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Does ion limitation select for pelvic reduction in threespine stickleback (*Gasterosteus aculeatus*)?

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ABSTRACT

Background: Resident freshwater threespine stickleback (*Gasterosteus aculeatus*) populations exhibit reduced bony armour structures (including the pelvis) that are always maintained in their marine or anadromous ancestors. Environmental ion and calcium limitation may select for armour reduction in freshwater habitats by increasing the energetic costs of importing ions from dilute fresh water. Armour reduction may allow increased somatic growth and reproductive effort.

Hypothesis: *Gasterosteus aculeatus* with reduced pelvic skeletons grow faster than those with robust pelvises in water with low total ion or calcium concentration.

Methods: We estimated growth rate (age-adjusted size) of field-caught *G. aculeatus* from four freshwater populations. Each of these populations is highly variable for the magnitude of pelvic expression, so we could tease apart the effects of pelvic phenotype and population statistically. We also estimated growth (size at 138 days post-fertilization) of specimens with large and small pelvic structures raised in the laboratory under contrasting total ion and calcium concentration regimes.

Results: Growth rates of field-caught *G. aculeatus* did not differ among individuals with small and large pelvic phenotypes within samples from each of four freshwater populations. Similarly, growth of laboratory-reared specimens from two of these populations did not differ among pelvic phenotypes at low total ion or low calcium concentration. Our comparisons provide no evidence that development and maintenance of a larger pelvis reduces growth under conditions of low ion availability either in nature or in the laboratory.

Keywords: selection agent, growth rate, calcium limitation, ion concentration, salinity, trade-off.

INTRODUCTION

A fundamental objective of evolutionary biology is to understand the causes of phenotypic divergence. Associations between phenotypes and habitat variables often provide the initial evidence for divergent natural selection and selection agents (Endler, 1986; Wade and Kalisz, 1990; MacColl, 2011). However, multiple environmental variables may vary together between the

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habitats where divergent phenotypes occur. For instance, freshwater habitats have lower total ion concentration (e.g. Conway, 1942; Kester *et al.*, 1967) and water density (e.g. Millero *et al.*, 1976), and greater diel and seasonal temperature fluctuations than saltwater habitats (e.g. Barrett *et al.*, 2011). Thus, teasing apart the environmental variables that cause divergence between related freshwater and marine populations would be a challenge, and correlations would be inadequate to confirm environmental variables that cause adaptive phenotypic divergence. Despite the value of phenotype–environment associations to suggest the action of natural selection and the identity of selection agents, experimental manipulation is necessary to test hypotheses for causation (Wade and Kalisz, 1990; Futuyma and Bennett, 2009; MacColl, 2011).

Numerous coastal, lowland freshwater habitats formed in the Holarctic after the most recent glacial retreat [i.e. 9.5–15 ka in Cook Inlet, Alaska, USA (Reger and Pinney, 1996)], which allowed colonization by marine or anadromous threespine stickleback, *Gasterosteus aculeatus* (e.g. McPhail and Lindsey, 1970; Bell and Foster, 1994). Subsequent adaptation to freshwater habitats resulted in parallel evolution of morphology, physiology, behaviour, and many other phenotypes in *G. aculeatus* populations (reviewed in Bell and Foster, 1994; Bell and Aguirre, 2013; Hendry *et al.*, 2013). Loss or reduction of bony armour, including lateral plates and the pelvic girdle, are among the most conspicuous of these changes. The pelvic skeleton, dorsal spines, and lateral plates of *G. aculeatus* help in post-capture escape from predatory fishes and birds (e.g. Hoogland *et al.*, 1957; Reimchen, 1983, 1991, 1992). *Gasterosteus aculeatus* erect their bilateral pelvic spines and three dorsal spines when threatened, which puncture the oral mucosa of predatory vertebrates and contribute to escape and survival after capture (Reimchen, 1991). The pelvic girdle, lateral plates, and dorsal spine supports form a ring of bone that protects the body from compression and laceration (Reimchen, 1983; Bell, 1988).

Pelvic reduction is a rare phenomenon compared with lateral plate reduction (reviewed in Klepaker *et al.*, 2013), but invariable presence of a robust pelvic skeleton in marine and anadromous stickleback (Klepaker *et al.*, 2013) suggests that pelvic reduction is maladaptive in the ocean but is either adaptive or has increased in frequency by drift in fresh water. The pattern of increase in the frequency of vestigial pelvic phenotypes through time in a fossil assemblage was probably due to directional selection over several thousand years (Bell *et al.*, 2006; Hunt *et al.*, 2008). Pelvic reduction has occurred primarily by *de novo* deletion mutations of the *Pel* enhancer, which controls expression of the *Pitx1* gene in the pelvic region, followed by selective sweeps that fixed alleles for pelvic reduction (Cresko *et al.*, 2004; Shapiro *et al.*, 2004; Bell *et al.*, 2007; Chan *et al.*, 2010). Thus, evidence suggests that pelvic reduction in *G. aculeatus* is an adaptation, but determining what selection agents favour this phenotypic evolution in fresh water is a more difficult problem.

While there is ample evidence that the pelvic apparatus is important for defence against gape-limited predation (Hoogland *et al.*, 1957; Hagen and Gilbertson, 1972; Gross, 1978; Reist, 1980; Bell *et al.*, 1993; Lescak and von Hippel, 2011), there are several proposed but poorly supported hypotheses for the selection agents for pelvic reduction. Many correlative studies suggest that armour is reduced when predatory fish are absent (e.g. Hagen and Gilbertson, 1972; Moodie and Reimchen, 1973; Bell, 1974, 1976; Reimchen, 1980, 1994; Bell *et al.*, 1993; Reimchen and Nosil, 2002; Reimchen *et al.*, 2013). In many cases, it appears that pelvic reduction can evolve only in lakes without native predatory fishes (e.g. Bell *et al.*, 1993; reviewed in Klepaker *et al.*, 2013). However, the simple lack of a selection agent that favours retention of the pelvis does not explain positive selection for pelvic reduction. Also, *G. aculeatus* with pelvic reduction co-occurs with predatory fishes in some instances, suggesting that their absence alone cannot account for the evolution of pelvic reduction (reviewed in Klepaker *et al.*, 2013).

