations have framed scenarios in which plasticity might shield genotypes from selection, and thus, delay evolution (de Jong 2005; Crispo 2008). Alternatively, a strong case for the importance of plasticity in evolutionary transitions can be made when adaptation to a new niche involves changes in both highly plastic and nonplastic traits (Price et al. 2003; Lande 2009). Some authors have even borrowed from Wright's legacy in arguing how plasticity may facilitate evolutionary change by bringing populations into the neighborhood of an adaptive peak (Price et al. 2003; Ghalambor et al. 2007). Others still have promoted plasticity as an initiator of evolutionary novelty (West-Eberhard 2005; Pigliucci et al. 2006; Pigliucci 2007). Many of these views remain controversial, thus, elucidating the role of plasticity and plastic traits in adaptive evolution represents an exciting frontier in evolutionary research.

Physiological traits may be particularly interesting given that their inherently labile and reversible nature may help to facilitate colonization of novel environments, whereas interindividual variability and associated bioenergetic costs and trade-offs may ultimately lead to adaptive evolution (Schulte 2001). In contrast to more complex morphological traits, many physiological processes are relatively simple biochemical reactions mediated by endogenously produced enzymes. Thus, being directly linked to transcriptional products, physiological processes may be more immediately susceptible to changes in the composition and/or conformation of proteins resulting from mutation in the coding DNA sequence. Moreover, simple mutations at regulatory regions can also affect the rate of transcription, rather than the physical properties of the protein itself. Such mutations may also be less deleterious than those affecting coding regions proper in the sense that transcriptional rate changes may be sublethal, while still resulting in different phenotypic variants upon which selection may act (Gibson and Wagner 2000; Cork and Purugganan 2004). Thus, physiology may capture elements of both developmental and regulatory (i.e., labile) plasticity, and as such, may be particularly germane to the question of plasticity's role in adaptation to environmental heterogeneity.

For aquatic organisms, the maintenance of plasma ion concentrations represents a unique set of physiological challenges, whether that be the loss of water and influx of salts in the marine environment, or the passive loss of ions to the external environment in freshwater. Osmoregulation under both conditions is energetically costly and necessitates active ion transport against a concentration gradient employing a variety of molecular pumps and channels, most of which are synthesized at the site of ion transfer (Perry 1997; Marshall 2002; Hwang and Lee 2007). Despite regulatory differences between environments, euryhaline species can acclimate to a range of salinities, and thus, might become distributed across a salinity gradient via their inherent physiological plasticity. Yet limits to plasticity are almost certainly imposed by energetic costs and trade-offs (van Tienderen 1997; DeWitt et al. 1998; van Kleunen and Fischer 2005). Moreover, many of the osmoregulatory mechanisms permitting permanent residency in the freshwater environment represent major evolutionary transitions (Lee and Bell 1999). Thus, locally adapted populations of euryhaline teleosts may serve as ideal models to determine the role of plasticity in evolutionary divergence.

The St. Lawrence River estuary represents a unique environment to explore the dynamics between processes constraining and promoting adaptive divergence on an ecological timescale. The entire drainage basin was ice covered during the last (Wisconsinan) glacial period. However, about 12 kaBP, the combination of glacial retreat and isostatic depression resulted in massive oceanic inflow and the formation of a large proglacial sea encompassing the entire St. Lawrence lowlands (Wassenaar et al. 1988; Richard and Occhietti 2005). The Champlain Sea persisted for approximately 4000 years, supporting a diverse community of marine life (Harington 1988), until the formation of an ice dam within a constriction in its center and the inflow of glacial meltwater from the proto-Great Lakes basin in the west caused a significant ecological shift: about 8–6 kaBP, salinity in the western basin dropped precipitously, ultimately resulting in the large freshwater Lake Lampsilis occupying that portion of the valley upstream of modern Québec City, Canada; downstream a marine environment persisted, the Goldthwait Sea (Hillaire-Marcel 1988; Wassenaar et al. 1988). Lake Lampsilis eventually drained, and by about 4 kaBP, the current hydrogeological features of the St. Lawrence River had been established. Today, the lower 540 km section of the river, the St. Lawrence estuary, is highly influenced by tidal processes, resulting in a gradient of physicochemical landscape features (Laprise and Dodson 1994; Vincent and Dodson 1999). Additionally, the estuary is characterized by relatively stable freshwater and saltwater zones located upstream and downstream, respectively, of a highly variable freshwater–saltwater transition zone that experiences diurnal salinity fluctuations (Vincent et al. 1996; Winkler et al. 2003).

Fossil evidence indicates that the euryhaline threespine stickleback (*Gasterosteus aculeatus*) has inhabited the estuary since the late Pleistocene, with well-preserved marine specimens found in Champlain era deposits in the far western region of the ancient

sea (McAllister et al. 1981; McAllister et al. 1988). Extant sticklebacks in this system are partitioned into two demes whose geographic ranges correspond to the freshwater/saltwater division of the estuary (McCairns and Bernatchez 2008). Genetic differentiation is weak ($F_{ST} \approx 0.006$; $P < 0.001$), yet temporally stable, and preliminary analyses suggest divergence from a common ancestral population corresponding to the same timeframe as the ecological division of the Champlain Sea into Lake Lampsilis and the Goldthwait Sea (R. J. S. McCairns and L. Bernatchez, unpubl. data). Unlike other euryhaline teleosts inhabiting the estuary (e.g., *Osmerus mordax*), in which multiple mitochondrial DNA (mtDNA) haplotypes suggest colonization from separate glacial refugia (Bernatchez 1997), only a single mtDNA haplotype has been found in stickleback sampled throughout the waters of the estuary and Gulf of St. Lawrence (J. J. Dodson, unpubl. data), perhaps not surprisingly given that both western European and eastern North American populations belong to a single clade, believed to be derived from the same refugial population (Ortí et al. 1994; Mattern 2004). Biogeographical patterns of stickleback distribution, compared to other fish in the region, are also indicative of colonization from a single Atlantic refuge (Crossman and McAllister 1986; Underhill 1986). Taken together, these observations all suggest that stickleback demes in the St. Lawrence estuary are most probably derived from a single ancestral population, split during a short-lived vicariant event in which the early ecological landscape was divided into freshwater and saltwater zones.

McCairns and Bernatchez (2008) have shown that ecological factors independent of geographic distance, particularly salinity, explained the greatest proportion of genetic variance among threespine sticklebacks inhabiting the St. Lawrence estuary. Although this is consistent with a model of "isolation-by-adaptation" (Nosil et al. 2008), it is not sufficient evidence to demonstrate adaptive divergence between demes. In this study, we test for adaptation to divergent osmoregulatory conditions by analyzing reaction norms of pure crosses reared in a reciprocal experimental microcosm, defined by limits of the natural salinity gradient. Additionally, we test if differentiation between demes might be facilitated by selection against hybrids. Finally, we seek to gain insights into the mechanisms underlying potential physiological adaptations by documenting the relative expression of putative candidate genes for osmoregulation. Differences in levels of gene expression have been well studied in their role in acclimation to environmental change; however, very few studies to date have investigated patterns of reaction norms for gene expression (but see Côté et al. 2007), and the role of quantitative changes in gene expression during adaptive evolutionary divergence is poorly known (Schulte 2004). To this end, we evaluate the potential adaptive value of candidate gene expression by estimating their narrow sense heritabilities and selection coefficients.

Materials and Methods

COMMON GARDEN EXPERIMENT

Broodstocks were obtained by sampling adults from two spawning sites within the St. Lawrence River estuary, each representing stable freshwater and maritime environments, respectively (see details in McCairns and Bernatchez 2008). Mature ova were stripped from females in situ and transported to wet laboratory facilities (LARSA, Université Laval), in sterile Holtfreiter's solution. Testes were also dissected in situ and transported in sterile Ginzburg's Fish Ringers solution. Gametes from both sites were stored for equal time periods at 4°C, and all crosses were performed within 24 h of sampling. Gamete sampling occurred once in early June 2005, with a second sampling from both sites approximately two weeks later.

Crosses followed a blocked factorial breeding design in which each female was mated with two males, one originating from her population of origin, and one from the other population (see Fig. S1 for a schematic). Each block yielded two pure and two hybrid crosses in which each full-sibling group had both a maternal and paternal half-sibling relation within its block. Eighty-eight families, comprising 22 independent factorial blocks, were established. First generation families were produced following modifications of zebrafish in vitro fertilization techniques adapted for stickleback research (University of Oregon Stickleback Research Site). A testis from each male was divided in half, and each half macerated in a separate 100 mm diameter petri dish. Ova were initially divided into four lots of approximately 50 eggs, and each lot mixed gently with one of the four macerated testis halves and one of two embryo media, either sterilized dechlorinated water, or a sterilized solution of artificial seawater at twenty parts-per-thousand salinity (20‰). However, due to low initial fertilization success (data not presented), crosses from the second sampling period were all initially established under optimal (5‰) salinity conditions (University of Oregon Stickleback Research Site). In all cases, unfertilized eggs were removed after 24 h, and fertilized eggs were incubated at 16°C. Mortalities were enumerated and removed, and embryo media changed, twice daily.

Upon hatching, fry from 5‰ salinity embryo medium were gradually acclimated to alternative salinity conditions. Experimental conditions consisted of all families raised under salinity regimes representative of the natal freshwater and maritime environments (McCairns and Bernatchez 2008). Each family was divided into two groups. In one group, salinity was increased by 5‰ per day to final experimental conditions (20‰). The second group was immediately transferred to freshwater medium (<1‰). Upon absorption of yolk sac and beginning of exogenous feeding, each family was transferred to an individual 2 L container. Fry were fed ad libitum twice daily with freshly hatched *Artemia* nauplii. At forty-five days posthatch, all larval fish were

photographed for length measurement. Eight factorial blocks, comprising 32 families with individuals in both salinity treatments, were selected for transfer to experimental aquaria (sample sizes for all families can be found in Table S1). Experimental tanks consisted of individual aquaria connected to one of two 1600 L recirculating systems, one maintained at 20‰ salinity, the other with freshwater (<1‰ salinity). Water quality was maintained with a biofiltration system, and through daily siphoning of waste materials in individual aquaria. Fish were fed ad libitum twice daily a mixture of flake food and commercial salmonid fry ration, in addition to once daily supplements of freeze-dried *Mysis relicta*, frozen chironomid larvae, and live *Artemia* nauplii. Each family was photographed an additional three times (120, 180, and 230 days posthatch), from which length-at-age measurements were extracted. Experiments continued until 230 days posthatch, at which time surviving individuals were sacrificed, a portion from which gill tissues were sampled and preserved at −80°C for subsequent RNA extraction.

DATA ANALYSES

Data consisted of traits both directly (survival) and indirectly (growth) related to fitness. Survival data included both larval and juvenile stages, wherein the former is defined as the period between hatching and 45 days posthatch, and the latter from 45 to 230 days posthatch. Data from both spawning periods were pooled for larval analyses; all other analyses were based on families from the second spawning, with one exception (details in Table S1). Growth-related measurements included specific growth rate for the larval period, standard length (SL) at 230 days post-hatch, and residual deviation from a "lifetime" growth model. Because size-at-age data over the course of the experiment exhibited asymptotic behavior, we modeled environment-specific mean SL as a function of the "von Bertalanffy" growth model (Bertalanffy 1957; Stamps et al. 1998). Individual, residual variation was calculated as deviation from this model, and served as a proxy for relative condition. To estimate relative fitness, we calculated the product of larval and juvenile survival for each family, and standardized this by the mean of all families within each environment. As a proxy for individual fitness, we combined all traits by principal component (PC) analyses. Thus, we were able to relate individual final size variables (SL and residual size) with family-by-environment means of early growth rate and absolute survival. Individual fitness was estimated as the PC score from the first eigenvector (PC1). To assess the potential efficacy of this metric, we tested for a significant positive correlation between PC1 score and relative survival.