It has been proposed that reduced bony armour in *G. aculeatus* increases manoeuvrability and burst swimming speed, enhancing pre-capture escape from predators (Nelson, 1969; Bell, 1974). Pre-capture escape would be especially important in habitats with refuge ['refuge use hypothesis' (Reimchen, 1992; Leinonen *et al.*, 2011)], and there is support for this hypothesis with respect to lateral plate reduction (Taylor and McPhail, 1986; Bergstrom, 2002; Leinonen *et al.*, 2011; Reimchen *et al.*, 2013). Andraso and Barron (1995) found that brook stickleback (*Culaea inconstans*) with larger pelvises had poor pre-capture escape performance compared with those with smaller pelvises.

Reimchen (1980) suggested that both dorsal and pelvic spines could be disadvantageous in some freshwater habitats due to insect predation. Dragonfly naiads (*Aeshna* spp.) and other insects may capture spined *G. aculeatus* more effectively because the spines offer surfaces for them to grasp. Correlative analyses provide some support for the hypothesis (Reimchen and Nosil, 2002), but experimental results using predatory insects are equivocal (some evidence for hypothesis in: Reist, 1980; Marchinko, 2009; Lescak *et al.*, 2012; and against: Reimchen, 1980; Zeller *et al.*, 2012; Mobley *et al.*, 2013). Myhre and Klepaker (2009) concluded that lateral plate reduction may be favoured for buoyancy reduction in freshwater *G. aculeatus*, but this hypothesis has not been tested for pelvic reduction.

Giles (1983) proposed that calcium deficiency in low-calcium lakes selects for pelvic reduction because of the high energetic cost of calcium ion transport. There is strong experimental evidence in support of total ion deficiency as a causal agent of selection for lateral plate reduction in *G. aculeatus* (Marchinko and Schluter, 2007; Barrett *et al.*, 2009). However, the only support for the ion deficiency hypothesis for pelvic reduction is based on phenotype–habitat associations. Giles (1983) found that all Scottish populations with pelvic reduction occurred in lakes with very low calcium concentration and pH. Bell *et al.* (1993) found an association between degree of pelvic reduction and calcium concentration among numerous Alaskan lakes that also lack native predatory fish. Low calcium, magnesium, silicon, pH, and reactive phosphorus were principal correlates of pelvic reduction and other skeletal traits in a reduced set of the same lakes used in Bell *et al.* (1993) (Bourgeois *et al.*, 1994). However, Lescak *et al.* (2013) noted that *G. aculeatus* from one Alaskan lake (Wallace Lake) with full pelvises did not appear to differ in body size relative to those with reduced pelvises, suggesting no growth advantage for pelvic reduction in this population. Unfortunately, this study did not include age differences within cohorts as a potential influence on size. Also, Klepaker *et al.* (2013) noted that pelvic reduction occurs in some Canadian lakes with very high calcium concentrations (>120 mg/L). There are no experimental tests of the hypothesis that pelvic reduction is caused by low ion or calcium concentrations.

The purpose of this study is to test the hypothesis that low ion or calcium concentration selects for pelvic reduction in *G. aculeatus*. Specifically, we hypothesize that *G. aculeatus* with severely reduced pelvic skeletons have a growth advantage over members of the same population with robust pelvic skeletons in freshwater environments in which total ion and calcium concentrations are low. This hypothesis follows from the possibility that mineralization of the pelvis with ions acquired by active transport competes with other metabolic costs, somatic growth, and reproductive effort for energy (Giles, 1983; Bell *et al.*, 1993; Marchinko and Schluter, 2007; Barrett *et al.*, 2009). First, we estimated growth rates (i.e. age-specific size) of field-caught, young-of-the-year *G. aculeatus* fry from four freshwater populations that are highly variable for pelvic phenotypes. Since these four lakes have low ionic and calcium concentrations, we predicted that wild fry with reduced pelvic structures would grow faster than those with more robust pelvises. Pelvic phenotypes in *G. aculeatus* populations from

each of these lakes range from full expression to absent, presenting the opportunity to detect a growth advantage for pelvic-reduced *G. aculeatus* within each of these natural populations. Populations with a wide range of pelvic phenotypes are rare, and most comparisons to date have used populations in which the majority of *G. aculeatus* have a narrower range of phenotypes (for exceptions, see Lescak and von Hippel, 2011; Lescak *et al.*, 2012, 2013). Second, we compared growth (size at 138 days post-fertilization) of *G. aculeatus* with large and small pelvises raised in the laboratory under contrasting salinity and calcium concentration regimes (2×2 factorial design). We predicted significant interactions between pelvic phenotypes and salt treatments (both salinity and calcium, or a significant three-way interaction among these factors), with fish with reduced pelvises growing larger than fish with larger pelvic structures at low total ion and calcium concentrations but not at high concentrations. This is the first study to experimentally test the hypotheses that low total ion and calcium concentrations are selection agents favouring the evolution of pelvic reduction in freshwater *G. aculeatus*.

METHODS

Growth in natural populations

We collected juvenile (young-of-the-year, 0+) *G. aculeatus* in August 2010 from Bruce, L-shape, Morvro, and Wallace lakes (Table 1) in the Matanuska-Susitna Borough, Alaska, USA, with minnow traps (⅛″ and ¼″ mesh) or a seine. They were euthanized using MS-222 and frozen at −20°C. We measured lake water conductivities (at 15°C; μS/cm) using a YSI-85 meter in June 2011 and obtained conductivities of 1990 water samples from the same lakes from Bell *et al.* (unpublished data), which were determined using a YSI-32 meter at 25°C (YSI Incorporated, Yellow Springs, OH, USA; Table 1). We converted conductivity measures to approximate salinities using appropriate polynomial equations relating salinity to the ratio of conductivities of our samples to known conductivities of a 35 ppt KCl standard at the corresponding sample temperature (Wooster *et al.*, 1969).

We measured standard lengths (SL, distance from the tip of the upper jaw to the end of the last vertebra) with digital calipers (±0.1 mm) and weights (±0.0001 g) of specimens, removed heads for otolith extraction, and preserved bodies in 10% formalin for phenotyping. A preliminary analysis of otolith increments indicated that all fish > 30 mm SL had annuli ($n = 39$, all populations represented in sample) and therefore were >1 year old. Thus, we used specimens ≤30 mm SL with low (PS = 0–2) or high pelvic scores (PS = 6–8) to contrast growth between these phenotypes during the first summer of life. We excluded any specimen ≤30 mm SL that had otoliths with annuli. Table 1 gives the sample sizes for growth comparisons for each population.