We analyzed data from two perspectives. The first was a comparison of reaction norms for pure crosses reared under reciprocal environmental salinities. The second approach consisted of tests comparing the mean performance of hybrid and foreign crosses to the native cross within each environment. This was accomplished by employing a planned contrast analysis in which both hybrid and foreign crosses were evaluated against the native pure cross in each respective salinity, that is, versus the FW–FW cross in freshwater (<1‰) and versus SW–SW in the simulated maritime environment (20‰). We used mixed-effects modeling via maximum likelihood estimation, with fitness data as response variables, for simultaneous optimization of both fixed and random model terms (Pinheiro and Bates 2000). All analyses were performed using the "lme4" package implemented in the R computing language (Bates 2007; R Development Core Team 2007).

For the reaction norm analysis, we modeled the effects of genotype (FW–FW vs. SW–SW), environment (<1‰ vs. 20‰), and genotype–environment interaction as fixed effects. Additionally, we modeled environmental effects separately for each pure cross. For all models, variation among families in both intercepts and slopes were treated as random effects. Because survival (larval and juvenile) consisted of proportional data, we used generalized mixed models (GLMM) assuming a logit link function and binomial error distribution. All other variables conformed to normality and homoscedasticity assumptions, after incorporation of random effects, so were analyzed using linear mixed-effects models (LME) with Gaussian error. The significance of fixed effects was evaluated by estimating the probability that model coefficients differed from zero based on Markov chain Monte Carlo (MCMC) sampling of their posterior distributions, conditional on random variation among families. Coefficients included simple differences between genotypes (i.e., deme-specific differences in intercepts; G), shared environmental effects (i.e., model slope; E), and genotype–environment interaction (i.e., differences between slopes; G × E). Interfamily variation in environmental effects (i.e., model slopes) was evaluated by testing for significant reductions in residual variance, based on Akaike's information criterion (AIC) and likelihood ratio tests against simpler models incorporating only random variation among family means. Contrast analyses employed the same model types, GLMM or LME, dependant upon the data; however, the factorial breeding design permitted random variation to be decomposed into separate dam and sire components. Analyses were also simpler, with significance evaluated by estimating the probability that contrast coefficients differed from zero, based on MCMC sampling of the conditional means for each cross.

CANDIDATE GENE EXPRESSION

Candidate genes for osmoregulation were selected based on a review of the physiological literature on teleosts, in which we identified molecular pumps and channels believed to be unique to osmoregulation in either the freshwater or saltwater environments (Marshall 2002; Hwang and Lee 2007). We targeted two genes associated with freshwater osmoregulation: a vacuolar hydrogen

Table 1. Gene transcripts amplified by real-time quantitative PCR.

Candidate gene	Ensembl gene ID (transcript ID)	Amplicon length	Genomic location			Primer sequences
			Chrom.	Start (exon)	End (exon)	
[1]EF1α	ENSGACG00000002182 (ENSGACT00000002893)	77 bp	X	1,567,954 (8)	1,568,030 (8)	F: CATTGTCACTTACCTGAATCACATGA R: TGTGGCATTTAACAACATTTCCA
[2]CFTR	ENSGACG00000009039 (ENSGACT00000011967)	65 bp	XIX	10,198,841 (10)	10,198,905 (10)	F: GCAGGCCTCTTCTTCACCAA R: TCCAGATAGAGGCTGATGTTCTTG
IGF	ENSGACG00000020042 (ENSGACT00000026526)	64 bp	IV	32,106,839 (3)	32,107,256 (4)	F: ACAGGAGCACAGAGCGTAGGA R: AACGGTCTCTTCTTGTTTTTTGTCTT
NAK	ENSGACG00000014324 (ENSGACT00000018945)	112 bp	I	21,699,651 (1)	21,699,762 (1)	F: ACTCCGGGCTGAGAGAGAGAG R: AGCCCCATGGTTGCAATG
VATP	ENSGACG00000017118 (ENSGACT00000022675)	60 bp	III	12,975,113 (7)	12,974,139 (8)	F: TGCACAGGAGCAGGAACTATTTC R: CGCCACACACTGGACGTACT

[1]Endogenous control used in relative quantitation.

[2]Transition at third nucleotide in the forward (F) primer sequence; guanine (G) reported in Ensembl stickleback genome.

ATPase (VATP; Gene Ontology annotation ATP6V1H) and a sodium–potassium ATPase (NAK; ATP1A1). Although NAK belongs to a multigene family, with many of its transcripts associated with Na^+ secretion in saltwater (Marshall 2002; Madsen et al. 2007), a number of isoforms also provide energy for ion uptake in freshwater (Bystriansky et al. 2006; Bystriansky et al. 2007; Nilsen et al. 2007). Moreover, the specific transcript amplified in this study is the product of a gene for which intronic single nucleotide polymorphisms have proven useful in discriminating between stickleback populations inhabiting a freshwater–saltwater gradient in an independent system (Jones et al. 2006). Thus, we hypothesized that any differential expression in this gene could be particularly informative regarding osmoregulatory divergence. As a candidate gene for saltwater adaptation, we selected the cystic fibrosis transmembrane regulator (CFTR; CFTR). Given its role in the smoltification process in salmonids (Sakamoto et al. 1993), we hypothesized that insulin-like growth factor (IGF; IGF1) might be a candidate gene for saltwater acclimation in *Gasterosteus* as well. Finally, as a reference gene, we selected the elongation factor EF1α (EEFA), for which mRNA expression in gills does not change in response to environmental salinity (Scott et al. 2004a,b).

Candidate gene expression was estimated based on relative quantitation of mRNA transcripts, assayed by real-time quantitative PCR (qPCR) using an Applied Biosystems (Foster City, CA) 7500 Real-Time PCR system. Primers and probes specific to each candidate gene were designed based on stickleback orthologs predicted from the annotated *Takifugu rubripes* genome (Ensembl Genome Browser). Putative transcript sequences were exported to PrimerExpress software (Applied Biosystems) to identify initial primer sequences. Initial primers were used to amplify and sequence candidate exon regions (200–300 bp) from five randomly selected test individuals. Sequence similarity was assessed by manual alignment, and consensus sequences exported to PrimerExpress software to identify primers and probes for qPCR. Predicted amplicons, in addition to primer/probe sequences individually, were compared against the stickleback genome using a BLAST search (Ensembl Genome Browser) to ensure that each targeted a unique transcript. Details regarding primer sequences, as well as amplicon sizes and genomic locations, can be found in Table 1.

A maximum of 10 surviving individuals per salinity treatment per family were selected at random; for groups with fewer than 10 survivors, all individuals were used. Gill arches were excised immediately and preserved in liquid nitrogen until RNA extraction. Frozen gills were transferred to RNA lysis buffer and mechanically disrupted and homogenized using a TissueLyser bead mill (QIAGEN, Valencia, CA). Total RNA was purified using PureLink silica-based membrane spin cartridges (Invitrogen Corporation, Carlsbad, CA), and eluted in nuclease free water. Total RNA concentration was estimated for each extraction based on UV absorbance at 260 nm, measured with a capillary spectrophotometer (GeneQuant, Pharmacia (now GE Healthcare), Piscataway, NJ). In total, high quality RNA was successfully extracted from 292 individuals (family specific details in Table S1).

Five nanograms of total RNA were treated with DNaseI to a final volume of 50 μL. Fifteen microlitres of this solution was used as a template for cDNA amplification from random primers (High-Capacity cDNA Archive Kit; Applied Biosystems). Each qPCR contained 5 μL of diluted (1:5) cDNA, 900 nM of forward and reverse primers, 250 nM FAM-labeled probe, and 12.5 μL TaqMan Universal PCR Master Mix (Applied Biosystems), to a final reaction volume of 25 μL. Candidate gene assays were performed in simplex reactions with three technical replicates per individual gene. Each 96-well plate contained all gene assay reactions for five randomly selected individuals, in addition to assay

reactions for a reference individual replicated across all plates and a single negative control for each reaction. Real-time PCR cycle threshold values (CT) for each technical replicate were calculated using 7500 Software (version 2.0.1; Applied Biosystems).

Analysis of relative transcription

We first validated that the amplification efficiencies of target and reference genes were approximately equal by calculating standard curves based on a logarithmic series of cDNA dilutions (Livak and Schmittgen 2001). Each target was normalized against the reference gene EF1α (Δ_{CT}). Target quantitation was estimated relative to transcription level differences of the reference individual replicated across all plates ($\Delta\Delta_{CT}$). Relative quantitation (RQ) was estimated based on the $2^{-\Delta\Delta CT}$ relationship (Livak and Schmittgen 2001). However, rather than basing calculations on the mean CT values from an individual's three technical replicates, we calculated bootstrapped values: one target and one reference CT value were sampled randomly from among the three technical replicates, in addition to one target and one reference CT value sampled from among all the multiplate data for the reference individual. Thus, one hundred bootstrap RQ values per candidate gene were calculated for each individual. Variation among plates and individuals was nested within each family and treated as random effects in LME models. Random variation in model intercepts and slopes was partitioned among all levels, and the significance of plate and slope effects evaluated via information theory (AIC) and likelihood ratio tests. Data were log transformed and analyzed as per procedures described for fitness traits, that is, reaction norm analysis of pure crosses and contrast analyses within each environment.

Finally, because adaptive divergence is a product of natural selection (Williams 1966; Endler 1986), and no trait can respond to selection without underlying heritable variation (Roff 1997), we deemed evidence for both heritability and selection coefficients as requisite to infer adaptive divergence between demes. We estimated genetic variance components for gene expression by restricted maximum likelihood (REML) within the framework of the animal model (Kruuk 2004; Thompson 2008). Employing the half-sibling pedigree structure for the entire dataset, and treating environmental salinity as a fixed effect, we used the program WOMBAT to estimate heritability and genetic correlations for transcription of all candidate genes (Meyer 2007). We used a multiple regression model, correlating gene expression with our proxy for individual fitness (PC1), to estimate potential selection gradients for the expression of osmoregulatory candidate genes (Lande and Arnold 1983). Log transformed RQ data were rescaled to a mean of zero, and were entered simultaneously in a multiple regression model as independent variables; PC1 scores were rescaled by their global mean (Lande and Arnold 1983). Gradients for directional and stabilizing selection were estimated as the simple linear and doubled quadratic coefficients, respectively (Stinchcombe et al. 2008). Estimates, their corresponding highest posterior density intervals, and *P*-values testing whether the estimates differed significantly from zero, were based on MCMC sampling of the posterior distribution of the multiple regression model, conditional on random dam and sire effects. Analyses were performed on the entire dataset, and separately for each experimental salinity.

Results

REACTION NORMS OF FITNESS TRAITS

Families from the freshwater deme (FW–FW) tended to have greater mean larval survival in freshwater (0.904) than in saltwater (0.876), although this environmental effect was not strictly significant (Fig. 1A; $P \approx 0.078$). We also detected significant variation among freshwater families in their response to environmental salinity, with up to 68.9% of model variance attributable to random variation in slopes (Table 2). In contrast, families originating from the maritime deme (SW–SW) exhibited a highly significant decrease in mean larval survival in freshwater (0.669 vs. 0.801; $P \approx 0.001$). Moreover, random variation in slopes among families was not significant in this group (Table 2), and their average reaction norm was significantly different from that of the freshwater deme ($P < 0.001$). Whereas survival was relatively high throughout the larval stage, juvenile survival appeared to be substantially reduced (Fig. 1B). Contrary to the trend observed for larval survival, FW–FW families exhibited greater juvenile survival at 20‰ salinity (0.130 vs. 0.208; $P \approx 0.012$), although there was also significant interfamily variation in model slopes (Table 2). SW–SW families also had low mean survival in freshwater (0.153), but did not differ significantly from the FW–FW mean in this environment ($P \approx 0.319$). However, the SW–SW cross had much higher mean survival in saltwater (0.540; $P < 0.001$), which was also reflected in a significant difference between slopes ($P < 0.001$).