We counted daily growth rings in sagittal otoliths to account for age and estimate growth rates of field-caught specimens. Daily deposition of sagittal growth rings was validated previously for *G. aculeatus* (Wright and Huntingford, 1993; von Hippel *et al.*, 2013). We dissected otoliths from both sides of the fish, cleaned them in household bleach (~6% sodium hypochlorite), rinsed them in de-ionized water, and dehydrated them with 75% ethyl alcohol. We mounted sagittae medial-side (sulcus) down on glass microscope slides with commercial superglue (cyanoacrolate) and sanded with fine sandpaper (Tamiya Finishing Abrasives P2000; Tamiya, Inc.) to make daily growth rings visible. We sanded most otoliths on both sides, according to the Otolith Research Laboratory at Bedford Institute of Oceanography (2013).

Table 1. Locations, collection dates (in August 2010), and number of specimens collected and studied from each *G. aculeatus* population to compare growth among specimens with large and small pelvic phenotypes under natural conditions

Lake name (acronym)	Latitude	Longitude	Collection dates	*n* (collected)	*n* (study)	Calcium 1990	Conductivity (salinity) 1990	Conductivity (salinity) 2011
Bruce Lake (BR)	61°36.622′N	149°33.341′W	18–19	1072	50	1.5	12 (0.015)	12 (0.012)
L-shape Lake (LS)	61°42.282′N	149°58.832′W	21–22	402	50	4.6	16 (0.016)	15 (0.014)
Morvro Lake (MO)	61°36.359′N	149°46.924′W	19	984	46	0.3	10 (0.014)	7 (0.010)
Wallace Lake (WL)	61°34.494′N	149°34.618′W	18–21	805	61	2.5	19 (0.017)	19 (0.016)

Note: Calcium concentrations (parts per thousand, ppt) were measured in 1990 (Bell *et al.*, unpublished data), and conductivities (μS/cm) and approximate salinities (ppt) converted from conductivities were measured at 25°C in 1990 and 15°C in 2011.

A single reader (J.L.R.) counted rings under a compound microscope at 400× magnification using transmitted light, and gave each otolith count a subjective 'confidence' score. When the confidence scores differed between left and right sagittae, we used the side with the higher confidence score and discarded the other count. We averaged the counts if their confidence scores were equal and discarded specimens for which rings could not be resolved using either otolith.

We transferred the formalin-fixed fish bodies to 50% isopropyl alcohol, stained them with Alizarin Red S in <2% KOH (wt./vol. of de-ionized water), de-stained them in <2% KOH, and returned them to 50% isopropyl alcohol for storage (Bell, 1979). We assigned stained fish a pelvic score between 0 and 8, with 0 representing absence of the entire pelvic apparatus and 8 representing presence of ascending branch, anterior process, posterior process, and pelvic spine on both left and right sides (Bell *et al.*, 1993; Bell and Ortí, 1994). We used fish with low pelvic scores (PS = 0–2; i.e. no pelvis or minimal vestige on one or both sides) and high pelvic scores (PS = 6–8; i.e. full pelvis and 0, 1, or 2 spines) for growth rate comparisons.

Instead of expecting an energetic cost to somatic growth of developing a costly mineralized structure like the pelvis, it is possible that there is an energetic trade-off between mineralizing the pelvis versus other parts of the skeleton during development. Such a trade-off may favour pelvic reduction if there are negative fitness consequences associated with the slower mineralization of other bony structures in fish that develop a robust pelvis. We looked for an effect of lateral plate number on pelvic areas to test for a trade-off between growth of the pelvis versus other bony structures at low ionic concentrations. We estimated the area of the ventral aspect of pelvic girdles using ImageJ® software [v.1.45s (Rasband, 1997–2006)] on pictures taken under a dissecting microscope with a PowerShot S45 camera (4.0 MegaPixels; Canon, Inc.). We summed left- and right-side pelvic areas for analysis. We counted lateral plates under a dissecting microscope (Hagen and Gilbertson, 1972) and summed counts for the left and right sides of each fish for analysis.

To determine whether fish with reduced pelvises have a growth advantage in freshwater populations, we performed a two-way ANCOVA of SL using SPSS® v.22 (IBM, Inc., Chicago, IL, USA), with population (BR, MO, LS, WL; see Table 1 for acronyms) and pelvic score categories (0–2, 6–8) as fixed effects and age as a covariate. We computed least-squares means using SPSS® and determined significance of pairwise mean differences among populations using the 95% confidence intervals for these estimates, which were confirmed by *post-hoc*, Bonferroni-corrected pairwise comparisons.

To test for a trade-off between growth of the pelvis and lateral plates, we performed a two-way ANCOVA of square root-transformed pelvic areas using SPSS®, with population and lateral plate number categories (0–2, 3–5, 6–7, 8–11) as fixed effects and SL as a covariate. We included only fish with pelvises in the ANCOVA because we could not test for an effect of lateral plate number on pelvic areas that did not exist. Owing to this exclusion of data, we also compared mean lateral plate counts between fish with and without pelvises using a Mann-Whitney *U*-test (Sokal and Rohlf, 1995).

Laboratory growth experiment

We performed two sets of crosses for each of two populations (i.e. BR, MO), one to produce mostly low-PS progeny and the other to produce mostly high-PS progeny, to increase our power to detect differences between these extremes when raised under different salinity and

calcium regimes. We collected reproductive adults from Bruce Lake and Morvro Lake on 13 and 14 June 2011, respectively, using unbaited minnow traps. We kept live fish in coolers partially filled with aerated lake water, and transported them to the University of Alaska Anchorage. One day after capture, parents were euthanized by over-anaesthesia in MS-222, and we performed *in vitro* fertilization according to Hagen (1967, 1973). The testes of 10 low- to intermediate-PS males (BR: one PS = 0, one PS = 2, two PS = 3, and six PS = 4; MO: seven PS = 0 and three PS = 2) were extracted and minced with razor blades or torn using forceps to release sperm. The mixed sperm was used to fertilize mixed egg clutches from 10 to 12 low- to intermediate-PS females from the same population (BR: one PS = 2, one PS = 3, four PS = 4, three PS = 5, three PS = 6; MO: ten PS = 0). Eggs were fertilized and held in Petri dishes with 3.5 ppt Instant Ocean® in deionized water (i.e. 10% sea water). From each population, we also performed *in vitro* fertilizations between ten males (BR: four PS = 6, two PS = 7, four PS = 8; MO: ten PS = 8) and ten females (BR: five PS = 6, one PS = 7, four PS = 8; MO: ten PS = 8) with high PS. We acclimated eggs in half of the dishes to a high salinity (12 ppt Instant Ocean, ~⅓ sea water), and those in the other half to slightly reduced salinity (2 ppt Instant Ocean), using four ~50% water changes over 48 h.