Indirect fitness traits (i.e., size and growth variables) proved to be uninformative when analyzed independently. Results, however, are available as supporting information (Fig. S2). Although there was evidence supporting an additive model incorporating genotypic and environmental effects describing trends in larval growth rate (Fig. S2A), we detected no significant patterns of G × E for any growth-related trait (Fig. S2), nor was there any significant family-level variation in trait plasticity (i.e., model slopes) for either deme (Table 2). PC analyses yielded an eigenvector (PC1) accounting for 47% of the variation in size and growth data, and positively correlated with relative survival (Fig. 2). Because this relationship was observed in both the freshwater ($r = 0.199$; $P = 0.031$) and saltwater treatments ($r = 0.266$; $P < 0.001$), we concluded that PC1 scores could serve as useful proxies for individual fitness.

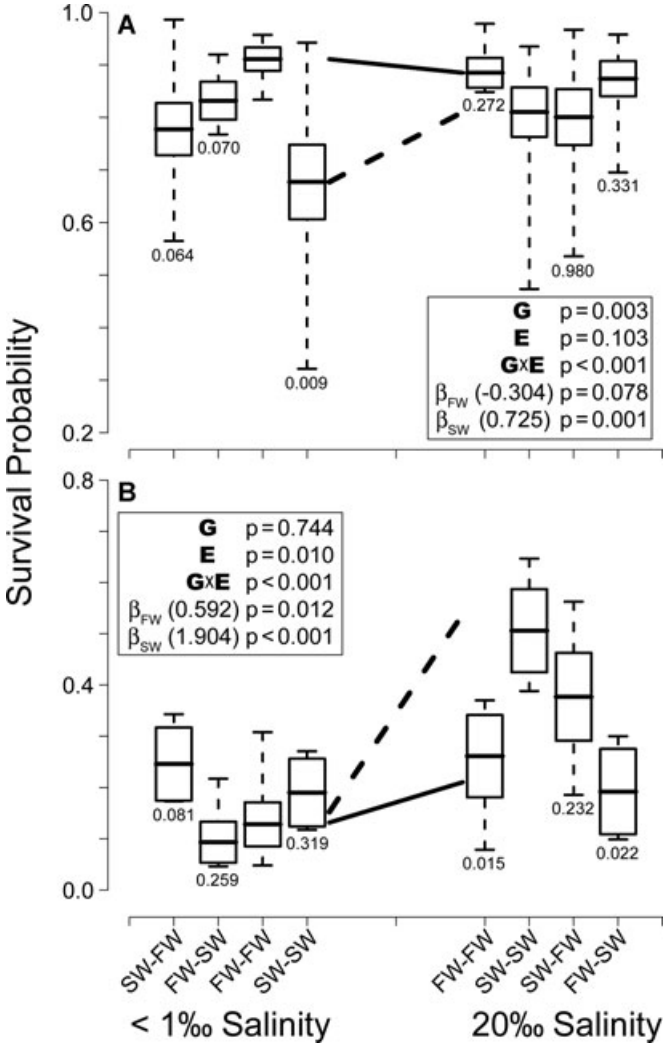

Figure 1. Larval (A) and juvenile (B) survival in freshwater (< 1‰) and saltwater (20‰) environments. Boxes represent estimated cross means ± standard error, conditional on random variation due to dam and sire effects; whiskers denote quartiles of the raw data. Significance of differences between hybrid and foreign crosses, contrasted with the pure cross native to each environment (i.e., FW–FW in freshwater; SW–SW in saltwater), are presented below the respective box-plots. Solid lines denote mean reaction norms for pure freshwater crosses (β_{FW}), conditional on random variation among families, whereas dashed lines correspond to those of pure maritime crosses (β_{SW}). Model results are offset in text boxes. Significance of coefficients from the reaction norm analyses, as estimated by MCMC sampling of their posterior distributions, is labeled accordingly. Independent estimates of reaction norm slopes (β) for each deme are presented in parentheses.

Overall fitness metrics suggested that crosses tended to perform better in their native salinity than in the reciprocal environment (Fig. 3); however, both the magnitude and significance of this effect varied. FW–FW crosses exhibited a trend toward reduced fitness in saltwater, but this was not significant for relative survival ($P \approx 0.543$), nor for the individual fitness proxy ($P \approx 0.769$). In contrast, SW–SW crosses exhibited a nearly significant reduction in relative survival when in freshwater ($P \approx 0.079$), with support for this interpretation also coming from evidence for G × E of relative survival ($P \approx 0.053$). Although the maritime deme did not differ from the native cross in the freshwater environment ($P \approx 0.839$), in saltwater the relative survival of the SW–SW group was significantly greater than that of the freshwater deme ($P \approx 0.007$). Similar trends were observed for the individual fitness proxy (Fig. 3B). Reduction of individual fitness in response to the freshwater environment was less equivocal in the maritime deme ($P \approx 0.037$), although mean values were not statistically different from the FW–FW crosses in either environment (Fig. 3B).

Hybrid comparisons

Larval survival in freshwater was suggestive of reduced hybrid fitness; however, these differences did not reach significance (Fig. 1A). In saltwater, neither hybrid crosses differed from the native deme in early life. Differences, however, began to emerge throughout the experiment, with hybrid juvenile survival in saltwater apparently less than that of the native cross (Fig. 1B), although only crosses with freshwater dams (FW–SW) exhibited significant differences in mortality ($P \approx 0.022$). Conversely, in freshwater neither hybrid crosses differed significantly from the native deme (Fig. 1B), although one showed a trend toward increased survival (SW–FW; $P \approx 0.081$). These same patterns were also reflected in relative fitness data (Fig. 3A): although apparent differences were less pronounced in freshwater, in saltwater, contrasts with the native deme were greater. However, no significant differences were observed for individual fitness (PC1) in either environment (Fig. 3B).

CANDIDATE GENES FOR OSMOREGULATION

Transcription of the CFTR gene was significantly influenced by environmental salinity in all crosses (Fig. 4A). CFTR was upregulated in freshwater, ranging from 1.5- to 2.3-fold greater; however, differences among crosses were not significant. IGF expression was also significantly upregulated in freshwater, but only for the FW–FW cross (Fig. 4B; $P \approx 0.009$). The slope describing the environmental effect in the SW–SW cross was not significant ($P \approx 0.204$), and was significantly different from the freshwater deme's reaction norm ($P \approx 0.007$). Interestingly, random variation in IGF plasticity was also significant in SW–SW, but not for FW–FW crosses (Table 2). NAK exhibited marked environmental effects in all crosses (Fig. 4C), with expression ranging from 1.7- to 2.8-fold greater in freshwater. Pure crosses also displayed a significant pattern of G × E ($P \approx 0.002$), although as with IGF, we also detected significant individual (within family) variation in NAK plasticity within the saltwater deme. Average gene

Table 2. Variance components of pure crosses used in reaction norm analyses testing for genotype–environment interactions. Random variation in model intercepts (mean) and slopes is partitioned among full-sibling families. Bootstrapped data of candidate gene expression also incorporates variation among plates and individuals, nested within families. Maximum likelihood estimates of variance are presented, in addition to the proportion of total variance (in parentheses). Significance of random variation in slopes (i.e., plasticity) was evaluated by information theory (AIC) and likelihood ratio tests (*P*-Value). Individual environmental effects are also modeled for each deme, separately.

Modeled variable	Variance component	G×E model			FW deme: environmental effect			SW deme: environmental effect		
		Variance (prop)	AIC	*P*-value	Variance (prop)	AIC	*P*-value	Variance (prop)	AIC	*P*-value
Fitness metrics										
larval	family (mean)	2.38 (0.411)	292.2		1.78 (0.208)	204.5		3.02 (0.761)	88.1	
survival	family (slope)	2.51 (0.434)	227.2	<0.001	5.88 (0.689)	127.4	<0.001	0.08 (0.020)	92.0	0.941
	residual	0.90 (0.155)			0.88 (0.103)			0.87 (0.219)		
juvenile	family (mean)	0.75 (0.334)	98.0		1.06 (0.429)	56.4		0.21 (0.104)	42.8	
survival	family (slope)	0.58 (0.258)	92.0	0.007	0.53 (0.213)	53.7	0.035	0.83 (0.414)	43.0	0.143
	residual	0.92 (0.408)			0.88 (0.357)			0.97 (0.482)		
relative	family (mean)	0.28 (0.380)	80.1		0.32 (0.493)	37.1		0.25 (0.286)	43.4	
survival	family (slope)	0.32 (0.436)	83.5	0.752	0.23 (0.349)	40.8	0.846	0.45 (0.517)	45.9	0.480
	residual	0.14 (0.185)			0.10 (0.159)			0.17 (0.198)		
PC1	family (mean)	0.17 (0.124)	444.3		0.19 (0.126)	220.6		0.17 (0.130)	227.6	
	family (slope)	0.33 (0.239)	445.3	0.228	0.40 (0.274)	222.0	0.272	0.24 (0.185)	230.9	0.683
	residual	0.88 (0.638)			0.89 (0.600)			0.88 (0.685)		
Size and growth variables										
larval	family (mean)	3.0×10^{-4} (0.470)	−9163.6		9.8×10^{-5} (0.238)	−6104.7		4.8×10^{-4} (0.556)	−3065.8	
growth	family (slope)	1.7×10^{-13} (0.000)	−9159.6	0.999	1.9×10^{-6} (0.005)	−6101.7	0.596	3.4×10^{-6} (0.004)	−3062.2	0.842
	residual	3.4×10^{-4} (0.530)			3.1×10^{-4} (0.757)			3.8×10^{-4} (0.441)		
final SL	family (mean)	28.86 (0.482)	984.1		6.19 (0.166)	472.6		50.23 (0.598)	510.6	
	family (slope)	7.31 (0.122)	984.9	0.203	8.27 (0.222)	475.2	0.499	9.20 (0.110)	511.8	0.254
	residual	23.65 (0.395)			22.79 (0.612)			24.52 (0.292)		
relative	family (mean)	2.01 (0.068)	940.6		2.36 (0.081)	456.0		0.65 (0.026)	486.5	
condition	family (slope)	5.74 (0.196)	942.7	0.387	6.74 (0.230)	458.5	0.461	1.00 (0.040)	490.4	0.971
	residual	21.53 (0.735)			20.22 (0.689)			23.51 (0.934)		

Continued.

Table 2. Continued.

Modeled variable	Variance component	G×E model			FW deme: environmental effect			SW deme: environmental effect		
		Variance (prop)	AIC	*P*-value	Variance (prop)	AIC	*P*-value	Variance (prop)	AIC	*P*-value
Candidate gene expression (relative mRNA transcription)										
CFTR	family (mean)	0.01 (0.079)	2350.7		0.02 (0.054)	1001.9		4.4×10^{-3} (0.016)	1347.3	
	family (slope)	3.1×10^{-11} (0.000)	2258.0		4.6×10^{-6} (0.000)	969.5		3.5×10^{-3} (0.012)	1295.7	
	individual (mean)	0.11 (0.585)	1437.1		0.22 (0.736)	812.3		0.21 (0.756)	626.8	
	individual (slope)	3.1×10^{-11} (0.000)	1444.6	0.925	3.1×10^{-11} (0.000)	820.3	>0.999	3.0×10^{-11} (0.000)	634.8	>0.999
	plate	3.1×10^{-11} (0.000)	1451.1	>0.999	—	—	—	—	—	—
	residual	0.06 (0.335)			0.06 (0.210)			0.06 (0.216)		
IGF	family (mean)	0.18 (0.240)	4772.5		0.06 (0.163)	1034.0		0.32 (0.328)	3403.3	
	family (slope)	0.16 (0.215)	3992.5		0.06 (0.140)	945.8		0.28 (0.283)	2837.4	
	individual (mean)	0.28 (0.370)	268.2		0.22 (0.553)	197.3		0.33 (0.333)	68.8	
	individual (slope)	0.07 (0.095)	264.8	0.010	1.1×10^{-5} (0.000)	204.3	0.908	2.8×10^{-11} (0.000)	66.2	0.032
	plate	4.2×10^{-3} (0.006)	268.1	0.442	—	—	—	—	—	—
	residual	0.06 (0.074)			0.06 (0.143)			0.06 (0.056)		
NAK	family (mean)	2.2×10^{-3} (0.011)	1812.4		3.2×10^{-3} (0.020)	698.3		4.8×10^{-3} (0.010)	1109.6	
	family (slope)	3.0×10^{-11} (0.000)	1602.1		2.5×10^{-3} (0.015)	550.2		3.0×10^{-11} (0.000)	1043.6	
	individual (mean)	0.08 (0.388)	927.8		0.10 (0.603)	427.2		0.23 (0.456)	502.9	
	individual (slope)	0.04 (0.169)	920.7	0.002	3.0×10^{-11} (0.000)	435.2	>0.999	0.21 (0.416)	499.2	0.020
	plate	0.03 (0.148)	929.8	0.995	—	—	—	—	—	—
	residual	0.06 (0.285)			0.06 (0.363)			0.06 (0.119)		
VATP	family (mean)	2.8×10^{-3} (0.027)	2056.9		3.3×10^{-11} (0.000)	718.3		4.1×10^{-3} (0.029)	1322.2	
	family (slope)	3.3×10^{-11} (0.000)	2035.1		3.2×10^{-11} (0.000)	712.3		3.3×10^{-11} (0.000)	1312.8	
	individual (mean)	0.03 (0.337)	2236.8		0.06 (0.500)	932.1		0.07 (0.510)	1305.1	
	individual (slope)	3.3×10^{-11} (0.000)	2234.3	0.015	3.2×10^{-11} (0.000)	940.1	>0.999	3.0×10^{-11} (0.000)	1313.1	>0.999
	plate	3.3×10^{-11} (0.000)	2250.8	>0.999	—	—	—	—	—	—
	residual	0.07 (0.636)			0.06 (0.500)			0.07 (0.461)		

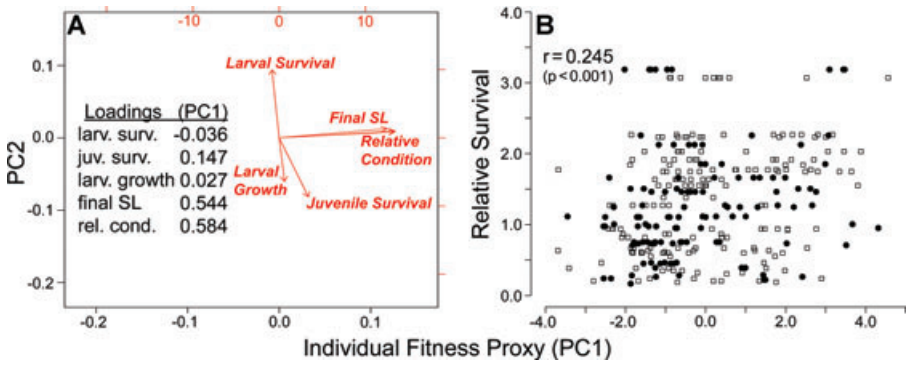

Figure 2. Principal components analysis of fitness related traits (A). The first eigenvector (PC1) captures 47% of the variation in component traits, for which loadings are presented. Individual scores for PC1 were used as a proxy for individual fitness. Correlation between this fitness proxy and family-specific relative survival (B). Individuals reared in freshwater (<1‰) are plotted with closed circles, whereas open squares denote individuals from saltwater (20‰).

expression in the SW–SW cross was 1.2-fold greater than that of the FW–FW cross in freshwater, although this difference was not significant ($P \approx 0.203$); however, significant upregulation (1.3-fold; $P \approx 0.033$) and a trend toward downregulation (0.8-fold; $P \approx 0.062$), relative to the native deme, was observed for hybrids of saltwater dams (SW–FW) and sires (FW–SW), respectively. In saltwater, FW–FW NAK expression was 1.2-fold greater ($P \approx 0.016$) than the native deme; hybrid expression did not differ significantly (Fig. 4C). VATP expression was unaffected by environmental salinity (Fig. 4D). There was a trend for greater levels of transcription in the SW–SW cross, although the apparent difference was not significant ($P \approx 0.129$), nor were hybrid crosses significantly different from pure crosses in their native environments, apart from a relative downregulation in saltwater of the SW–FW cross (0.89-fold; $P \approx 0.037$).

Heritability and selection gradients

With the exception of CFTR, we detected significant additive genetic variance for relative transcription rates of all candidate genes, which allowed calculating narrow sense heritabilities (h^2) that excluded zero for IGF, NAK, and VATP (Table 3). Genetic correlations between CFTR and other candidate genes were negative; however, because h^2 was not significant for CFTR, we could not calculate sampling errors for the estimates. As such, these correlations must be assumed to be nonsignificant. In contrast, all other pairwise genetic correlations were estimated to be positive. However, only the correlation between IGF and VATP expression was significant, that is, the difference between the estimate and the sampling error excluded zero (Table 3). We also estimated significant directional selection gradients for both CFTR and NAK expression, whereas only VATP exhibited a significant quadratic (stabilizing) selection coefficient (Table 4). When data corresponding to the different rearing environments were analyzed separately, we observed that these relationships were indeed environment specific. The directional selection gradient estimated for NAK was not significant in saltwater, and equivocal in the freshwater environment ($P \approx 0.077$; however, the 95% HPD interval excluded zero, ranging between 0.001 and 0.109). In contrast, a significant negative selection coefficient for CFTR was only observed in saltwater ($P \approx 0.003$; 95% HPD: -0.091–-0.031).

Discussion

Genotype–environment interaction for fitness is often indicative of adaptive differentiation, and the precise form of this interaction may be useful to infer the specific nature of the divergence. For example, crossing reaction norms represent the unequivocal satisfaction of a "local versus foreign criterion," wherein resident genotypes in their respective environments have higher relative fitness than genotypes originating from other habitats, and thus, signify local adaptation. Indeed, it has been argued that satisfaction of the local versus foreign criterion is the requisite test necessary to infer local adaptation (Kawecki and Ebert 2004). However, given that environment-specific fitness trade-offs may not be a universal characteristic of all locally adapted demes (Hereford 2009), dogmatic adoption of this strict definition might exclude many informative examples of local adaptation. Moreover, other patterns of divergent fitness reaction norms, although perhaps equivocal regarding local adaptation per se, have been indispensable in revealing more cryptic examples of adaptation, such as the phenomenon of counter gradient variation (Conover and Schultz 1995). Thus, reaction norms for fitness are clearly indicative of adaptive differentiation between freshwater and maritime stickleback demes. Genotype–environment interaction for relative survival is effectively significant ($P \approx 0.053$), and model slopes suggest that each deme's fitness is reduced in their nonnative salinity (Fig. 3A), although only that of the saltwater deme truly approaches significance ($P \approx 0.079$). Patterns in component data (i.e., absolute survival) are equally revealing, in which the

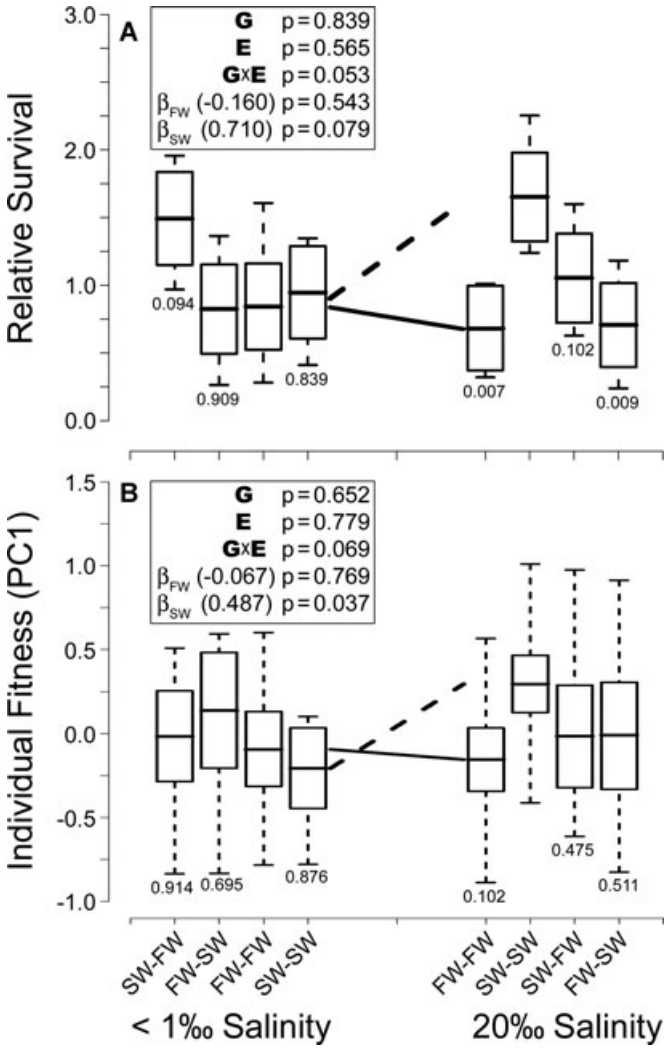

Figure 3. Environment-specific relative survival (A) and individual fitness (B), as estimated from the first eigenvector score combining all fitness related traits (see Methods for details). Boxes represent estimated group means and their 95% posterior density intervals, conditional on random variation due to dam and sire effects; whiskers denote quartiles of the raw data. Reaction norms, *P*-values, and modeling results are as per Figure 1.

resident freshwater deme exhibits a relatively small decrease (3.1%) in larval survival in saltwater, whereas the effect of freshwater on the maritime deme is more severe (16.4%). Moreover, this steep fitness cost in freshwater persists throughout the juvenile stage (Fig. 1B).

Poor juvenile survival in freshwater, however, does raise some concern regarding the generality of our conclusions. Stickleback reared under laboratory conditions are known to perform poorly in freshwater (Benjamin 1974); consequently, best practices for their husbandry typically involve rearing in slightly brackish water (3‰–5‰), regardless of the natal environment of source populations (University of Oregon Stickleback Research Site 2008). However, the salinities used in this experiment were selected specifically to mimic those of the natural environments of the two demes (McCairns and Bernatchez 2008). Furthermore, water used in the wet laboratory, although treated and dechlorinated, originated from the same source as that of the freshwater deme. Maintenance in brackish water instead of freshwater would likely have lead to better overall survival, given that all crosses tended to exhibit positive model slopes in analyses of juvenile survival (Fig. 1B; see also Table S2). However, we contend that any improvement in survival would have come at the expense of increased ambiguity in model interpretations, particularly given that our ultimate objective was to test for adaptation to the freshwater environment. Moreover, apart from the SW–SW cross, juvenile survival in saltwater is only marginally better than that in freshwater: in freshwater, average survival ranges between 10% and 25%, as opposed to 12% and 28% in saltwater. Therefore, although results should be interpreted cautiously, there is no reason to suggest that they are merely experimental artefacts. Indeed, exploration of variance components suggests a role for maternal effects: the dam component of mean larval and juvenile survival in both environments appears to capture a substantially higher proportion of variance than the sire component (Table S2), although we do not wish to overinterpret this observation given the lack of intrademe half-sibling crosses for comparison. Nevertheless, given that mitochondria-rich cells are crucial for ion exchange (Marshall 2002; Evans et al. 2005; Varsamos et al. 2005), it would not be surprising to discover that maternal contributions play an important role in salinity acclimation and/or adaptation.