On 17 June, we chilled the embryos to 4–7°C and transported them to Stony Brook University, where we housed embryos in an incubator at 19°C. The fry hatched between 21 and 23 June and began feeding on live brine shrimp nauplii after yolk absorption was completed on 27 June. On 2 July, the fry were moved to 56.78-litre (15-gallon) aquaria, each containing one of four salinity and calcium treatments (see Tables S1 and S2 at evolutionary-ecology.com/data/2881Appendix.pdf). We transferred half of the fry raised initially in 12 ppt Instant Ocean to aquaria with high salinity and high calcium (HS/HC), and we transferred the other half to aquaria with high salinity and low calcium (HS/LC). Similarly, we transferred half of the fry raised initially in 2 ppt Instant Ocean to aquaria with low salinity and high calcium (LS/HC), and the other half to aquaria with low salinity and low calcium (LS/LC). Aquaria initially contained between 50 and 80 fry, and we reduced these numbers by randomly culling specimens to ~40 per aquarium at day 60 and ~25 per aquarium at day 90 (12 aquaria per salt treatment).

We determined the salinity and calcium treatments (see 2881Appendix.pdf) using the seawater recipe of Kester *et al.* (1967), which was based on true estimates of the concentration of the major constituents of sea water. Our treatments are similar to those of Spence *et al.* (2012). The high salinity treatments were approximately ⅓ sea water (12.03 ppt for HS/HC and 11.61 ppt for HS/LC; Table S2, 2881Appendix.pdf) because the ionic concentration of fish body fluids is about 30% sea water (e.g. Holmes and Donaldson, 1969; Evans, 1979), and the cost of osmoregulation should be minimal at this salinity. Low salinity treatments were 0.66% sea water. We adjusted the amount of $MgCl_2$ $6H_2O$ relative to $CaCl_2$ $2H_2O$ to keep salinities nearly equivalent between calcium treatments within salinity blocks (Table S1, 2881Appendix.pdf) (Spence *et al.*, 2012). High calcium treatments contained the concentration of calcium found in sea water (Kester *et al.*, 1967). Low calcium treatments initially contained 0 ppt calcium (as in Spence *et al.*, 2012). However, a week after transfer to 0 ppt calcium, many of the HS/LC and some of the LS/LC treatment fish died suddenly. Since insufficient dietary calcium was the probable cause of these mortalities, we administered 0.1 ppt calcium for a week to provide sufficient calcium for the fish to recover. All surviving fish appeared healthy, and we saw no apparent differences in growth relative to fish in HC treatments. After a week of recovery, we gradually reduced the calcium concentration to 0.00701 ppt

(Table S2, 2881Appendix.pdf) using six 25% water changes with 0.00701 ppt replacement water over 6 days, and then resumed weekly 25% water changes with 0.00701 ppt replacement water thereafter. We covered aquaria with clear, plastic lids to limit evaporation of water and salt loss. We performed weekly 25% water changes to maintain water quality and salt concentrations.

We fed fish brine shrimp nauplii twice daily until they were large enough to eat adult brine shrimp. Then they were fed defrosted adult brine shrimp twice daily to satiation. We euthanized fish using an overdose of MS-222 120 days after transfer to aquaria and 138 days post-fertilization, blotted and weighed them immediately, and fixed them in pH-neutral 4% paraformaldehyde in phosphate-buffered saline solution (Sambrook and Russell, 2001). We measured SL using digital calipers to the nearest 0.01 mm. We stained fish with Alizarin Red S in <2% KOH (wt./vol.), and we determined pelvic scores, pelvic areas, and lateral plate numbers as described above.

To determine whether fish with reduced pelvises have a growth advantage over those with large pelvises in water with a low total ion or calcium concentration, we performed mixed-model ANOVA on log (SL + 1) using SAS® (PROC MIXED, SAS Institute Inc., 2009), with population (BR, MO), salinity treatment (low, high), calcium treatment (low, high), and pelvic score categories (0–2, 3–5, 6–8) as fixed effects and blocks (representing horizontal rows of aquaria at varying distances from the overhead light source) as a random effect. We included in the model all interactions for fixed effects. We performed *post-hoc* Tukey-Kramer tests for significance of pairwise mean differences according to Sokal and Rohlf (1995).

We tested for a trade-off between growth of the pelvis and lateral plates (mineralized structures) using a mixed-model ANOVA on the residuals of a regression of square root-transformed pelvic areas [sqrt (PA + 1)] on log-transformed SL [log (SL + 1); we transformed residuals to fit normality as $(\text{residuals} + 1)^{20}$] in SAS® (PROC MIXED, SAS Institute Inc., 2009), with population, salinity treatment, calcium treatment, and plate count categories (0–2, 3–5, 6–7, 8–10) as fixed effects and blocks as a random effect. We included in the model all interactions for fixed effects. We performed Tukey-Kramer tests for significance of pairwise mean differences according to Sokal and Rohlf (1995). We used a Mann-Whitney *U*-test to compare mean lateral plate numbers between fish with and without pelvises (Sokal and Rohlf, 1995).

RESULTS

Growth in natural populations

Age estimates of field-caught specimens from all populations ranged from 43.25 to 83 days, with a mean of 63.52 and a standard deviation of 7.20 days ($n = 200$). The ANCOVA analysed differences in SL at the mean age of 63.52 days. The results from a two-way ANOVA on growth rates (SL divided by age; square-root transformed) did not differ qualitatively from the ANCOVA results reported here. Contrary to our expectations, SL did not differ significantly between fish with high ($n = 122$) and low ($n = 85$) pelvic scores ($P = 0.06$; Table 2). Indeed, the least-squares mean SL was nominally higher in high pelvic score fish (Table 3), and for the mean growth rates of three of the four intra-population samples (Fig. 1). Age-adjusted SL differed significantly among samples from different populations ($P < 0.0001$; Table 2). Although SL did not differ between Bruce and Morvro

Table 2. Results from a two-way, unbalanced ANCOVA to test the effects of pelvic score and population on standard length of field-caught, young-of-the-year *G. aculeatus*

Effect	d.f.	Sums of squares	Mean square	*F*-value	*P*-value
Pelvic score (PS)	1	1.036	1.036	0.159	0.690
Population	3	555.860	185.287	28.458	< **0.0001**
PS × Population	3	42.993	14.331	2.201	0.089
Age	1	64.876	64.876	9.964	**0.002**
Error	191	1243.595	6.511		
Total	199	0.462			

Note: Age was a covariate. **Bold** *P*-values indicate significant effects at $P < 0.05$.