THE FATE OF HYBRIDS

Given the lack of physical barriers to dispersal within the St. Lawrence estuary, there is considerable potential for movement and interbreeding between demes, potentially at the expense of locally adapted gene complexes. However, theoretical and empirical work has demonstrated that adaptive differentiation in the face of gene flow is possible if the strength of postzygotic selection exceeds the effective rate of migration (García-Ramos and Kirkpatrick 1997; Hendry et al. 2001; Lenormand 2002). Thus, selection against hybrids could help to maintain adaptive differentiation if gene flow were prevalent between demes. Of particular relevance is the fate of hybrids in the novel environment. Given the global colonization history of the species (Bell and Foster 1994; Ortí et al. 1994), and particularly the paleoecology of the St. Lawrence estuary, we can safely assume that freshwater represents the novel habitat type. Data for larval hybrids in freshwater suggest that absolute survival is 5.8% and 11.2% less than the native deme for families with maritime sires (FW–SW) and dams (SW–FW), respectively (Fig. 1A). However, these differences may only be judged significant if one were to hypothesize reduced larval survival a priori, thus, justifying treatment of data under a one-tailed analysis. Trends in juvenile survival are less

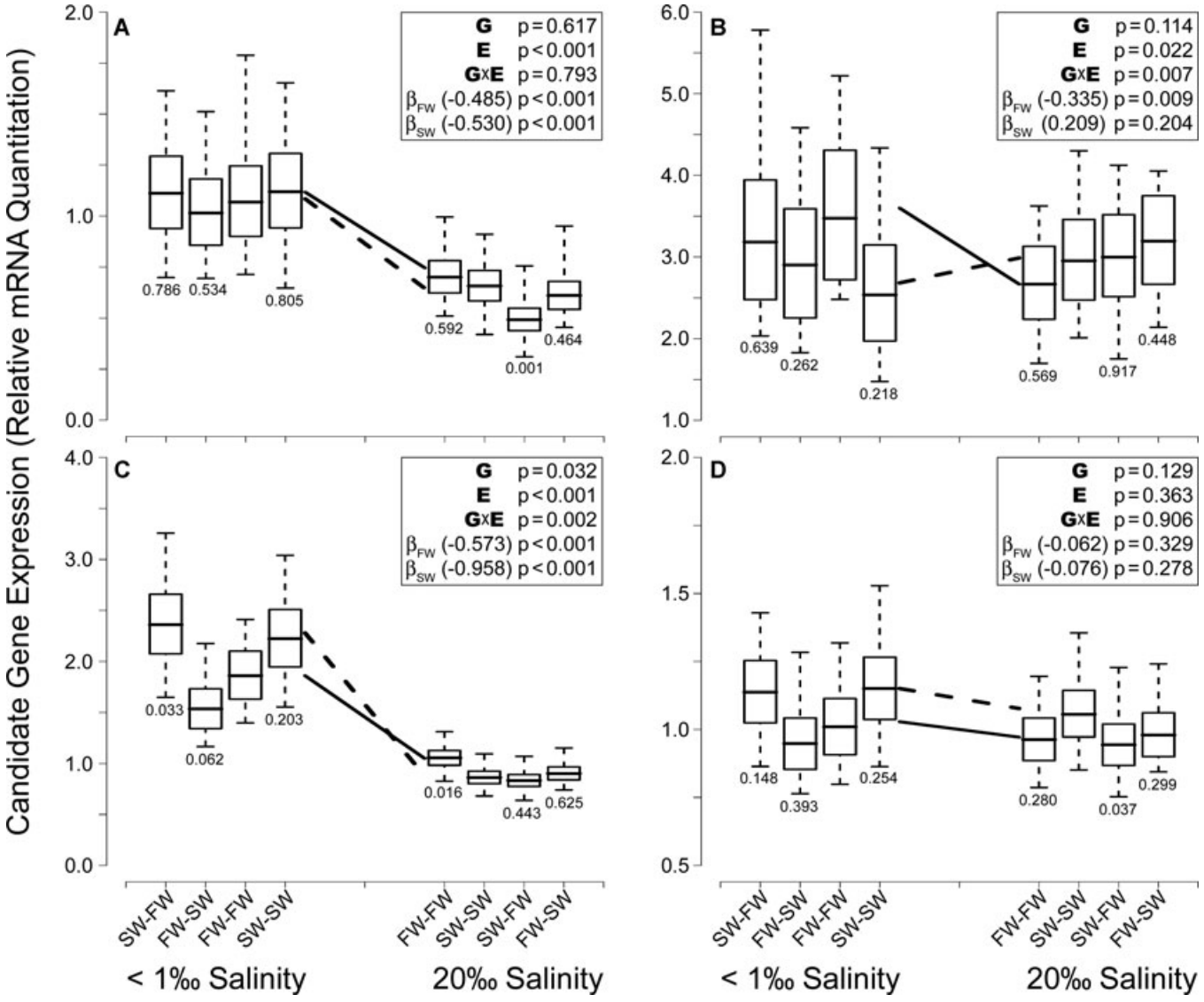

Figure 4. Relative quantitation of mRNA transcription for CFTR (A), IGF (B), NAK (C) and VATP (D). Data are normalized against EF1α, and quantitation estimated relative to a control individual replicated across all plates (see Methods for details). Box-plots, reaction norms, *P*-values, and modeling results are as per Figure 3.

pronounced (Fig. 1B), suggesting a slight decrease for FW–SW crosses (3.5%), and even increased survival for SW–FW hybrids (11.8%). Recent evidence, however, suggests that dispersal among stickleback populations may be male biased (Cano et al. 2008). If this is true, then hybrids derived from maritime dams (SW–FW) are less likely to exist in freshwater, and we may focus on the relative fitness of those with a maritime paternal lineage (FW–SW). By standardizing survival relative to that of the native FW deme (ω), we can estimate selection coefficients ($s = 1 - \omega$) in the novel environment (Orr 2009), suggesting reduced fitness

Table 3. Estimated heritabilities and genetic correlations. Diagonal elements list the heritabilities for candidate gene expression, with associated sampling errors in parentheses. Genetic correlations are presented in the lower triangle, with their corresponding sampling errors in parentheses. Significant estimates (i.e., those which exclude zero) are presented in bold type.

	CFTR	IGF	NAK	VATP
CFTR	0.031 (0.062)			
IGF	−0.958 (n.a.)	**0.161 (0.089)**		
NAK	−0.407 (n.a.)	0.348 (0.456)	**0.095 (0.076)**	
VATP	−0.427 (n.a.)	**0.655 (0.305)**	0.241 (0.451)	**0.197 (0.107)**

Table 4. Normalized multiple regression coefficients modeling the relationship between relative transcription levels of candidate genes for osmoregulation and a proxy for individual fitness (PC1). Coefficients define both linear (β) and quadratic (β^2) relationships simultaneously. Estimates and their corresponding 95% highest posterior density intervals (HPD) are based on MCMC sampling of the posterior distribution of the multiple regression model, conditional on random dam and sire effects. Data unique to each salinity treatment were also analyzed separately, for which point estimates and their significance are reported.

Gene	Coef.	All data		FW (<1‰)	SW (20‰)
		Est. (*P*-value)	HPD interval	Est. (*P*-value)	Est. (*P*-value)
CFTR	β	−0.039 (0.011)	−0.064 – −0.014	−0.016 (0.461)	−0.054 (0.003)
	β^2	0.025 (0.178)	−0.005 – 0.055	0.042 (0.175)	−0.027 (0.305)
IGF	β	−0.016 (0.143)	−0.035 – 0.003	−0.030 (0.116)	−0.018 (0.223)
	β^2	−0.009 (0.444)	−0.028 – 0.011	−0.018 (0.329)	0.016 (0.368)
NAK	β	0.049 (0.006)	0.019 – 0.078	0.057 (0.079)	0.019 (0.390)
	β^2	−0.001 (0.959)	−0.032 – 0.029	0.002 (0.979)	0.037 (0.084)
VATP	β	−0.019 (0.133)	−0.040 – 0.002	−0.036 (0.211)	−0.017 (0.357)
	β^2	0.032 (0.021)	0.009 – 0.055	0.026 (0.306)	0.032 (0.056)

in both larval ($s = 0.065$) and juvenile ($s = 0.273$) stages of FW–SW hybrids. Moreover, previous modeling of divergence with gene flow between these demes suggest that a selection differential greater than 0.006 would be sufficient to maintain adaptive divergence (McCairns and Bernatchez 2008). Thus, even these modest decreases in hybrid survival could be sufficient for persistent local adaptation in the face of gene flow.

INSIGHTS FROM CANDIDATE GENE EXPRESSION

Although fitness data support the hypothesis of adaptive divergence, some observations from the qPCR experiment were antithetical to our original expectations, and as such, warrant special consideration. The most striking was the observed upregulation of CFTR in freshwater (Fig. 4A). CFTR expression has been studied in a number of euryhaline teleosts, and in all instances, transcription in gill tissue is reportedly increased in response to saltwater (Scott et al. 2004a; Madsen et al. 2007; Tang and Lee 2007). However, nearly all studies we have examined in the physiological literature are based upon abrupt transfer of individuals from one environment to another, and focus on acute time periods (e.g., 1–48 h). Moreover, relatively longer-term studies (i.e., 30 days) suggest that increases may be transient, with expression levels tending to decline to control (i.e., freshwater) levels after 30 days (Singer et al. 2002; Mackie et al. 2007). As such, previous results may be more indicative of the physiological mechanisms underlying acclimation to dynamic salinity changes, as opposed to evolved responses to novel environments. Nevertheless, one comparative study of *Fundulus heteroclitus* populations known to differ in their tolerance to freshwater may shed light upon these unexpected observations. Scott and colleagues (2004b) demonstrated that transfer from brackish (10‰) to freshwater resulted in predicted decreases in CFTR expression; however, after 14 days, transcription levels posttransfer were comparable to those in brackish water, but only in fish from the more freshwater adapted population. These observations may lend some support to the largely untested hypothesis of a possible involvement of CFTR in freshwater ion transport (Marshall 2002; Marshall et al. 2002; Hwang and Lee 2007). Certainly future study is required to improve our generally poor understanding of freshwater osmoregulation, particularly regarding mechanisms underlying chloride ion influx (Perry 1997; Marshall 2002; Tresguerres et al. 2006).

In most reported cases, VATP is upregulated in freshwater (Piermarini and Evans 2001; Kaneko and Katoh 2004), and downregulated in saltwater (Reis-Santos et al. 2008). Thus, the lack of environmental effects on VATP expression was also unexpected (Fig. 4D), but serves to highlight the importance of transcript and/or isoform identification in candidate gene studies. VATP is composed of at least six subunits, but it is the "B" subunit that is coupled to sodium transport (Boesch et al. 2003; Kane 2005). More specifically, it is the B1 or "kidney" isoform whose kinetic properties have been linked to proton transfer through epithelial membranes, whereas the B2 isoform is involved in acidification of intracellular vesicles (Boesch et al. 2003; Schredelseker and Pelster 2004). However, a BLAST search of the stickleback genome revealed no region annotated as the B1 isoform; moreover, the closest stickleback ortholog to the well-studied zebrafish B1 sequence (GenBank accession no. AF472614) was annotated as the B2 form. Consequently, we targeted the H subunit, a domain critical to proper VATP function (Kane 2005). However, given VATP's alternate role in acid–base equilibration, it is likely that our target was too general to detect VATP expression unique to its putative role in freshwater ion influx.

IGF expression exhibited the crossing reaction norms indicative of locally adapted demes (Fig. 4B); however, even these results were surprising. The role of IGF as an osmoregulatory protein is perhaps best known from its association with the

process of smoltification in salmonids (Sakamoto and Hirano 1993; Sakamoto et al. 1995; McCormick 1996). Observations from other euryhaline species have also demonstrated transient increases in gill expression after saltwater transfer (Mancera and McCormick 1998; Tipsmark et al. 2007). Yet, rather than upregulation in saltwater, we observed a significant decrease in IGF expression unique to the pure FW–FW cross (Fig. 4B). Consequently, we have rejected IGF as a likely candidate gene for saltwater acclimation in sticklebacks. However, IGF is also thought to regulate prolactin production (Fruchtman et al. 2000), which in turn may serve as a hormonal regulator of proteins associated with ion uptake (McCormick 2001; Manzon 2002; Hirose et al. 2003). This pathway does seem plausible in light of the results of one study in which long-term cultures of marine sticklebacks could only be maintained successfully in freshwater with continued prolactin injections (Benjamin 1974). Thus, future research into freshwater osmoregulation in sticklebacks should consider both prolactin and IGF expression.

NAK expression is 1.7- to 2.8-fold higher in freshwater (Fig. 4C). This is consistent with studies of *F. heteroclitus*, in which an orthologous isoform exhibits both increased mRNA transcription and enzyme activity after freshwater transfer (Scott et al. 2005), and with kinetic modeling suggesting that NAK alone can provide sufficient energy to promote sodium uptake against an unfavorable electrochemical gradient (Kirschner 2004). Given significant heritable variation for its expression (Table 3), in addition to a positive correlation with fitness, particularly in freshwater (Table 4), we predict that NAK is likely involved in adaptation to the freshwater environment. It is somewhat perplexing then, that relative NAK quantitation does not differ between demes in the freshwater environment ($P \approx 0.203$). However, their reaction norms are significantly different, and in saltwater, NAK expression for the freshwater deme is 1.2-fold greater than the maritime deme ($P \approx 0.016$). Interestingly, this pattern may be suggestive of adaptation mediated by ancestral plasticity, a model that has also emerged to explain morphological divergence within the species (Wund et al. 2008).

ADAPTIVE ANCESTRAL PLASTICITY

Although reaction norms for fitness components can be diagnostic for local adaptation, it is likely erroneous to discuss plasticity in survival per se. Rather, the environmental effects described in reaction norms for survival are indicative of the overall functional plasticity of a given group. From this perspective, the freshwater deme might appear to be the more functionally plastic, given that survival differed only marginally between environments (Fig. 1). This is surprising because the freshwater region of the estuary not only represents a novel habitat type, but also represents a more stable salinity regime: within the freshwater, fluvial estuary there is no influx of saltwater, whereas nearly half of the maritime deme's range experiences diurnal salinity fluctuations from 5‰ to 30‰ (Vincent and Dodson 1999; McCairns and Bernatchez 2008). Furthermore, salinity within the tidal marshes of the maritime deme's breeding/nursery sites may also vary depending on the relative precipitation in any given year. Thus, maritime sticklebacks are likely to experience periods of reduced salinity, if not freshwater, at some point in their lifecycle, whereas freshwater individuals will be exposed to constant, hypo-osmotic conditions. Interestingly, both demes exhibited increased juvenile survival in response to environmental salinity, although this environmental effect was substantially greater in the maritime deme. Moreover, a cumulative selection estimate, accounting for both the positive salinity effect and decreased survival relative to the native deme, suggests a 22% reduction of relative survival ($s = 0.217$) for the freshwater deme in saltwater. Yet within the freshwater environment, juvenile survival was similar between demes. This suggests that functional plasticity within the ancestral group may have facilitated survival in the novel environment, but subsequent adaptation to freshwater appears to have come at the cost of reduced fitness in the ancestral environment (Ghalambor et al. 2007).

Trait plasticity also appears to have been reduced in the derived population. The absolute values of the freshwater deme's reaction norm slopes were less than that of the saltwater deme in three of the four candidate genes studied (Fig. 4); however, only three of the targeted transcripts are likely involved in freshwater osmoregulation, and only two exhibited statistically significant differences. Yet the same trend was also observed for all growth-related traits (Fig. S2); thus, in six of seven traits, the maritime deme exhibited a trend toward greater plasticity than that of the freshwater deme (binomial exact test; $P = 0.063$). This begs the question of whether the relatively constant freshwater environment presents a reduction of selective pressures favoring plasticity, thereby allowing for drift and eventual loss (Masel et al. 2007), although large effective population sizes suggest drift may not be the likeliest explanation (McCairns and Bernatchez 2008). Conversely, could selection actually favor increased plasticity in the maritime deme? Certainly individual components of a reaction norm (e.g., the slope), and thus plasticity, may be subject to selection given intragenerational environmental heterogeneity and character (i.e., trait) lability within an organisms' lifetime (Via et al. 1995). Candidate gene expression conform to these conditions, although variable trait plasticity and environmental heterogeneity alone are not sufficient evidence to infer an adaptive value for trends in reaction norm slopes. Nevertheless, we must consider the potential adaptive value of such physiological plasticity itself; and although we do not have the data to test these hypotheses explicitly, that is, measures of individual plasticity to correlate with individual fitness, all hinge on the controversial assumptions underlying the argument for adaptive plasticity.

There is a general consensus that under certain demographic/ecological conditions plasticity can be advantageous, that is, "adaptive" in a broad sense (Gotthard and Nylin 1995; Via et al. 1995; Dudley and Schmitt 1996; Hollander 2008); however, there is considerable debate as to whether plasticity can be considered a sensu stricto "adaptation" to environmental heterogeneity. This controversy stems from two related and largely unresolved issues: is plasticity actually a character state unto itself, separate from mean trait values across environments; and if so, is there underlying additive genetic variance associated with its differential expression? Via (1993) has argued that interpopulation differences in reaction norm shape can result from directional selection on trait means in divergent environments. Moreover, treating plasticity as an independently evolving character necessitates the assumption of separate genetic control, although such "plasticity genes" may exist as regulatory elements within the genome (Schlichting and Pigliucci 1995; Schlichting and Smith 2002). For reaction norms to evolve, there must also be genetic variation independent of mean population-level environment effects (Via and Lande 1985; Gomulkiewicz and Kirkpatrick 1992). Theory suggests that additive variance for plasticity is possible (Scheiner and Lyman 1989), although it is generally weak (Scheiner 1993), and has been exceedingly difficult to quantify.

Recent studies have proposed a solution to the difficulty of estimating additive variance for plasticity by using REML mixed-model analysis, in which mean effects of cohort/population and environmental interactions are controlled as fixed model terms, and variation among families are incorporated as random effects (Brommer et al. 2005; Nussey et al. 2005a,b; Charmantier et al. 2008). Additive variance in plasticity is inferred by random variation in the coefficient describing environmental effects, and its significance can be evaluated via likelihood ratio test. Using the same analytical framework, we detected no significant random variation in reaction norm slopes for CFTR or VATP (Table 2). NAK and IGF exhibited significant interfamily and individual variation in slopes, but only within the saltwater deme (Table 2). These estimates based on full-sibling family groups, however, represent broad-sense genetic variation, and likely include both nonadditive and maternal sources of variation. This appears to be confirmed in the full analysis of all crosses, in which variation in NAK plasticity is effectively partitioned only into the dam component (Table S2). In contrast, variation among individuals within families, which is analogous to additive variance within an animal model, remains significant for IGF. Thus, IGF appears to be the only trait for which additive variance in plasticity is likely. Ironically, this is also the only trait exhibiting greater plasticity in the freshwater deme, yet additive variance is most likely only present within the saltwater deme, which showed no significant plasticity (Fig. 4B). Additionally, environment-specific selection gradients for NAK and CFTR suggest differential selection in freshwater and saltwater, thus, corresponding to Via's (1993) alternative explanation for reaction norm differences. Altogether, these observations do not support the hypothesis that plasticity could respond to selection, thus, it is unlikely that plasticity has increased in the maritime deme. Consequently, we are left to consider the loss of plasticity in the derived population.

Genetic assimilation and the loss of plasticity

Genetic assimilation is a form of canalization in which an environmentally induced trait becomes genetically entrained, that is, no longer dependent upon the environmental stimulus for its expression in subsequent generations (Waddington 1942; Crispo 2007). Although originally rejected as a curiosity unique to artificial environments during the framing of the Modern Synthesis, recent interest has grown due to its hypothesized role as a potential source of both phenotypic and genetic novelty (West-Eberhard 2005). Although this remains a matter of debate, its theoretical underpinnings have proven plausible (Eshel and Matessi 1998; Price et al. 2003), and as in the case of adaptive plasticity, the most cogent hypotheses confer an important role to regulatory genes (Eshel and Matessi 1998; Behera and Nanjundiah 2004). Empirical studies demonstrating the effect are rare, and the best-documented evidence of the phenomenon involve physiological induction (Waddington 1953; Waddington 1959; Chapman et al. 2000), pathways perhaps most reliant upon regulatory genes. The one element common to both theoretical and empirical work is that genetic assimilation necessarily leads to a loss of plasticity (de Jong 2005; Crispo 2007; Lande 2009). When applied to quantitative traits, this should be reflected by less acute reaction norms in the derived population.

Given the marine origins of *G. aculeatus* (Bell and Foster 1994; Ortí et al. 1994), and no support for the hypothesis of increased plasticity via selection in the maritime estuary, SW–SW reaction norms should be a good representation of ancestral osmoregulatory plasticity. If this is so, then observed differences in reaction norms are indicative of a loss of plasticity in the novel freshwater environment. With the exception of IGF, both demes exhibited common direction of reaction norm slopes, thereby suggesting similarity in osmoregulatory capacity and/or pathways. If colonization of the freshwater environment was facilitated by ancestral osmoregulatory plasticity, the extant pattern of reduced plasticity could be indicative of adaptation via genetic assimilation (Crispo 2007). Furthermore, given the canalizing nature of genetic assimilation, trait means in the ancestral environment are predicted to be shifted in the direction of trait means in the novel/inducing environment. Thus, for genes upregulated in response to freshwater, a comparison between derived and ancestral groups in saltwater should reveal greater mean expression in the derived group. In the case of our most likely candidate gene conferring adaptation to the novel freshwater environment (NAK),

when measured in the ancestral environment (20‰), mRNA transcription in the derived group (FW–FW) was 1.2-fold greater than in the putatively ancestral deme.

Finally, it must be noted that our interpretations are contingent upon the assumption that inadvertent artificial selection in the laboratory crosses has not unduly influenced survival, and consequently biased the patterns inferred from reaction norm analyses. We have no reason to suspect this source of bias, but our inability to rigorously refute it warrants an explicit caveat. Furthermore, similar interpretations of process (i.e., genetic assimilation) from pattern (i.e., reduced plasticity) have been thoroughly and reasonably criticized on the grounds that offering only "indirect support" is not equivalent to direct testing, and that alternative quantitative genetic models (i.e., selection on plasticity) can also account for such patterns (de Jong 2005). We readily admit that our interpretation offers only such indirect support for the hypothesis of genetic assimilation. Perhaps more direct support could be gleaned by comparing additive genetic variance for trait expression between ancestral and derived groups. Unfortunately, given our pedigree design we cannot rigorously estimate deme-specific heritabilities. However, we have clearly considered and rejected the requisite hypothesis of additive variance for plasticity. As such, we are lead to consider the possibility of genetic assimilation, although further study will be required to test this hypothesis explicitly. Our limited, post hoc interpretation is included with the aim of providing a heuristic example for future research, to help identify potential sources of phenotypic and genotypic variance, and their related environmental stimuli, that might lead to better, explicit tests for an hypothesis seeking its place within the Modern Synthesis (West-Eberhard 2005; Pigliucci 2007).

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LITERATURE CITED

Alpert, P., and E. L. Simms. 2002. The relative advantages of plasticity and fixity in different environments: when is it good for a plant to adjust? Evol. Ecol. 16:285–297.

Bates, D. M. 2007. lme4: linear mixed-effects models using S4 classes. R package version 0. 99875–99879.

Behera, N., and V. Nanjundiah. 2004. Phenotypic plasticity can potentiate rapid evolutionary change. J. Theor. Biol. 226:177–184.

Bell, M. A., and S. A. Foster. 1994. Introduction to the evolutionary biology of the threespine stickleback. Pp. 1–27 *in* S. A. Foster, ed. The evolutionary biology of the threespine stickleback. Oxford Univ. Press, Oxford, U.K.

Benjamin, M. 1974. Seasonal changes in the prolactin cell of the pituitary gland of freshwater stickleback, *Gasterosteus aculeatus*, form Leiurus. Cell Tissue Res. 152:93–102.

Bernatchez, L. 1997. Mitochondrial DNA analysis confirms the existence of two glacial races of rainbow smelt *Osmerus mordax* and their reproductive isolation in the St Lawrence River estuary (Quebec, Canada). Mol. Ecol. 6:73–83.