Table 3. Least-squares (LS) mean standard length (at 63.52 days of age), lower and upper limits of 95% confidence interval, and sample sizes for field-caught, young-of-the-year *G. aculeatus* with low (PS = 0–2) and high (PS = 6–8) pelvic scores, and from four different freshwater populations

	LS mean	95% CI (lower limit)	95% CI (upper limit)	*n*
Pelvic score				
0–2	23.88	23.28	24.48	85
6–8	24.03	23.57	24.50	122
Population				
Bruce Lake	26.12^a	25.25	26.99	50
Morvro Lake	25.09^a	24.29	25.89	46
Wallace Lake	23.31^b	22.66	23.96	61
L-shape Lake	21.30^c	20.57	22.02	50

Note: Different superscript letters next to LS means indicate significant differences after Bonferroni correction (experiment-wise $\alpha = 0.05$).

lake fish, they both were significantly larger than Wallace Lake fish, which were larger than L-shape Lake fish (Table 3, Fig. 1).

Pelvic areas (accounting for the effect of size) did not differ significantly in relation to lateral plate number or among populations ($n = 164$; Table 4), suggesting the lack of an energetic trade-off between development of the pelvis and other mineralized structures. Fish with pelvises (mean rank = 109, $n = 164$) had more lateral plates than those without them (mean rank = 63, $n = 36$; Mann-Whitney *U*-test, $P < 0.0001$).

Laboratory growth experiment

The results using laboratory-reared fish are consistent with those for field-caught fish. Three- and two-way interactions among PS, salinity treatments, and calcium treatments (PS × salinity × calcium, PS × salinity, and PS × calcium) did not affect growth significantly (SL measured at 138 days post-fertilization; $n = 691$; Table 5). The population × PS interaction was significant ($P < 0.01$; Table 5). Tukey-Kramer tests comparing pairwise means between PS categories within populations showed that for Bruce Lake, PS = 3–5 (unadjusted mean = 23.43 mm) specimens grew significantly larger than PS = 0–2 specimens

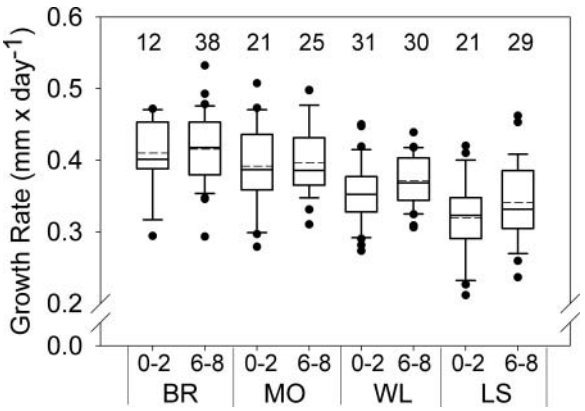

Fig. 1. Box-and-whisker plots of growth rates for young-of-the-year *G. aculeatus* with low (PS = 0–2) and high (PS = 6–8) pelvic scores collected from each of four populations: Bruce (BR), Morvro (MO), Wallace (WL), and L-shape (LS) lakes. Solid and dashed lines in boxes are medians and means, respectively. Boxes are inter-quartile ranges (IQR), whiskers are 10th and 90th percentiles, and dots are outliers. Sample sizes are shown above the entry for each sample.

Table 4. Results from a two-way, unbalanced ANCOVA to test the effects of lateral plate number and population on pelvic areas of field-caught, young-of-the-year *G. aculeatus*

Effect	d.f.	Sums of squares	Mean square	*F*-value	*P*-value
Lateral plate number (LP)	3	0.013	0.004	1.209	0.309
Population	3	0.012	0.004	1.074	0.362
LP × Population	7	0.039	0.006	1.525	0.163
Standard length	1	0.030	0.030	8.192	**0.005**
Error	149	0.538	0.004		
Total	163				

Note: Standard length was a covariate. **Bold** *P*-values indicate significant effects at $P < 0.05$.

(unadjusted mean = 21.39 mm; $P < 0.05$), but neither of these PS categories differed in size from PS = 6–8 fish (unadjusted mean = 22.33 mm) (Fig. 2a). For Morvro Lake fish, size differences were not significant at 138 days post-fertilization among fish in different PS categories ($P > 0.05$; Fig. 2a).

The effect of calcium ($P < 0.0001$) and the interaction between calcium and population on SL at 138 days post-fertilization were significant ($P < 0.01$; Table 5). Thus, we performed Tukey-Kramer tests for differences between calcium treatment means within populations. Morvro Lake progeny raised in low calcium (unadjusted mean = 23.55 mm) grew larger than those in high calcium (unadjusted mean = 21.23 mm; $P < 0.05$), but the effect of calcium was not significant for Bruce Lake progeny ($P > 0.05$; Fig. 2b). Fish grew larger in high salinity than in low salinity treatments (unadjusted mean SL for high salinity = 21.89 mm, for low salinity = 21.85 mm; $P < 0.05$; Table 5). Although the mean SL for fish grown in high salinity was only 0.04 mm larger than that for those grown in low salinity, these values were not least-squares means, which account for all other significant factors from the main model, because these means could not be estimated due to the large number of factors in the

Table 5. Results from a mixed-model, unbalanced ANOVA to test the effects of pelvic score, calcium, salinity, and population on growth (standard length in mm measured at 138 days post-fertilization) of laboratory-grown *G. aculeatus* raised in water with different salinity and calcium concentrations

Effect	d.f.	*F*-value	*P*-value
Pelvic score (PS)	2	0.84	0.4320
Calcium	1	18.60	**<0.0001**
Salinity	1	6.26	**0.0126**
Population	1	0.32	0.5747
PS × Calcium	2	0.37	0.6927
PS × Salinity	2	0.22	0.8029
PS × Population	2	4.85	**0.0081**
Calcium × Salinity	1	0.05	0.8165
Calcium × Population	1	9.75	**0.0019**
Salinity × Population	1	0.70	0.4028
PS × Calcium × Salinity	2	0.01	0.9885
PS × Calcium × Population	2	0.28	0.7526
PS × Salinity × Population	2	0.04	0.9630
Calcium × Salinity × Population	0		
PS × Calcium × Salinity × Population	0		

Note: **Bold** *P*-values indicate significant effects at $P < 0.05$. Only fixed effects are shown (block was a random effect in the model and represented horizontal rows of aquaria at different distances from an overhead light source). d.f. = degrees of freedom; denominator degrees of freedom = 666.

model. Thus, the true difference after accounting for other significant factors may be larger than the raw means without accounting for these factors. All other factors in the ANOVA were not significant or could not be estimated (Table 5).