Bertalanffy, L. V. 1957. Quantitative laws in metabolism and growth. Q. Rev. Biol. 32:217–231.

Boesch, S. T., B. Eller, and B. Pelster. 2003. Expression of two isoforms of the vacuolar-type ATPase subunit B in the zebrafish *Danio rerio*. J. Exp. Biol. 206:1907–1915.

Brommer, J. E., J. Merilä, B. C. Sheldon, and L. Gustafsson. 2005. Natural selection and genetic variation for reproductive reaction norms in a wild bird population. Evolution 59:1362–1371.

Bystriansky, J. S., J. G. Richards, P. M. Schulte, and J. S. Ballantyne. 2006. Reciprocal expression of gill Na^+K^+-ATPase alpha-subunit isoforms alpha 1a and alpha 1b during seawater acclimation of three salmonid fishes that vary in their salinity tolerance. J. Exp. Biol. 209:1848–1858.

Bystriansky, J. S., N. T. Frick, J. G. Richards, P. M. Schulte, and J. S. Ballantyne. 2007. Wild Arctic char (*Salvelinus alpinus*) upregulate gill Na^+K^+-ATPase during freshwater migration. Physiol. Biochem. Zool. 80:270–282.

Cano, J. M., H. S. Makinen, and J. Merilä. 2008. Genetic evidence for male-biased dispersal in the three-spined stickleback (*Gasterosteus aculeatus*). Mol. Ecol. 17:3234–3242.

Chapman, L. J., F. Galis, and J. Shinn. 2000. Phenotypic plasticity and the possible role of genetic assimilation: hypoxia-induced trade-offs in the morphological traits of an African cichlid. Ecol. Lett. 3:387–393.

Charmantier, A., R. H. McCleery, L. R. Cole, C. Perrins, L. E. B. Kruuk, and B. C. Sheldon. 2008. Adaptive phenotypic plasticity in response to climate change in a wild bird population. Science 320:800–803.

Conover, D. O., and E. T. Schultz. 1995. Phenotypic similarity and the evolutionary significance of countergradient variation. Trends Ecol. Evol. 10:248–252.

Cork, J. M., and M. D. Purugganan. 2004. The evolution of molecular genetic pathways and networks. BioEssays 26:479–484.

Côté, G., G. Perry, P. Blier, and L. Bernatchez. 2007. The influence of gene-environment interactions on GHR and IGF-I expression and their association with growth in brook charr, *Salvelinus fontinalis* (Mitchill). BMC Genet. 8

Crispo, E. 2007. The Baldwin effect and genetic assimilation: revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. Evolution 61:2469–2479.

———. 2008. Modifying effects of phenotypic plasticity on interactions among natural selection, adaptation and gene flow. J. Evol. Biol. 21:1460–1469.

Crossman, E. J., and D. E. McAllister. 1986. Zoogeography of freshwater fishes of the Hudson Bay drainage, Ungava Bay and the Arctic Archipelago. Pp. 53–104 *in* C. H. Hocutt and E. O. Wiley, eds. The Zoogeography of North American freshwater fishes. John Wiley & Sons, New York, NY.

de Jong, G. 2005. Evolution of phenotypic plasticity: patterns of plasticity and the emergence of ecotypes. New Phytol. 166:101–117.

DeWitt, T. J., A. Sih, and D. S. Wilson. 1998. Costs and limits of phenotypic plasticity. Trends Ecol. Evol. 13:77–81.

Dudley, S. A., and J. Schmitt. 1996. Testing the adaptive plasticity hypothesis: density-dependent selection on manipulated stem length in *Impatiens capensis*. Am. Nat. 147:445–465.

Endler, J. A. 1986. Natural selection in the wild. Princeton Univ. Press, Princeton, NJ.

Ensembl Genome Browser. 2008. Stickleback genome (*Gasterosteus aculeatus*). Available at http://www.ensembl.org/index.html. Accessed April 6, 2008.

Eshel, I., and C. Matessi. 1998. Canalization, genetic assimilation and preadaptation: a quantitative genetic model. Genetics 149:2119–2133.

Evans, D. H., P. M. Piermarini, and K. P. Choe. 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85:97–177.

Fruchtman, S., L. Jackson, and R. Borski. 2000. Insulin-like growth factor I disparately regulates prolactin and growth hormone synthesis and secretion: studies using the teleost pituitary model. Endocrinology 141:2886–2894.

Gabriel, W. 2006. Selective advantage of irreversible and reversible phenotypic plasticity. Arch. Hydrobiol. 167:1–20.

García-Ramos, G., and M. Kirkpatrick. 1997. Genetic models of adaptation and gene flow in peripheral populations. Evolution 51:21–28.

Ghalambor, C. K., J. K. McKay, S. P. Carroll, and D. N. Reznick. 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. Funct. Ecol. 21:394–407.

Gibson, G., and G. Wagner. 2000. Canalization in evolutionary genetics: a stabilizing theory? BioEssays 22:372–380.

Gomulkiewicz, R., and M. Kirkpatrick. 1992. Quantitative genetics and the evolution of reaction norms. Evolution 46:390–411.

Gotthard, K., and S. Nylin. 1995. Adaptive plasticity and plasticity as an adaptation: a selective review of plasticity in animal morphology and life-history. Oikos 74:3–17.

Harington, C. R. 1988. Marine mammals of the Champlain Sea, and the problem of whales in Michigan. Pp. 225–240 *in* N. R. Gadd, ed. The late quaternary development of the champlain sea basin. Geological Association of Canada, St. John's, Newfoundland, Canada.

Hendry, A. P., T. Day, and E. B. Taylor. 2001. Population mixing and the adaptive divergence of quantitative traits in discrete populations: a theoretical framework for empirical tests. Evolution 55:459–466.

Hereford, J. 2009. A quantitative survey of local adaptation and fitness tradeoffs. Am. Nat. 173:579–588.

Hillaire-Marcel, C. 1988. Isotopic composition (^{18}O, ^{13}C, ^{14}C) of biogenic carbonates in Champlain Sea sediments. Pp. 177–194 *in* N. R. Gadd, ed. The late quaternary development of the champlain sea basin. Geological Association of Canada, St. John's, Newfoundland, Canada.

Hirose, S., T. Kaneko, N. Naito, and Y. Takei. 2003. Molecular biology of major components of chloride cells. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 136:593–620.

Hoffmann, A. A., C. M. Sgro, and S. H. Lawler. 1995. Ecological population genetics: the interface between genes and the environment. Annu Rev. Genet. 29:349–370.

Hollander, J. 2008. Testing the grain-size model for the evolution of phenotypic plasticity. Evolution 62:1381–1389.

Hwang, P. P., and T. H. Lee. 2007. New insights into fish ion regulation and mitochondrion-rich cells. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 148:479–497.

Jones, F. C., C. Brown, J. M. Pemberton, and V. A. Braithwaite. 2006. Reproductive isolation in a threespine stickleback hybrid zone. J. Evol. Biol. 19:1531–1544.

Kane, P. M. 2005. Close-up and genomic views of the yeast vacuolar H^+-ATPase. J. Bioenerg. Biomembr. 37:399–403.

Kaneko, T., and F. Katoh. 2004. Functional morphology of chloride cells in killifish *Fundulus heteroclitus*, a euryhaline teleost with seawater preference. Fish. Sci. 70:723–733.

Kawecki, T. J., and D. Ebert. 2004. Conceptual issues in local adaptation. Ecol. Lett. 7:1225–1241.

Kirschner, L. B. 2004. The mechanism of sodium chloride uptake in hyperregulating aquatic animals. J. Exp. Biol. 207:1439–1452.

Kruuk, L. E. B. 2004. Estimating genetic parameters in natural populations using the 'animal model'. Phil. Trans. R. Soc. Lond. B 359:873–890.

Lande, R. 2009. Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. J. Evol. Biol. 22:1435–1446.

Lande, R., and S. J. Arnold. 1983. The measurement of selection on correlated characters. Evolution 37:1210–1226.

Laprise, R., and J. J. Dodson. 1994. Environmental variability as a factor controlling spatial patterns in distribution and species-diversity of zooplankton in the St-Lawrence estuary. Mar. Ecol. Prog. Ser. 107:67–81.

Lee, C. E., and M. A. Bell. 1999. Causes and consequences of recent freshwater invasions by saltwater animals. Trends Ecol. Evol. 14:284–288.

Lenormand, T. 2002. Gene flow and the limits to natural selection. Trends Ecol. Evol. 17:183–189.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-DDCT} method. Methods 25:402–408.

Mackie, P. M., K. Gharbi, J. S. Ballantyne, S. D. McCormick, and P. A. Wright. 2007. $Na^+/K^+/2Cl^-$ cotransporter and CFTR gill expression after seawater transfer in smolts (0+) of different Atlantic salmon (*Salmo salar*) families. Aquaculture 272:625–635.

Madsen, S. S., L. N. Jensen, C. K. Tipsmark, P. Kiilerich, and R. J. Borski. 2007. Differential regulation of cystic fibrosis transmembrane conductance regulator and Na^+,K^+-ATPase in gills of striped bass, *Morone saxatilis*: effect of salinity and hormones. J. Endocrinol. 192:249–260.

Mancera, J. M., and S. D. McCormick. 1998. Osmoregulatory actions of the GH/IGF axis in non-salmonid teleosts. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 121:43–48.

Manzon, L. A. 2002. The role of prolactin in fish osmoregulation: a review. Gen. Comp. Endocrinol. 125:291–310.

Marshall, W. S. 2002. Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. J. Exp. Zool. 293:264–283.

Marshall, W. S., E. A. Lynch, and R. R. F. Cozzi. 2002. Redistribution of immunofluorescence of CFTR anion channel and NKCC cotransporter in chloride cells during adaptation of the killifish *Fundulus heteroclitus* to sea water. J. Exp. Biol. 205:1265–1273.

Masel, J., O. D. King, and H. Maughan. 2007. The loss of adaptive plasticity during long periods of environmental stasis. Am. Nat. 169:38–46.

Mattern, M. Y. 2004. Molecular phylogeny of the Gasterosteidae: the importance of using multiple genes. Mol. Phylogenet. Evol. 30:366–377.

Mayr, E. 1963. Animal Species and Evolution. Harvard Univ. Press, Cambridge, MA.

McAllister, D. E., S. L. Cumbaa, and C. R. Harington. 1981. Pleistocene fishes (*Coregonus, Osmerus, Microgadus, Gasterosteus*) from Green Creek, Ontario, Canada. Can. J. Earth Sci. 18:1356–1364.

McAllister, D. E., C. R. Harington, S. L. Cumbaa, and C. B. Renaud. 1988. Paleoenvironmental and biogeographic analyses of fossil fishes in peri-Champlain Sea deposits in eastern Canada. Pp. 241–258 *in* N. R. Gadd,

ed. The late quaternary development of the champlain sea basin. Geological Association of Canada, St. John's, Newfoundland, Canada.

McCairns, R. J. S., and L. Bernatchez. 2008. Landscape genetic analyses reveal cryptic population structure and putative selection gradients in a large-scale estuarine environment. Mol. Ecol. 17:3901–3916.

McCormick, S. D. 1996. Effects of growth hormone and insulin-like growth factor I on salinity tolerance and gill Na^+K^+ATPase in Atlantic salmon (*Salmo salar*): interaction with cortisol. Gen. Comp. Endocrinol. 101:3–11.

———. 2001. Endocrine control of osmoregulation in teleost fish. Am. Zool. 41:781–794.

Meyer, K. 2007. WOMBAT—A tool for mixed model analyses in quantitative genetics by restricted maximum likelihood (REML). J. Zhejiang Univ. Sci. B 8:815–821.

Nilsen, T. O., L. O. E. Ebbesson, S. S. Madsen, S. D. McCormick, E. Andersson, B. T. Bjornsson, P. Prunet, and S. O. Stefansson. 2007. Differential expression of gill Na^+/K^+-ATPase alpha- and beta-subunits, $Na^+/K^+/2Cl^-$ cotransporter and CFTR anion channel in juvenile anadromous and landlocked Atlantic salmon *Salmo salar*. J. Exp. Biol. 210:2885–2896.