The effect of lateral plate number and its interaction with either calcium or salinity on pelvic area were not significant ($n = 404$; all $P > 0.05$; Table 6). The interaction between population and calcium treatments on pelvic area was significant ($P < 0.01$; Table 6), but all Tukey-Kramer pairwise tests of means within the levels of each factor were not significant. Pelvic areas of Morvro Lake fish grown in low calcium and Bruce Lake fish grown in high calcium treatments were nominally larger (Fig. 3). No other factors in the ANOVA were significant (Table 6). Fish with pelvises (mean rank = 345, $n = 403$) had more lateral plates relative to those without pelvises (mean rank = 286, $n = 241$; Mann-Whitney *U*-test, $P < 0.0001$).

DISCUSSION

Environmental ion limitation has been proposed to select for armour reduction in *G. aculeatus* from low ionic strength, freshwater environments, decreasing the energetic costs of importing ions from dilute fresh water to mineralize the skeleton and allowing increased somatic growth and reproductive effort (Heuts, 1947; Giles, 1983; Bell *et al.*, 1993). Experimental studies have shown that low ion concentrations reduce somatic growth in *G. aculeatus* with more bony lateral plates (Marchinko and Schluter, 2007; Barrett *et al.*, 2009), and negative correlations between the size and complexity of pelvic phenotypes and ion concentrations across environments lend some support for ion, especially calcium ion, limitation as a selection

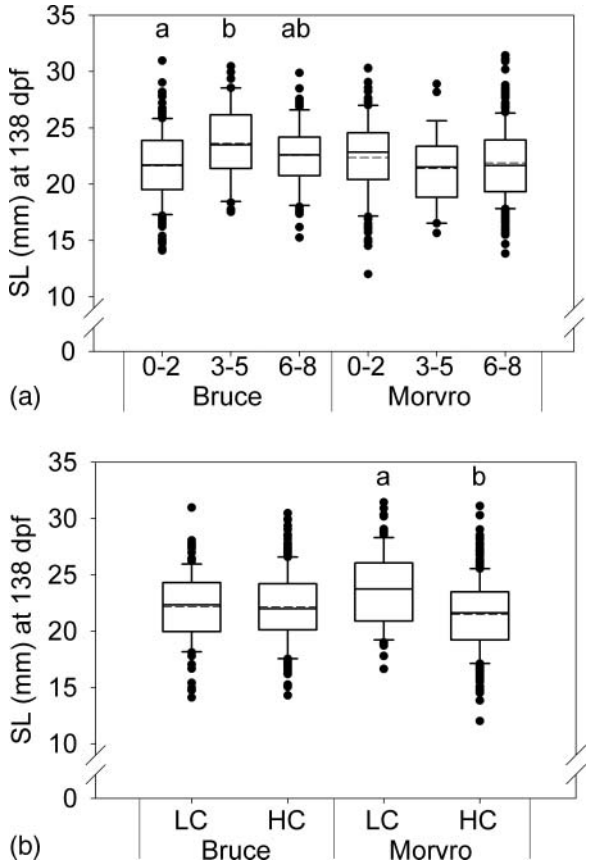

Fig. 2. Box-and-whisker plots of standard length (SL) at 138 days post-fertilization (dpf) of laboratory-grown *G. aculeatus* with (a) low (PS = 0–2), intermediate (PS = 3–5), and high (PS = 6–8) pelvic scores, and (b) low and high calcium treatments. Bruce [(a) PS = 0–2, $n = 184$; PS = 3–5, $n = 48$; PS = 6–8, $n = 89$; (b) low calcium, $n = 99$; high calcium, $n = 222$] and Morvro lake [(a) PS = 0–2, $n = 139$; PS = 3–5, $n = 26$; PS = 6–8, $n = 204$; (b) low calcium, $n = 88$; high calcium, $n = 281$] progeny were raised under contrasting salinity and calcium regimes. Different letters above bars indicate significant differences at $P < 0.05$ (Tukey-Kramer tests). Lines, boxes, whiskers, and dots are as in Fig. 1.

agent for pelvic reduction in *G. aculeatus* (Giles, 1983; Bell *et al.*, 1993; Bourgeios *et al.*, 1994). While correlative studies across habitats provide hypotheses for the environmental factors that select for a phenotype, experimental analysis is needed to test such hypotheses (Wade and Kalisz, 1990; Futuyma and Bennett, 2009). Previous experimental studies have found evidence that sticklebacks with larger, more complex pelvic structures may be more vulnerable to predatory insects [*C. inconstans* (Reist, 1980); *G. aculeatus* (Reimchen and Nosil, 2002; Marchinko, 2009; Lescak *et al.*, 2012; but see Reimchen, 1980; Zeller *et al.*, 2012; Mobley *et al.*, 2013)]. Until now, however, other hypotheses for selection agents of pelvic reduction have not been tested experimentally. The goals of our study were to assess experimentally whether ion or calcium limitation favours reduced pelvic expression by estimating growth rates of *G. aculeatus* with large and small pelvic

Table 6. Results from a mixed-model, unbalanced ANOVA to test the effects of lateral plate number, calcium, salinity, and population on residuals from a regression of pelvic area on growth (standard length in mm at 138 days post-fertilization) of laboratory-grown *G. aculeatus* raised in water with different salinities and calcium concentrations

Effect	d.f.	*F*-value	*P*-value
Lateral plate number (LP)	3	0.34	0.7964
Calcium	1	0.85	0.3565
Salinity	1	1.27	0.2614
Population	1	1.94	0.1650
LP × Calcium	3	0.17	0.9135
LP × Salinity	3	0.68	0.5626
LP × Population	3	0.11	0.9542
Calcium × Salinity	1	1.28	0.2577
Calcium × Population	1	8.79	**0.0032**
Salinity × Population	1	0.54	0.4647
LP × Calcium × Salinity	2	0.30	0.7437
LP × Calcium × Population	2	1.20	0.3034
LP × Salinity × Population	2	0.31	0.7346
Calcium × Salinity × Population	0		
LP × Calcium × Salinity × Population	0		

Note: **Bold** *P*-values indicate significant effects at $P < 0.05$. Only fixed effects are shown (block was a random effect in the model, and represented horizontal rows of aquaria at different distances from an overhead light source). d.f. = degrees of freedom; denominator degrees of freedom = 375.

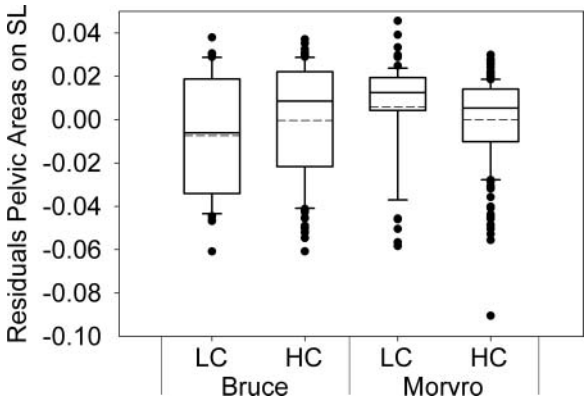

Fig. 3. Box-and-whisker plots of residuals from a regression of pelvic area on standard length (SL; mm) measured at 138 days post-fertilization of laboratory-grown *G. aculeatus* in low and high calcium treatments. Bruce (low calcium, $n = 99$; high calcium, $n = 222$) and Morvro (low calcium, $n = 88$; high calcium, $n = 281$) lake progeny were raised under different salinity and calcium regimes. Although ANOVA indicated a significant interaction between population and calcium treatment, Tukey-Kramer tests did not detect significant differences among pair-wise means. Lines, boxes, whiskers, and dots are as in Fig. 1.

structures among laboratory-reared specimens and also to test whether growth differences among pelvic phenotypes consistent with our predictions could be detected in nature under low ion and calcium conditions.

Our results do not support hypotheses that low total ion or calcium concentrations select for reduced pelvic structures in *G. aculeatus*. Age-adjusted size (SL) of field-caught *G. aculeatus* did not differ between specimens with large and small pelvic phenotypes within each of four variable freshwater populations (Tables 2 and 3, Fig. 1). In addition, at 138 days post-fertilization, the size of laboratory-raised *G. aculeatus* from two of these populations did not differ between extreme pelvic phenotypes at low total ion or calcium concentration (Table 5); interactions between pelvic score and salinity, calcium, or both salinity and calcium were not significant. Results from neither field-caught nor laboratory-reared specimens indicate that development of a larger pelvis reduces growth under conditions of low ion or calcium availability. While we detected growth differences among the pelvic phenotypes of Bruce Lake specimens in the laboratory, those with the smallest pelvic skeletons did not grow fastest, and these differences were independent of total ion or calcium concentrations (Fig. 2a).

Our results differ from those of previous correlative studies. Bell *et al.* (1993) found a correlation between mean pelvic phenotypes and calcium ion concentrations across lake populations of Cook Inlet, Alaska, provided that native predators were absent. In a multivariate analysis on a reduced dataset from Bell *et al.* (1993), Bourgeois *et al.* (1994) found that ion composition explained a relatively large proportion of the variation in mean pelvic scores across lakes (11.4%). However, our results did not detect a somatic growth difference between fish with contrasting pelvic phenotypes in low calcium or total ion concentrations during the first year of life. This discrepancy suggests that: (1) the effect of ion or calcium concentrations found in previous correlative studies is confounded with some other variable(s), which alone or due to some interaction with ionic composition, favours pelvic reduction; (2) ionic concentrations differentially affect fitness of fish with small or large pelvises, but somatic growth is the wrong fitness proxy; or (3) a pelvic phenotype-dependent effect of calcium or total ion concentration on somatic growth in *G. aculeatus* is too small to be detected with moderate sample sizes. We now consider each hypothesis in turn.

First, the results of Bourgeois *et al.* (1994) suggest that several environmental variables contribute to variation in pelvic phenotypes, and that they are inter-correlated. Correlations among environmental variables only explain the levels to which those variables are confounded and together affect the occurrence of pelvic phenotypes, but not how they interact with each other to affect this occurrence. Therefore, factors that covary with calcium or ion concentration among habitats may select for pelvic reduction, or a combination of variables or the way in which they interact may favour pelvic reduction. Indeed, we found growth differences among populations (Tables 2 and 3) and significant interactions of population with other factors (Table 5) on growth in our field and experimental studies, respectively, suggesting that environmental and genetic differences among populations make a large contribution to somatic growth compared with the effect of pelvic phenotype.

Second, ion concentrations may differentially affect the fitness of *G. aculeatus* with small or large pelvic structures, but not in the way that Giles (1983) had proposed. Perhaps instead of an energetic cost some other fitness component (e.g. overwinter survival, fecundity) is responsible. Instead of affecting total somatic growth, use of ions to grow the pelvis may delay development of other bony structures or affect the size or integrity of those structures (reviewed in Arendt, 1997). To test for a trade-off between other mineralized structures versus

the pelvis in low ionic strength water, we determined whether lateral plate number affected the body size-adjusted area of pelvises. In most specimens of both the field-caught (average age of 63.52 days) and the laboratory-raised *G. aculeatus* (138 days post-fertilization), lateral plates had not fully developed because specimens were <21.5 or 30 mm SL, the size at which the addition of lateral plates is complete in low-plated *G. aculeatus* [Bell (1981) and Hagen and Gilbertson (1972), respectively; specimens had between 0 and 11 plates, with a mode of 6 in both data sets]. Since the potential for a mineralization trade-off existed as long as both the pelvis and lateral plates were still developing, we used lateral plate number as an indicator of the amount of resources (calcium and phosphate ions) allotted to the other bony structures relative to the pelvis. Lateral plate number did not affect pelvic areas in specimens that had pelvises (Tables 4 and 6), suggesting that ion limitation does not differentially affect growth among bony structures. Also, *G. aculeatus* with pelvises had significantly greater lateral plate numbers (on average) than those without pelvises (Mann-Whitney *U*-tests, $P < 0.0001$ in both data sets), which is the opposite of what we would expect if a trade-off existed.