Nosil, P., S. R. Egan, and D. J. Funk. 2008. Heterogeneous genomic differentiation between walking-stick ecotypes: "isolation by adaptation" and multiple roles for divergent selection. Evolution 62:316–336.

Nussey, D. H., T. H. Clutton-Brock, D. A. Elston, S. D. Albon, and L. E. B. Kruuk. 2005a. Phenotypic plasticity in a maternal trait in red deer. J. Anim. Ecol. 74:387–396.

Nussey, D. H., E. Postma, P. Gienapp, and M. E. Visser. 2005b. Selection on heritable phenotypic plasticity in a wild bird population. Science 310:304–306.

Orr, H. A. 2009. Fitness and its role in evolutionary genetics. Nat. Rev. Genet. 10:531–539.

Ortí, G., M. A. Bell, T. E. Reimchen, and A. Meyer. 1994. Global survey of mitochondrial-DNA sequences in the threespine stickleback: evidence for recent migrations. Evolution 48:608–622.

Perry, S. F. 1997. The chloride cell: structure and function in the gills of freshwater fishes. Annu. Rev. Physiol. 59:325–347.

Piermarini, P. M., and D. H. Evans. 2001. Immunochemical analysis of the vacuolar proton-ATPase B-subunit in the gills of a euryhaline stingray (*Dasyatis sabina*): effects of salinity and relation to Na^+/K^+-ATPase. J. Exp. Biol. 204:3251–3259.

Pigliucci, M. 2007. Do we need an extended evolutionary synthesis? Evolution 61:2743–2749.

Pigliucci, M., C. J. Murren, and C. D. Schlichting. 2006. Phenotypic plasticity and evolution by genetic assimilation. J. Exp. Biol. 209:2362–2367.

Pinheiro, J. C., and D. M. Bates. 2000. Mixed-effects models in S and S-Plus. Springer, New York, NY.

Price, T. D., A. Qvarnstrom, and D. E. Irwin. 2003. The role of phenotypic plasticity in driving genetic evolution. Proc. R. Soc. Lond. B 270:1433–1440.

R Development Core Team. 2007. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Reis-Santos, P., S. D. McCormick, and J. M. Wilson. 2008. Ionoregulatory changes during metamorphosis and salinity exposure of juvenile sea lamprey (*Petromyzon marinus* L.). J. Exp. Biol. 211:978–988.

Richard, P. J. H., and S. Occhietti. 2005. ^{14}C chronology for ice retreat and inception of Champlain Sea in the St. Lawrence Lowlands, Canada. Quat. Res. 63:353–358.

Roff, D. A. 1997. Evolutionary quantitative genetics. Springer, New York, NY.

Rogers, S. M., V. Gagnon, and L. Bernatchez. 2002. Genetically based phenotype-environment association for swimming behavior in lake whitefish ecotypes (*Coregonus clupeaformis* Mitchill). Evolution 56:2322–2329.

Russell, A. P., and A. M. Bauer. 2005. Variation in structure and its relationship to function: correlation, explanation and extrapolation. Pp. 399–434 *in* B. Hallgrímsson and B. K. Hall, eds. Variation. Elsevier Academic Press, Burlington, MA.

Sakamoto, T., and T. Hirano. 1993. Expression of insulin-like growth factor-I gene in osmoregulatory organs during seawater adaptation of the salmonid fish: possible mode of osmoregulatory action of growth hormone. Proc. Natl. Acad. Sci. USA 90:1912–1916.

Sakamoto, T., S. D. McCormick, and T. Hirano. 1993. Osmoregulatory actions of growth hormone and its mode of action in salmonids: a review. Fish Physiol. Biochem. 11:155–164.

Sakamoto, T., T. Hirano, S. S. Madsen, R. S. Nishioka, and H. A. Bern. 1995. Insulin-like growth factor-I gene expression during parr-smolt transformation of coho salmon. Zool. Sci. 12:249–252.

Scheiner, S. M. 1993. Genetics and evolution of phenotypic plasticity. Annu. Rev. Ecol. Syst. 24:35–68.

Scheiner, S. M., and R. F. Lyman. 1989. The genetics of phenotypic plasticity I. Heritability. J. Evol. Biol. 2:95–107.

Schlichting, C. D., and M. Pigliucci. 1995. Gene regulation, quantitative genetics and the evolution of reaction norms. Evol. Ecol. 9:154–168.

Schlichting, C. D., and H. Smith. 2002. Phenotypic plasticity: linking molecular mechanisms with evolutionary outcomes. Evol. Ecol. 16:189–211.

Schredelseker, J., and B. Pelster. 2004. Isoforms vatB1 and vatB2 of the vacuolar type ATPase subunit B are differentially expressed in embryos of the zebrafish (*Danio rerio*). Dev. Dyn. 230:569–575.

Schulte, P. M. 2001. Environmental adaptations as windows on molecular evolution. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 128:597–611.

———. 2004. Changes in gene expression as biochemical adaptations to environmental change: a tribute to Peter Hochachka. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 139:519–529.

Scott, G. R., J. G. Richards, B. Forbush, P. Isenring, and P. M. Schulte. 2004a. Changes in gene expression in gills of the euryhaline killifish *Fundulus heteroclitus* after abrupt salinity transfer. Am. J. Physiol. Cell Physiol. 287:C300–C309.

Scott, G. R., J. T. Rogers, J. G. Richards, C. A. Wood, and P. M. Schulte. 2004b. Intraspecific divergence of ionoregulatory physiology in the euryhaline teleost *Fundulus heteroclitus*: possible mechanisms of freshwater adaptation. J. Exp. Biol. 207:3399–3410.

Scott, G. R., J. B. Claiborne, S. L. Edwards, P. M. Schulte, and C. M. Wood. 2005. Gene expression after freshwater transfer in gills and opercular epithelia of killifish: insight into divergent mechanisms of ion transport. J. Exp. Biol. 208:2719–2729.

Sinervo, B., and E. Svensson. 2002. Correlational selection and the evolution of genomic architecture. Heredity 89:329–338.

Singer, T. D., K. M. Clements, J. W. Semple, P. M. Schulte, J. S. Bystriansky, B. Finstad, I. A. Fleming, and R. S. McKinley. 2002. Seawater tolerance and gene expression in two strains of Atlantic salmon smolts. Can. J. Fish. Aquat. Sci. 59:125–135.

Skúlason, S., S. S. Snorrason, D. Ota, and D. L. G. Noakes. 1993. Genetically based differences in foraging behavior among sympatric morphs of Arctic charr (Pisces, Salmonidae). Anim. Behav. 45:1179–1192.

Stamps, J. A., M. Mangel, and J. A. Phillips. 1998. A new look at relationships between size at maturity and asymptotic size. Am. Nat. 152:470–479.

Stinchcombe, J. R., A. F. Agrawal, P. A. Hohenlohe, S. J. Arnold, and M. W. Blows. 2008. Estimating nonlinear selection gradients using quadratic regression coefficients: double or nothing? Evolution 62:2435–2440.

Tang, C. H., and T. H. Lee. 2007. The effect of environmental salinity on the protein expression of Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter, cystic fibrosis transmembrane conductance regulator, anion, exchanger 1, and chloride channel 3 in gills of a euryhaline teleost, *Tetraodon nigroviridis*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 147:521–528.

Thompson, R. 2008. Estimation of quantitative genetic parameters. Proc. R. Soc. Lond. B 275:679–686.

Tipsmark, C. K., J. A. Luckenbach, S. S. Madsen, and R. J. Borski. 2007. IGF-I and branchial IGF receptor expression and localization during salinity acclimation in striped bass. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292:R535-R543.

Tresguerres, M., F. Katoh, E. Orr, S. K. Parks, and G. G. Goss. 2006. Chloride uptake and base secretion in freshwater fish: a transepithelial ion-transport metabolon? Physiol. Biochem. Zool. 79:981–996.

Underhill, J. C. 1986. The fish fauna of the Laurentian Great Lakes, the St. Lawrence lowlands, Newfoundland and Labrador. Pp. 105–136 *in* C. H. Hocutt and E. O. Wiley, eds. The Zoogeography of North American Freshwater Fishes. John Wiley & Sons, New York, NY.

University of Oregon Stickleback Research Site. 2008. Crossing and rearing protocols. Available at http://stickleback.uoregon.edu.

van Kleunen, M., and M. Fischer. 2005. Constraints on the evolution of adaptive phenotypic plasticity in plants. New Phytol. 166:49–60.

van Tienderen, P. H. 1997. Generalists, specialists, and the evolution of phenotypic plasticity in sympatric populations of distinct species. Evolution 51:1372–1380.

Varsamos, S., C. Nebel, and G. Charmantier. 2005. Ontogeny of osmoregulation in postembryonic fish: a review. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 141:401–429.

Via, S. 1993. Adaptive phenotypic plasticity: target or by-product of selection in a variable environment. Am. Nat. 142:352–365.

Via, S., and R. Lande. 1985. Genotype-environment interaction and the evolution of phenotypic plasticity. Evolution 39:505–522.

Via, S., R. Gomulkiewicz, G. de Jong, S. M. Scheiner, C. D. Schlichting, and P. H. Vantienderen. 1995. Adaptive phenotypic plasticity: consensus and controversy. Trends Ecol. Evol. 10:212–217.

Vincent, W. F., and J. J. Dodson. 1999. The St. Lawrence River, Canada-USA: the need for an ecosystem-level understanding of large rivers. Jpn. J. Limnol. 60:29–50.

Vincent, W. F., J. J. Dodson, N. Bertrand, and J. J. Frenette. 1996. Photosynthetic and bacterial production gradients in a larval fish nursery: the St Lawrence river transition zone. Mar. Ecol. Prog. Ser. 139:227–238.

Waddington, C. H. 1942. Canalization of development and the inheritance of acquired characters. Nature 150:563–565.

———. 1953. Genetic assimilation of an acquired character. Evolution 7:118–126.

———. 1959. Canalization of development and genetic assimilation of acquired characters. Nature 183:1654–1655.

Wassenaar, L., U. Brand, and J. Terasmae. 1988. Geochemical and paleoecological investigations using invertebrate macrofossils of the late Quaternary Champlain Sea, Ontario and Quebec. Pp. 195–205 *in* N. R. Gadd, ed. The late quaternary development of the champlain sea basin. Geological Association of Canada, St. John's, Newfoundland, Canada.

West-Eberhard, M. J. 2003. Developmental plasticity and evolution. Oxford Univ. Press, New York, NY.

———. 2005. Developmental plasticity and the origin of species differences. Proc. Natl. Acad. Sci. USA 102:6543–6549.

Williams, G. C. 1966. Adaptation and natural selection: a critique of some current evolutionary thought. Princeton Univ. Press, Princeton, NJ.

Winkler, G., J. J. Dodson, N. Bertrand, D. Thivierge, and W. F. Vincent. 2003. Trophic coupling across the St. Lawrence River estuarine transition zone. Mar. Ecol. Prog. Ser. 251:59–73.

Wright, S. 1931. Evolution in Mendelian populations. Genetics 16:0097–0159.

Wund, M. A., J. A. Baker, B. Clancy, J. L. Golub, and S. A. Fosterk. 2008. A test of the "Flexible stem" model of evolution: ancestral plasticity, genetic accommodation, and morphological divergence in the threespine stickleback radiation. Am. Nat. 172:449–462.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Blocked factorial breeding design.

Figure S2. Larval growth rate (A), final standard length (B), and relative body condition (C), as estimated by individual residual variation from the von Bertallanfy growth model fit to average size-at-age data (see Methods for details), in freshwater (<1‰) and saltwater (20‰) environments.

Table S1. Details of the blocked factorial breeding design outlining which families comprise each block.

Table S2. Variance components for reaction norm analyses of all crosses (pure and hybrid), and for contrast analyses comparing hybrid and foreign crosses with the native deme in each rearing environment.

Supporting Information may be found in the online version of this article.

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