Also, many studies have shown that growth and survival are positively correlated (e.g. Toneys and Coble, 1979; Sogard, 1997), and growth can only be assessed for fish that survived to be measured. A larger fraction of high PS fish (relative to low PS fish) may have grown slowly in low ion or calcium treatments and died as a result. These fish would not have been measured in our study [the 'invisible fraction' (Grafen, 1988; Hadfield, 2008)], and if they had, our results would have supported the hypothesis that high PS fish have reduced growth and survival (relative to low PS fish) in low ion or calcium concentration. Other studies have shown a negative correlation between fast growth and survival, so growth may be a bad fitness proxy in some cases (e.g. DiBattista *et al.*, 2007; Carlson *et al.*, 2008). Thus, our study shows that low ion and calcium concentrations do not appear to reduce somatic growth, but that does not prove that these are not agents of selection for pelvic reduction since they may influence fitness components, such as survival, that we did not consider in this study.

Third, perhaps we could not detect a fitness effect of ion concentrations between pelvic phenotypes because the selective advantage is so small that the effects on growth were not significant. This explanation is plausible, since it took about 6000 years (3000–6000 generations) for pelvic reduction to evolve (Bell *et al.*, 2006) in response to probable directional selection in a fossil stickleback lineage (Hunt *et al.*, 2008).

Alternatively, we may not have detected an effect in this study because our low salinity treatments were slightly brackish (Table S2, evolutionary-ecology.com/data/2881Appendix.pdf). The total ion concentrations in the low salinity treatments were elevated because we used a high concentration of calcium in the high calcium treatments (equivalent to the amount present in sea water) and a large concentration of magnesium in the low calcium treatments to compensate for the elevated salinity caused by the elevated calcium concentration in the high calcium treatments. In natural populations, salinities are lower (0.010–0.017 ppt for our lakes, Table 1) than those used in our low salinity treatments (between 0.097 and 1.39 ppt; Table S2, 2881Appendix.pdf). Perhaps our low-concentration treatments were not low enough to cause a significant interaction between pelvic phenotypes and ion concentrations that may exist in nature. However, we did not find an effect of pelvic phenotypes on somatic growth in field-caught, freshwater *G. aculeatus*, either. Contrary to our expectations, field-caught, young-of-the-year *G. aculeatus* with robust pelvic skeletons did not grow significantly slower than those with small vestiges (Tables 2 and 3, Fig. 1).

Our choice of populations is yet another possibility. We chose them because the wide range of pelvic phenotypes within each population (Bell and Ortí, 1994) allows intra-population comparison of the components of fitness related to this phenotype. But they represent cases in which a wide range of pelvic phenotypes (from full expression to absent pelvis) has been maintained for at least 21 years (from 1990 to 2011). Our laboratory experiments show that Morvro Lake fish grew larger by 138 days post-fertilization in low than in high calcium concentrations, and Bruce Lake fish grew equally well in both low and high calcium treatments (Table 5, Fig. 2b). Perhaps they are adapted to low calcium environments that initially favoured reduced pelvic phenotypes. Evolution of more efficient ion uptake or retention may have occurred and eliminated the selective advantage of pelvic reduction in the existing population. These alternative mechanisms may have evolved only after pelvic reduction had proceeded to its present polymorphic state. Alternatively, polymorphism may imply weak selection for pelvic reduction, disruptive selection, assortative mating according to pelvic phenotypes, or that selection has not had enough time to cause fixation of pelvic reduction. It is unlikely that gene flow from populations with robust pelvises is the cause of the high degree of polymorphism in these populations – there are no inlet or outlet tributaries for all four of our seepage lakes, and these lakes would probably not receive runoff from others during flood events based on topographic maps. If there is disruptive selection, assortative mating, or limited time for evolution of pelvic reduction, then we should still have been able to show an effect, for our laboratory-raised specimens, of ion or calcium concentration on growth if one existed. However, if pelvic reduction is under weak selection in these populations, we would expect the selective advantage to be small and perhaps undetectable in our experiments (e.g. Mobley *et al.*, 2013).

The selection agents that favour reduced pelvic expression in *G. aculeatus* are still unknown. The survival value of the full pelvis in post-capture defence against gape-limited predators is understood (e.g. Hoogland *et al.*, 1957; Reimchen 1983, 1991, 1992), and pelvic reduction tends to occur in the absence of native predatory fishes (Bell *et al.*, 1993; reviewed in Klepaker *et al.*, 2013). Patterns of change in the fossil record (Hunt *et al.*, 2008) and evidence for selective sweeps using DNA sequence data (Chan *et al.*, 2010) both indicate that pelvic reduction results from positive selection. Our study found that *G. aculeatus* with reduced pelvic structures did not have a detectable growth advantage compared with those with robust pelvises in water with low total ion or calcium concentrations. Therefore, despite the association of reduced ion and calcium concentrations with pelvic reduction (Giles 1983; Bell *et al.*, 1993), these environmental factors may not be the actual selection agents for pelvic reduction in *G. aculeatus*.

Broader implications

Experimental tests to infer selection agents are important for determining how and why traits evolve and for predicting the direction of trait evolution in different environmental contexts, and they have been performed for numerous traits in a wide range of taxa (MacColl, 2011). In general, it is possible to develop strong experimental evidence to identify the selection agent for a trait, but there may be a sampling bias; selection agents may tend to be identified in cases that seem *a priori* to be amenable for analysis because of the nature of the trait or prior evidence for the existence of high selection coefficients, which should facilitate identification of the selection agent. However, it may often be impossible to use experiments to detect a fitness effect for many traits, even if the hypothesis for the selection agent is correct and if one measures the right proximate variable for fitness. This may be due to weak

selection acting over many generations (e.g. Hunt *et al.*, 2008) or negative pleiotropic effects at different developmental stages. In experimental ponds, selection against the low *Eda* allele (Eda^L), which causes reduction in the number of lateral plates, occurs prior to development of lateral plates, and is strong enough to counteract selection in favour of Eda^L once plates begin to develop (Barrett *et al.*, 2008). The environmental factors causing selection against the Eda^L allele prior to plate development are unknown, but negative pleiotropic effects may be prevalent in nature (e.g. Leroi *et al.*, 2005) and affect our ability to detect selective advantages measured at a single point in ontogeny. Future research should investigate whether ion or calcium availability differentially affect components of fitness other than somatic growth between large- and small-pelvic phenotypes. Experimental tests of other hypothesized agents of selection for pelvic reduction are necessary, but it appears that identification of the selection agent for pelvic reduction will be difficult.

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